Plant Physiol. (1971) 47, 465–477

Biogenesis and Degradation of Starch

I. THE FATE OF THE AMYLOPLAST MEMBRANES DURING MATURATION AND STORAGE OF POTATO TUBERS[1]

Received for publication July 31, 1970

ITZHAK OHAD, ILAN FRIEDBERG, ZVI NE'EMAN, AND MICHAEL SCHRAMM
Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

ABSTRACT

Storage of mature or developing potato tubers (*Solanum tuberosum* "Up-to-Date" variety) at 4 C causes a reduction in the starch content and the elevation in the level of free sugars. This phenomenon is not observed when the tubers are stored at 25 C. Changes in the morphology of cells from developing or mature tubers after storage at 4 or 25 C have been followed by electron microscopy. During all stages of the tuber development the starch granules are surrounded by a membrane derived from the plastid envelope. Storage in the cold induces disintegration of this membrane. A membrane fraction isolated from starch granules of tubers stored at 4 C has a lower buoyant density, and the electrophoretic pattern of its proteins is different from that of a similar membrane fraction obtained from tubers stored at 25 C. It is suggested that the cold-induced changes in the starch and sugar content during storage of potato tubers might be correlated with damage to the membranes surrounding the starch granules and changes in their permeability to degradative enzymes and substrates.

Storage of potato tubers at 4 C causes degradation of starch and accumulation of free sugars (sucrose, glucose, and fructose). These changes are minimized when the tubers are stored at 25 C (31). Because of the economic importance of the phenomenon, much attention was paid to its possible causes. In general, it was assumed that activation of degradative enzymes might play the main role in the degradation process, and much work concerning the activities of enzymes involved in the pathways of starch synthesis and degradation has accumulated (4, 21, 22, 25, 31).

It is, however, possible that the process of degradation of starch is not related to a significant increase in some enzymatic activity but rather to a change in the distribution of different enzymes or substrates within the subcellular compartments of the potato tuber. Thus, if the starch granules were surrounded by a semipermeable membrane, permeability changes in this membrane induced by cold storage might bring in contact the degradative enzymes and their substrates. Although it is known that the formation of starch granules in plant tissue occurs within a plastid whose boundaries consist of a double membrane, little attention has been paid so far to the role of the plastid membrane in the processes of synthesis and degradation of the starch granule within the plastid.

It has apparently not been established whether in the mature potato tuber the plastid membrane is still present and what its fate is during the normal process of tuber maturation. In addition, if the membrane is present around the starch granules in the mature tuber, one should expect that isolated starch granules will be partially or entirely surrounded by this membrane. However, little information is presently available on this point.

As a part of a more extensive study of the process of cold-induced starch degradation in potato tubers we have decided to follow the development of the starch granules during the maturation stages of the potato tubers with special attention to the plastid mmbranes; to establish whether there is a surrounding intact membrane around the mature starch granule; and, if such a membrane is present, to characterize it chemically and morphologically, to find out whether storage at different temperatures will induce changes in the structure and chemical composition of the membranes which might be correlated with the process of starch degradation.

For the specific purpose of this work a method for the determination of starch suitable for the quick analysis of numerous samples has been adapted and a method for the isolation of a membrane fraction connected with the starch granules has been developed. The results of this work indicate that the mature starch granule is surrounded by the remnant of the plastid membrane which seems to be specifically damaged during storage in the cold.

MATERIALS AND METHODS

Mature, fresh white potato tubers (*Solanum tuberosum*, "Up-to-Date" variety) from the spring harvest were obtained from the market 1 or 2 days after harvesting. The potato tubers were stored in air at 4 or 25 C, in the dark. Several batches were stored at 4 C under an atmosphere of nitrogen or 5% CO_2 in air.

For the study of chemical and morphological changes during development the same white potato variety of the autumn harvest was supplied directly from the field, from Kibbutz "Urim," 1 day after harvesting. The potatoes were sown on August 12, 1969, and germinated after 12 days. Potato tubers were supplied from the same field 46, 54, 64, 71, and 80 days after germination.

After harvest the tubers were stored at 4 or 25 C in air, in the dark.

Determination of Starch Content in Potato Tubers. Potato tubers were cut into slices about 2 mm thick and dried in an aerated oven at 80 C to constant weight. The dry material was ground in a mortar to a fine powder. The powder was sifted through a fine sieve (400 mesh) and 50 mg were weighed into a glass test tube. Hot, 80% (v/v) ethanol was added (10 ml) for

[1] Supported by United States Department of Agriculture grant FG-Is-280 to M. Schramm and I. Ohad.

the extraction of soluble sugar and other low molecular weight material. The ethanol was decanted after centrifugation (3,000*g*, 5 min), and the extraction was repeated four times. The final sediment was suspended in 5 ml of water and the starch was solubilized in a boiling water bath for 15 min (14). The resulting suspension was cooled to 70 C, and an α-amylase preparation (bacterial α-amylase, Fluka) was added to a final concentration of 10 μg/ml. Incubation was continued at 70 C. The suspension was cleared within a few minutes. After 15 min of incubation, the sample was centrifuged at 3,000*g* for 5 min to sediment cell walls, coagulated protein, and other cellular debris, and the sugar content of the clear supernatant solution was determined with the phenol-sulfuric acid reagent (9). Both the sediment and the clear supernatant solution did not contain undigested starch as tested by I_2-KI. The protein content of the supernatant solution was determined according to Lowry *et al.* (19) and the RNA content by the method of Mejbaum (23). These constituted less than 0.5% (w/w) of the sugar content in all samples tested.

The possible interference of protein or RNA present in the sample with the determination of the sugar content was considered, and the following tests were performed: (*a*) Protein and RNA were precipitated by addition of trichloroacetic acid to a final concentration of 10%; (*b*) protein (bovine serum albumin) and RNA (yeast) were added to the solubilized starch in amounts equivalent to those present in the samples. The changes in the sugar content value as determined by the phenol-sulfuric acid reagent (9) were measured. In both cases it was found that the results differed only by a few per cent from those obtained by direct measurement of the sugar content. Thus, it was concluded that interference is insignificant and all further measurements were performed directly on the cleared solubilized starch solution.

Sampling Procedure for Sugar Determination. Tubers were peeled, washed, and weighed. Samples of about 20 g were taken from several tubers for sugar determination (6). The samples were cut to pieces and homogenized in a glass Waring Blendor for 2 min in the presence of 65 ml of 80% (v/v) hot ethanol. The ethanolic extract was decanted after 5 min standing, and three additional extractions were performed, each with 60 ml of 80% hot ethanol. The supernatant solutions were pooled together, and the final volume was measured. Sugars were assayed in the extract, after proper dilution. Total carbohydrate content was estimated with the phenol-sulfuric acid reagent (9). Reducing sugars were assayed according to the method of Somogyi and Nelson (32, 34), before and after acid hydrolysis, which was performed by incubation for 15 min in 0.1 M HCl in a boiling water bath. Glucose was estimated with glucose oxidase (Glucostat × 4 reagent, Worthington Biochemical Corp.) (15). Fructose, free or bound, was determined with the orcinol reagent (29). Sucrose was determined either colorimetrically with the orcinol reagent (29) or enzymatically by digestion with invertase (16) and estimation of the glucose released by glucose oxidase reagent (15).

Protein was assayed according to Lowry *et al.* (19). Protein determination in starch preparations was performed according to Frydman (12).

Lipids were extracted with chloroform-methanol, 2:1 (v/v) (28). The solvent was evaporated in a stream of nitrogen, and the lipids were redissolved in a small volume of the same solvent. Total ester bonds in the lipid fraction were determined by the hydroxamate reagent (33).

Thin layer chromatography of the lipids was carried out on silica gel plates (DC cards, Si, Riedel de Haen, West Germany) using as solvent a mixture of chloroform-methanol-water, 65:25:4 (v/v). Two-dimensional thin layer chromatography was carried out on the same plates, with the above solvent for the first dimension and diisobutyl ketone-acetic acid-water, 80:50:10 (v/v), for the second dimension (18). The spots were revealed by iodine vapor (30) and were determined by the ninhydrin reagent for the phosphatidyl ethanolamine and phosphatidyl serine; modified Dragendorff reagent (5) for choline-containing lipids; and with the use of Dittmer reagent for phospholipids (7).

Total phosphate was measured after the oxidation of the sample with $Mg(NO_3)_2$ (1), and assay of inorganic phosphate was according to Fiske and SubbaRow (10).

Preparation of Membrane Fraction. A total membrane fraction of potato tubers was prepared as follows. The tubers were peeled, washed and weighed. The tubers were cut into small pieces in tris-chloride buffer, 0.5 M, pH 7.2, with addition of sodium dithionite (0.5 g/100 g of fresh potato) and homogenized in the same solution in a glass Waring Blendor for 1 min at 0 C. The homogenate was filtered through four layers of gauze and was centrifuged for 5 min at 3,000*g* at 4 C in order to remove starch granules, cell walls, and other cellular debris. The supernatant fluid was centrifuged at 100,000*g* for 90 min. at 4 C, and the sediment consisting of all types of cellular membranes was suspended in 50 mM tris-chloride buffer, pH 7.2, to a final concentration of 20 to 40 mg of protein per ml and stored at −20 C.

Isolation of Membrane Fraction Connected with Starch Granules. For examination of the composition, properties, and changes in the membrane surrounding the starch granules, a method for isolation of starch granule membranes was developed.

Potato tubers were peeled, washed, and weighed. The tubers were gently homogenized by grinding on a glass grater. Sodium dithionite was added to the mesh (4 g/kg mesh) (6, 12). The mesh was filtered through four layers of gauze and resuspended in a large volume of 2.5 mM tris-chloride, pH 7.4, containing 7.5 mM NaCl and 0.5 mM β-mercaptoethanol. The suspension was refiltered through gauze as above. The filtrates were kept for several hours at 4 C for sedimentation of the starch granules. The starch granules were washed four times in the same buffer, to remove other cell components, especially other membranes, and then were broken in a stainless steel Waring Blendor, by 50 strokes of 15 sec each. The ground starch was centrifuged at 200*g*. The sediment was washed three times with the same buffer, and the supernatant fluids were collected together. After a centrifugation at 3,000*g* for 5 min to remove broken starch granules and other cellular debris, the membrane fraction was sedimented by centrifugation at 60,000*g* for 45 min in a Spinco model L ultracentrifuge. The membrane fraction was resuspended in a small volume of the buffer.

Samples were taken for examination by electron microscopy and for determination of protein, lipids, buoyant density, and electrophoresis on polyacrylamide gels. The membranes were stored at −20 C.

Density gradient centrifugation of the membrane fraction was performed either with an Urografin (Schering AG, Berlin, Germany) or sucrose linear gradient of density intervals 1.05 to 1.25 g/ml. Samples of 1 to 2 ml of membrane suspension (40–80 mg protein) were layered on a gradient in a tube, containing 25 to 27 ml of Urografin or sucrose solution. Centrifugation was for 90 min at 25,000 rpm, in a Spinco model L ultracentrifuge, with the SW-25 rotor at 0 C. The location of each band which appeared in the gradient was measured, and the density at the center position of the band was calculated. Protein and lipid were determined in the various fractions of the gradient.

Gel electrophoresis of the proteins was performed according to Razin *et al.* (27).

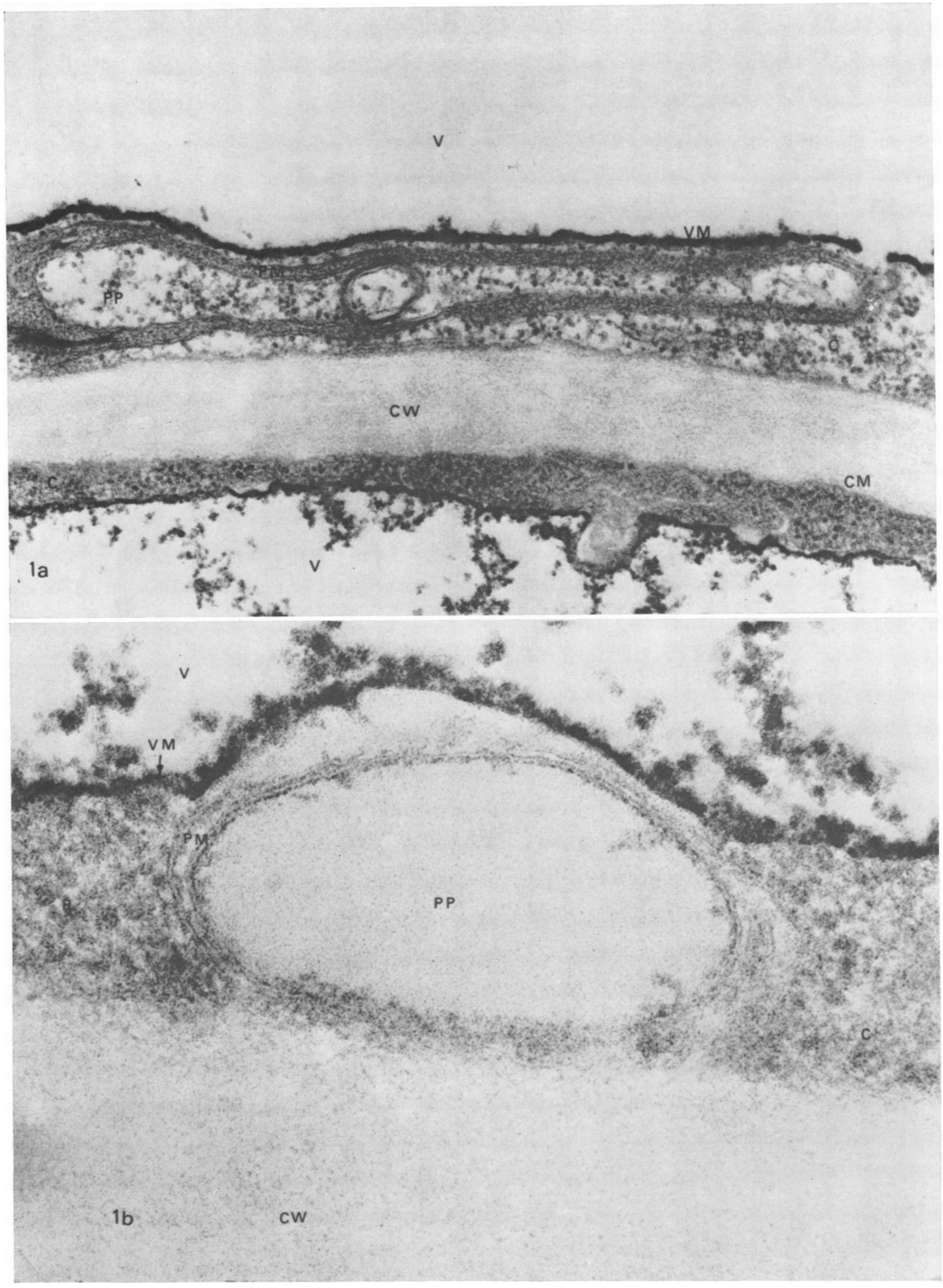

FIG. 1. Section through a cell of a potato tuber harvested 46 days after germination. In the heavy cell wall (cw) rows of parallel fibers are discernible. A thin layer of cytoplasm (c) underlines the cell wall from each side. The cytoplasmic membrane (cm) toward the cell wall is only slightly delineated, whereas the vacuolar membrane (vm), around the vacuole (v), which occupies most of the cell volume, is heavily stained. Within the cytoplasm one can distinguish ribosome-like particles (r) and proplastids (pp) surrounded by a double membrane (pm), which is better seen at the higher magnification shown in part b. a: × 48,000; b: × 135,000. For details of preparation see "Materials and Methods."

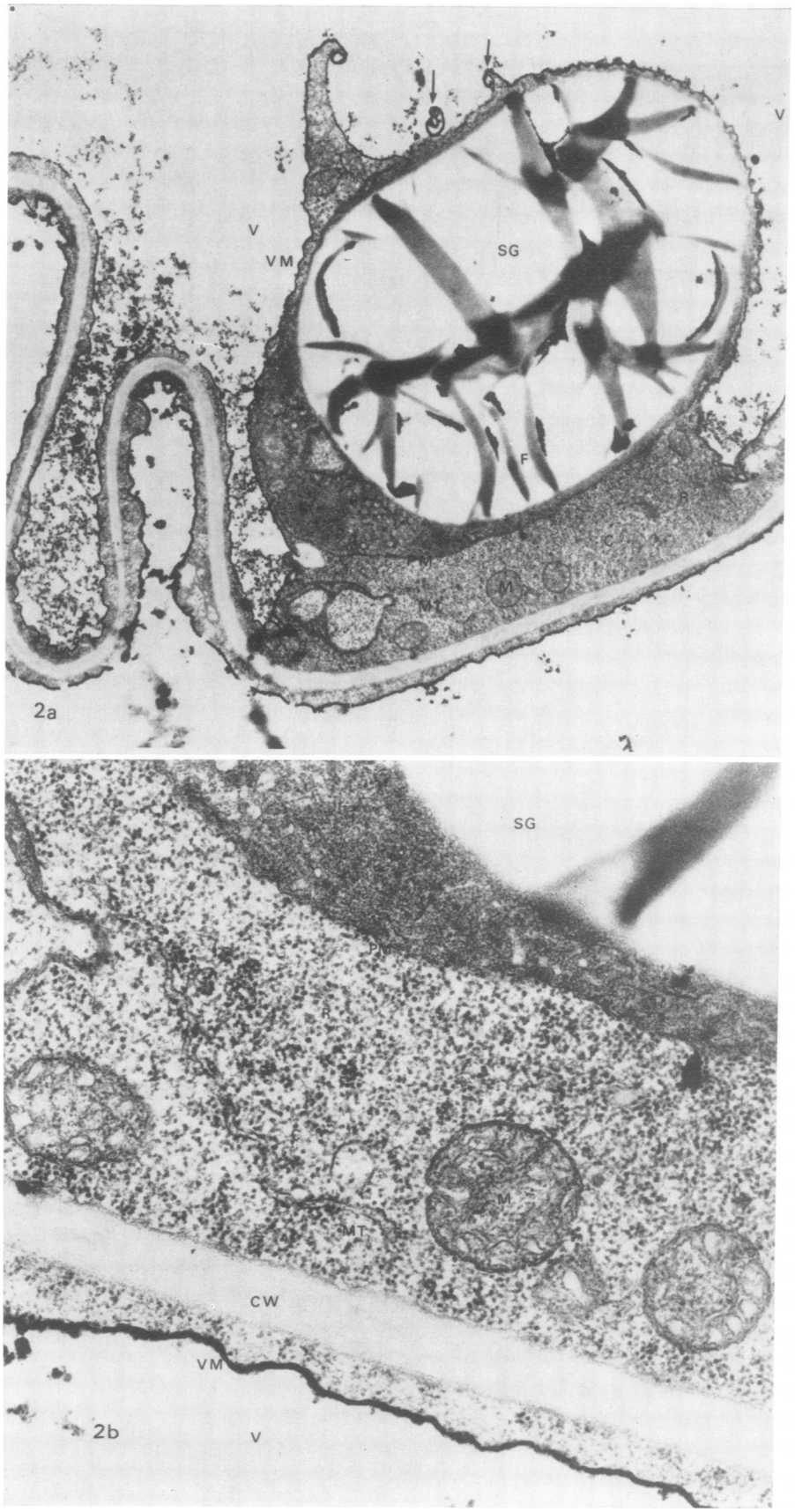

FIG. 2. Same material as in Figure 1. Notice the starch granule (sg) contained within the plastid protruding into the vacuolar space (v). The plastid membrane (pm) is intact. The vacuolar membrane (vm) seems to be broken or peeled at several points (arrows). The matrix of the plastid is denser and less uniform as compared with the cytoplasm (c). The cytoplasm (c) contains several intact mitochondria (m), microtubuli (mt), and numerous ribosomes (r). The section of the starch granule is folded in several places giving the image of patches of a dense material (f). This situation is very often encountered in potato tuber sections. a: × 7,100; b: (higher magnification of part of part a) × 32,900.

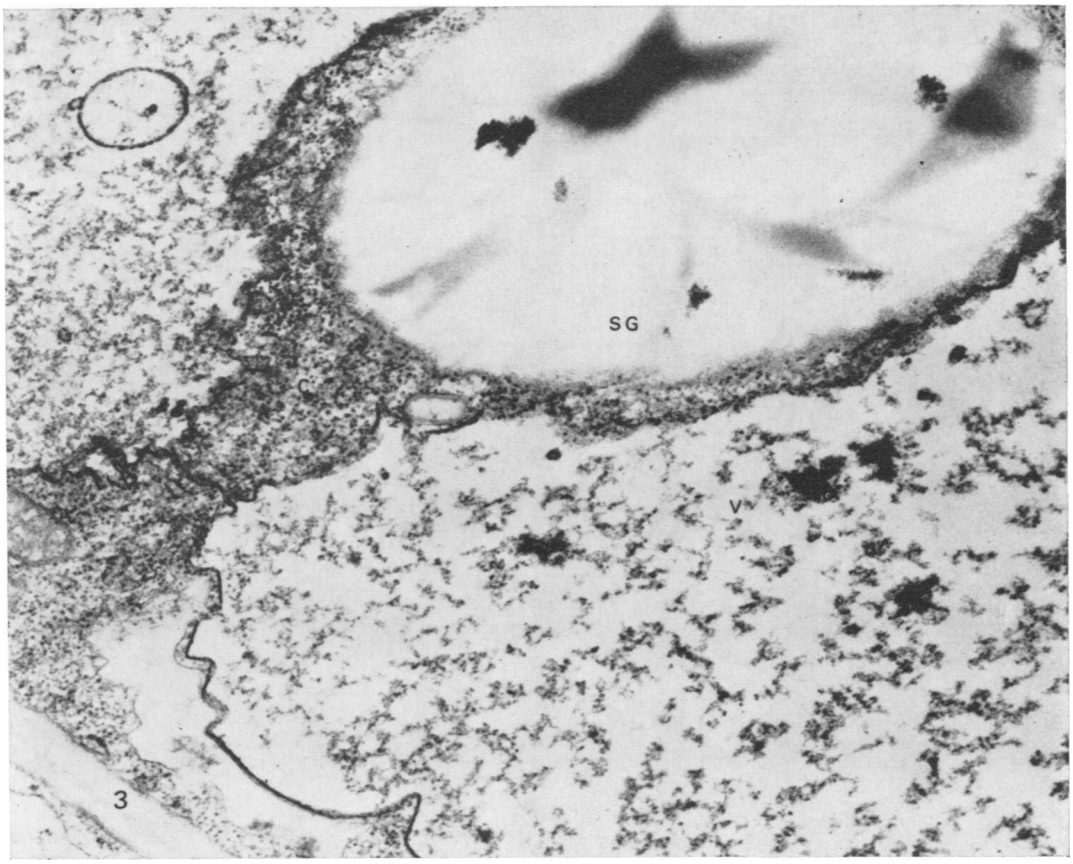

FIG. 3. Same material as in Figures 1 and 2. A starch granule (sg) is shown within a plastid protruding into the vacuole (v) and being still connected with the cytoplasm body (c) by a cytoplasmic stalk or bridge. × 22,700.

Electron Microscopy. Samples of potato tubers were submerged in 0.1 M phosphate buffer, pH 7.4, and cut into small cubes, 1 mm thick. The cubes were transferred into a solution of 4% glutaraldehyde (Ladd), in the above buffer for 3 hr at room temperature. The fixed material was washed by gentle decantation in four changes of the same buffer and transferred to a solution of 2% OsO_4 in the same buffer, and fixation was continued for an additional 3 hr at 4 C (26). The material was again washed in phosphate buffer, then in 28 mM veronal-acetate buffer adjusted to pH 3.9 with acetic acid and was finally fixed in 0.5% uranyl acetate in veronal-acetate buffer as above, for ½ hr at 4 C (35). The fixed tissue was dehydrated in graded concentrations of ethanol and finally kept in absolute ethanol overnight. The samples were transferred to two changes, 30 min each, of propylene oxide and then to a solution of Epon in propylene oxide as described by Luft (20). Infiltration with the plastic monomer was continued for 2 days and then the propylene oxide was evaporated in a stream of air for 24 hr. Membrane preparations were fixed in 4% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, for 4 hr at 0 C, then washed with the phosphate buffer and postfixed with 2% OsO_4 in the same buffer, for 8 hr. The material was embedded in Epon according to Luft (20). The fixed material was transferred to polyethylene capsules and polymerized at 60 C for 24 hr and at 90 C for an additional 24 hr. Thin sections were cut with a diamond knife using an LKB Ultrome III. The sections were stained in uranyl acetate and lead citrate as described by Reynolds (28) and examined in a Phillips EM-300 electron microscope operated at 60 or 80 kv.

RESULTS

MORPHOLOGICAL CHANGES DURING GROWTH OF THE POTATO TUBER

The development of the starch granules and their membranes in the growing potato tubers was followed by electron microscopy, beginning with young tubers, 46 days after germination (mean weight 9.4 g), and continued with batches that were harvested at 54, 64, 71, and 80 days after germination. The mean weight of the tubers at the last harvest was 100 g. In the first batch, 46 days after germination, proplastids were frequently observed in the preparations (Fig. 1). Young starch granules were present and were contained within the plastid membanes. It seems that during growth of the starch granule the amyloplast extrudes into the vacuolar space (Fig. 2, a, b) while still connected to the cytoplasmic region by a "stalk" (Fig. 3). After additional growth of the tuber (54 days after germination) it seems that the stalk between the amyloplast and the cytoplasmic region is broken, the vacuolar membrane is torn, and the granule, still coated by the intact plastid membrane and at least partially by the vacuolar membrane, "falls" into the vacuole (Fig. 4, a, b, c). At these early stages of development, the starch granules are surrounded by the plastid and by the cytoplasmic matrix. During growth of the tuber this matrix becomes thinner and thinner, as the starch granules grow, until it disappears almost completely, as demonstrated in tubers after an additional 4 weeks of growth. In tubers 80 days after germination the shape of the starch granules is similar to

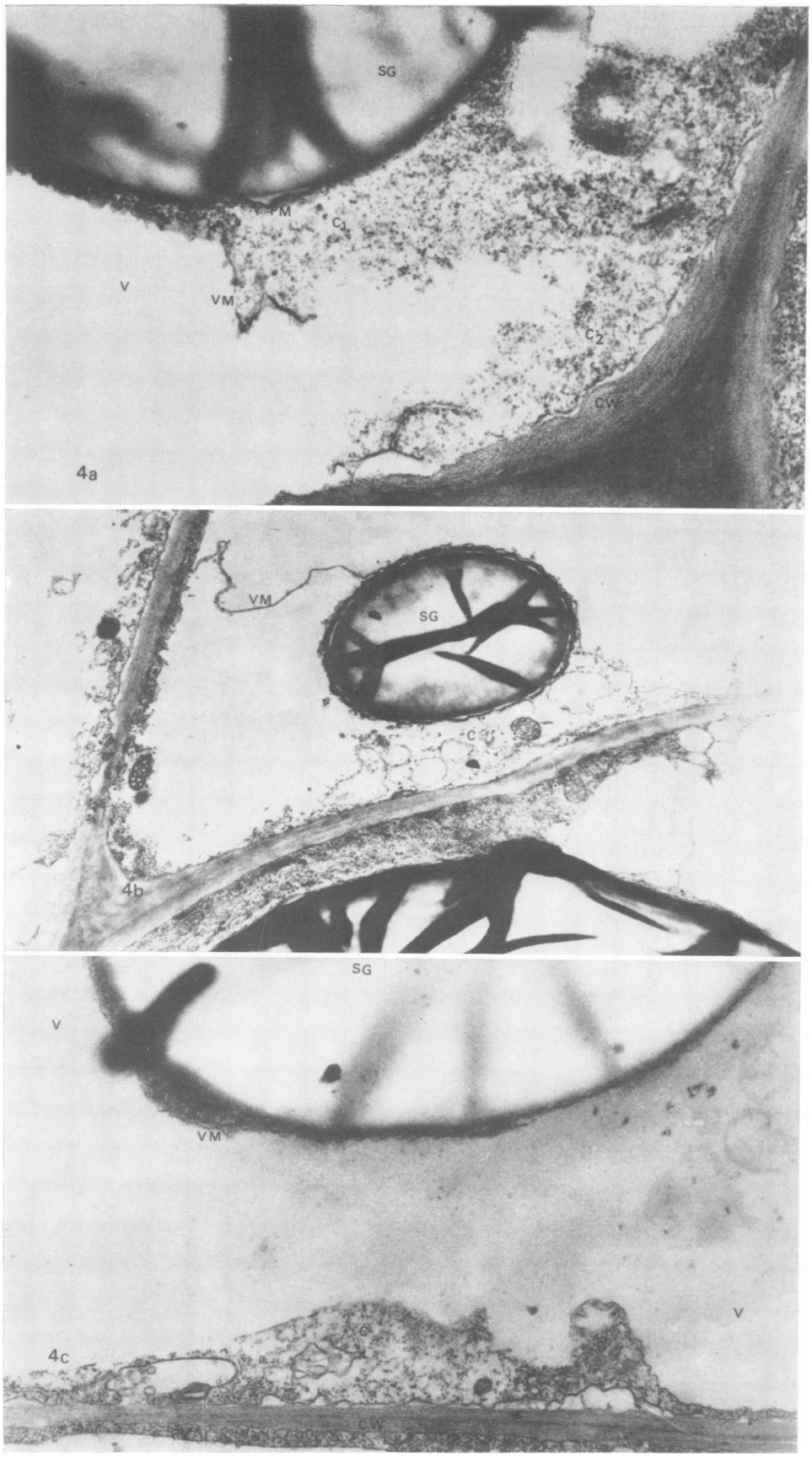

FIG. 4. Sections through cells of potato tubers harvested 54 days after germination. a: A starch granule (sg) contained within the plastid membrane (pm) extends into the vacuolar space (v). The vacuole membrane (vm) is broken and the cytoplasmic bridge connecting the cytoplasmic layer around the plastid (c_1) with the main cytoplasmic region (C_2) around the cell wall (cw) is torn away. × 19,500. b: Same material as in part a. Notice the vacuole membrane (vm) and the plastid containing the starch granule (sg) in the process of separation from the main cytoplasmic body of the cell (c). × 6,800. c: Same material as in part a. The starch granule (sg) is found inside the vacuole (v) while part of the vacuole membrane (vm) and a thin layer of cytoplasm (c) still surrounds the granule. Notice that the remnant of the cytoplasmic body of the cell along the cell wall (cw) does not spread or disperse inside the vacuole (v). × 15,000.

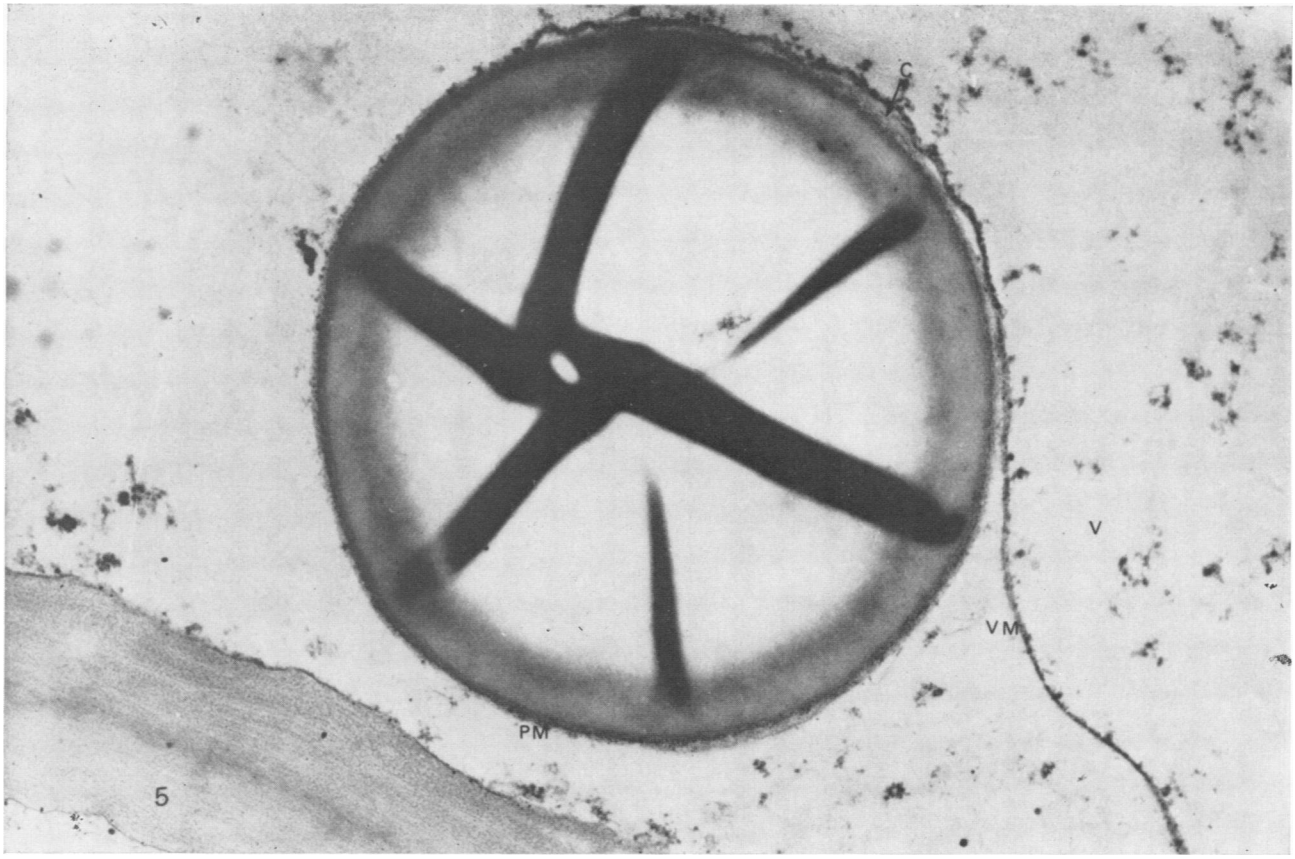

FIG. 5. Section through a cell of a potato tuber 80 days after germination. A starch granule is seen surrounded by the plastid membrane (pm) and the vacuolar membrane (vm), free within the vacuolar space (v). Notice the scarcity of the plastid matrix (c) around the starch grain. ×23,500.

that of mature tubers. The granules are still surrounded by two intact membranes, and in most cases no cytoplasmic or plastid matrix was observed between the granule and the coating membranes (Fig. 5).

Starch granules continue to increase in size during all the above stages, indicating that the enzymes connected with the synthesis of the starch are contained within the plastid space (12).

MORPHOLOGICAL CHANGES DURING STORAGE AT DIFFERENT TEMPERATURES OF DEVELOPING POTATO TUBER

Young potato tubers, 46 days after germination, were harvested and stored either at 4 C or at 25 C, in the dark. After 8, 18, 25, and 34 additional days of growth, new batches were harvested from the same field and were stored under the same conditions for 30 days. Samples were taken for observation in the electron microscope from every batch, before storage and after 30 days of storage. Changes of the starch granules and their membranes as a function of storage at different temperatures were followed.

Storage at 4 C results in disintegration and disappearance of the membranes around the starch granules, whereas during storage at 25 C the majority of the membranes remained intact. The morphology of young potato tubers after 31 days of storage at 4 C or at 25 C is shown in Figure 6, b and c, respectively. A similar picture was obtained with the batches which have been harvested afterwards and stored under the same conditions.

CHEMICAL CHANGES INDUCED BY DIFFERENT STORAGE CONDITIONS IN POTATO TUBERS OF DIFFERENT AGES

Dry Mass. During growth the dry mass of the tubers increased, from 12% (at 46 days after germination) to 20% (at 80 days after germination). During storage of the tubers harvested at different ages, no significant changes were observed in the dry mass whether the storage temperature was 4 or 25 C.

Free Sugar Content. The free sugar content of the potato tubers was determined as a function of the tuber age, storage period, and temperature of storage. Determination of total free sugar content (reducing sugars after acid hydrolysis) indicated that the initial level of free sugar immediately after harvest is rather constant in tubers of all ages tested, and it was found to be 4 to 6% of the dry weight.

Storage at 4 C induced an increase in the total free sugar content in all the batches after 4 days. However, only a slight increase was found when the tubers were stored at 25 C.

Determination of reducing sugars in the ethanolic extracts before and after acid hydrolysis permitted the estimation of glucose, fructose, and sucrose. In tubers stored at 4 C, it was found that the sucrose level increase after 4 days of storage, reached a maximum at 8 days, and decreased afterwards. The glucose and fructose level started to increase only after 4 days of storage. This behavior was found in all batches irrespective of their age.

The increase in the relative free sugar content after 31 days of storage at 4 C was higher in the younger tubers (27% wt/dry wt) as compared to older tubers (20% wt/dry wt).

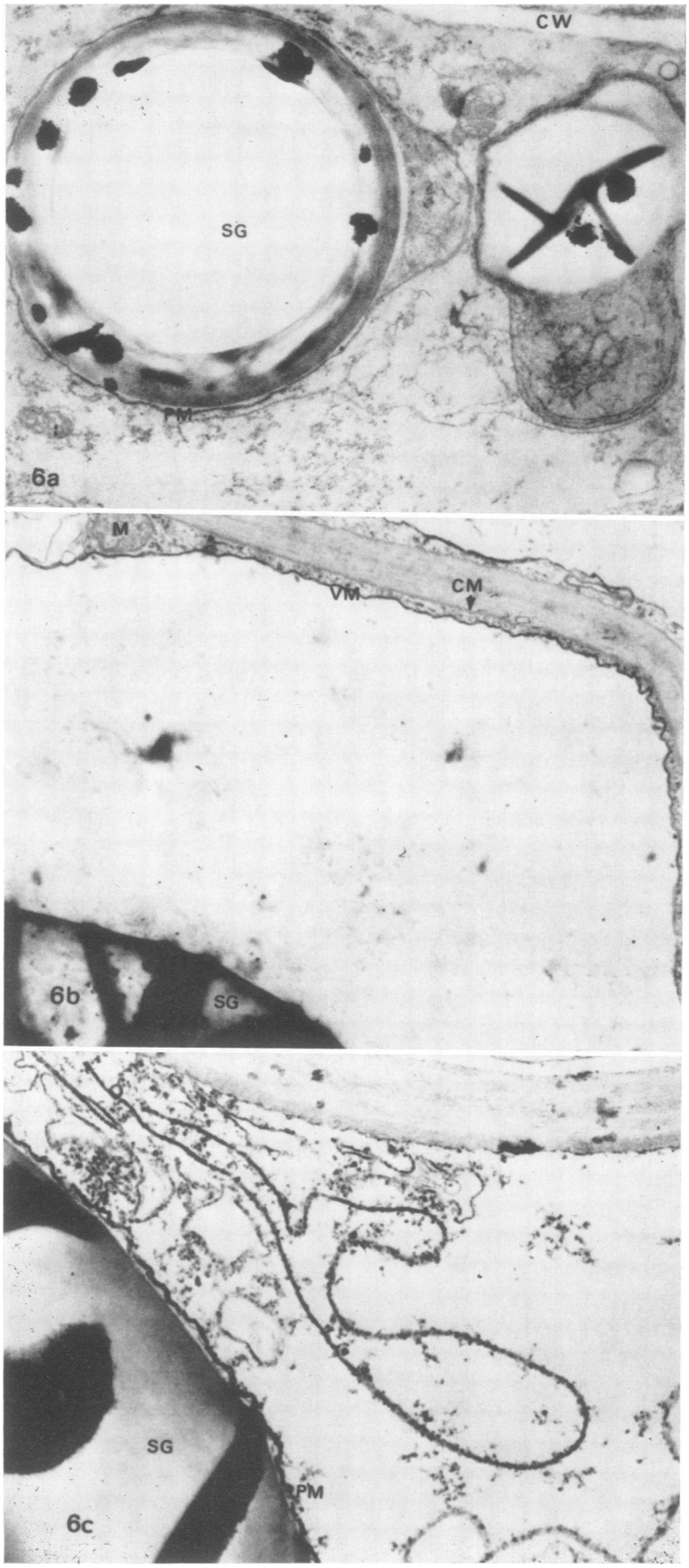

FIG. 6. Section through cells of potato tubers harvested 64 days after germination. a: Fresh potato tuber, immediately after harvesting. Starch granules (sg) are surrounded by apparently intact membranes (pm). cw: cell wall. × 10,000. b: The tubers were stored at 4 C for 31 days. No membranes are observed around the starch granules (sg), whereas the vacuolar membrane (vm), the cytoplasmic membrane (cm) and mitochondria (M) remained, apparently, intact. × 10,500. c. The tubers were stored at 25 C for 31 days. The membrane (pm) around the starch granule (sg) remained apparently intact. × 22,000.

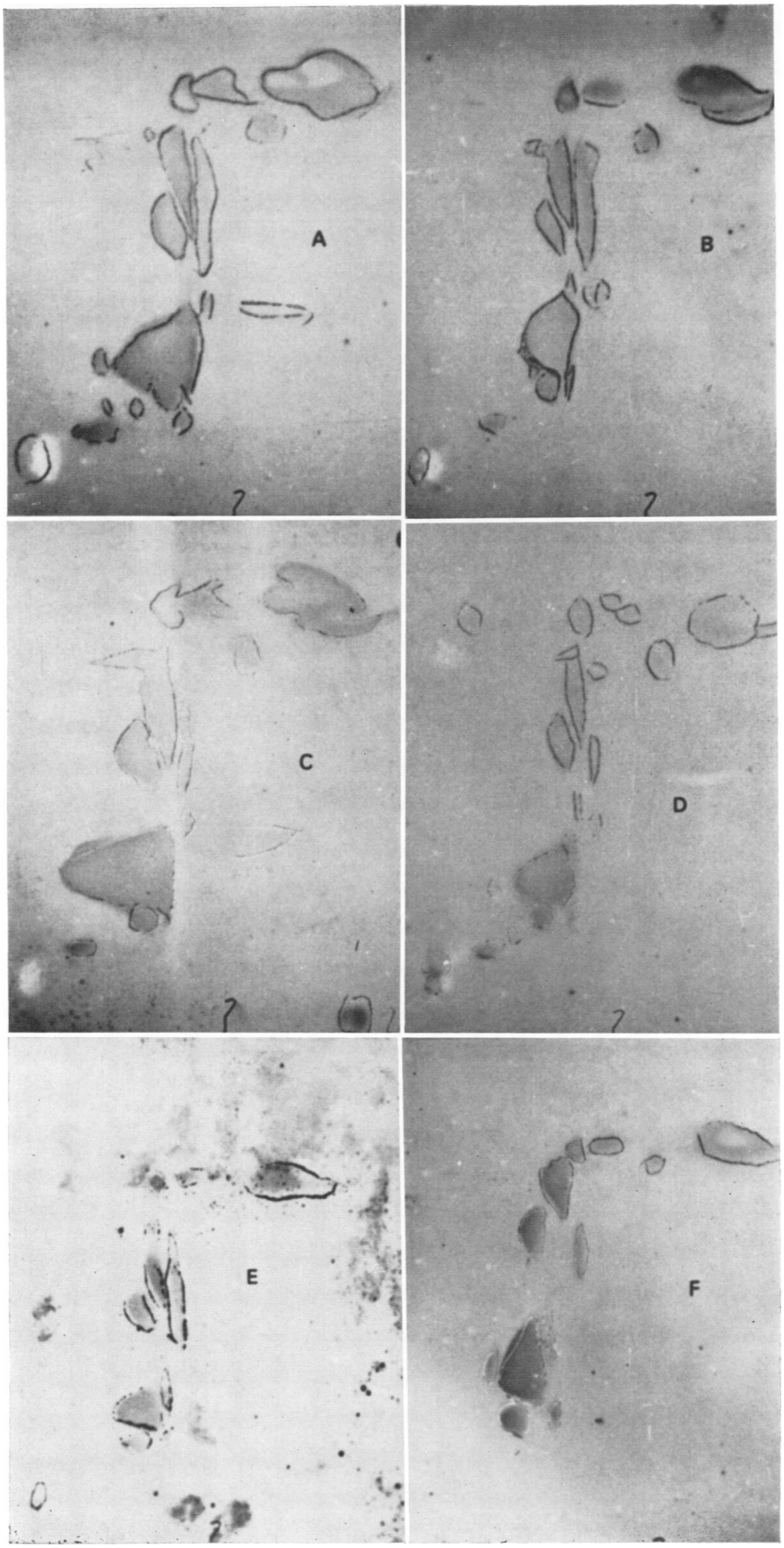

FIG. 7. Thin layer chromatographic pattern of total lipid extract of potato tubers as a function of age and storage conditions. A, B, C: Potato tubers were harvested 46 days after germination and analyzed at harvesting (A) and after 31 days of storage at 4 C (B) or 25 C (C) in air. D, E, F: Potato tubers were harvested 71 days after germination and analyzed at harvesting (D) and after 31 days of storage at 4 C (E) or 25 C (F) in air. For experimental procedure, see "Materials and Methods." The origin of each chromatogram is in the left lower corner.

Table I. *Sugar, Starch and Solid Content in Mature Potato Tubers as Function of Time and Storage Conditions*

Conditions of Storage		Sugar Content				Starch	Solid Content
At 25 C	At 4 C	Glucose	Fructose	Sucrose	Sum of sugars		
weeks		*% of dry wt*				*% of wet wt*	
0	0	0.01	0.08[1]	—	0.09	—	—
5		0.02	0.00	0.18	0.20	17.4	26.7
10		0.06	0.06	0.24	0.36	18.0	23.9
	5	0.46	0.44	0.22	1.12	14.2	25.8
	10	0.76	0.68	0.48	1.91	10.7	19.2
4[2] → 1		0.06	0.00	0.40	0.47	14.9	22.9
4 → 6		0.62	0.67	0.66	1.94	13.0	23.1
1 ← 4		0.41	0.33	0.00	0.75	15.8	24.3
6 ← 4		0.47	0.19	0.26	0.92	15.3	23.5

[1] Total fructose (including sucrose).

[2] Arrow indicates transfer from one set of conditions to another.

Changes in Starch Content. During storage at 4 C, the starch content of young potato tubers decreased markedly, resulting in a loss of 26% of the initial starch content after 17 days of storage. During maturation of the tubers this effect seems to be slightly reduced, the loss being only 19% after a similar storage period. On the contrary, during storage at 25 C, there were practically no changes in the starch content of the tubers.

Protein Content. The protein content of tubers of all ages varied between 2.8 and 4.2% of dry mass and was not affected by storage for a month at 4 C or 25 C.

Lipid Content. The relative amount of lipids decreased during growth of the potato tubers from 24.5 to 13.0 μeq of ester per dry weight, as expected from the increase of starch content. Fluctuations of the relative lipid content during the storage period were observed, but practically there was no significant change in the relative amount of lipids after 31 days of storage, neither at 4 C nor at 25 C.

Analysis of the lipid composition by thin layer chromatography showed that there were small changes in the relative amount of different lipids, but the same lipid constituents were found in tubers of all ages, before and after storage at 4 or 25 C (Fig. 7).

MORPHOLOGICAL AND CHEMICAL CHANGES IN MATURE POTATO TUBERS DURING STORAGE AT DIFFERENT TEMPERATURES

Storage of mature potato tubers at 4 C caused a reduction in starch content and elevation of free sugar level, whereas these effects were reduced markedly during storage at 25 C. The experimental results are shown in Table I.

Isolation of Membrane Fractions and Their Properties. Membrane fractions were isolated either from the whole tuber or from isolated starch granules during storage at different temperatures. Further fractionation of the membranes was performed by gradient centrifugation on Urografin and sucrose linear gradients. Nine bands differing in density were observed after centrifugation of the total membrane fraction of the tuber. Most of the material, however, was concentrated in two major bands (Fig. 8). The membrane fraction isolated from starch granules showed only one major band (Fig. 9), indicating the homogeneity of this fraction and its purification according to the buoyant density criterion.

The density of the main bands, containing the majority of the total membrane fraction, was different at different storage temperatures. The total membrane fraction of potato tubers stored at 4 C was heavier than that from tubers stored at 25 C (Fig. 8). On the other hand, the membranes of isolated starch granules from tubers stored at at 4 C were lighter than those from tubers stored at 25 C (Fig. 9). These density changes might indicate changes of the lipid to protein ratio in the membrane. Storage of potato tubers at 4 C causes a reduction in the lipid content in the whole membrane fraction, as compared to tubers stored at 25 C. The opposite was found in the membranes of the isolated starch granules.

Changes in the protein composition were examined by the gel electrophoresis method. Changes in the band pattern of proteins from the total membrane fraction were observed during storage period (from 21 to 75 days of storage, but no stor-

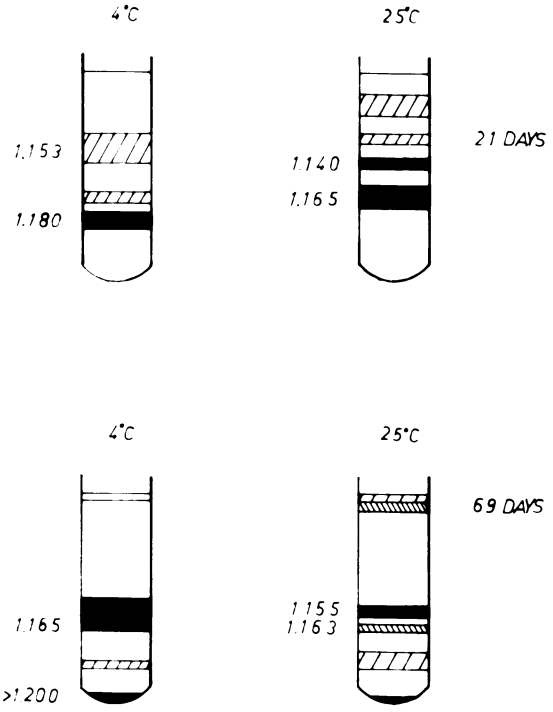

FIG. 8. The distribution of total membrane fraction from potato tubers on a linear, continuous density gradient. Membrane fractions of the tubers were isolated after 21 and 69 days of storage at 4 or 25 C and centrifuged as described in "Materials and Methods." The numbers indicate the average density of the band.

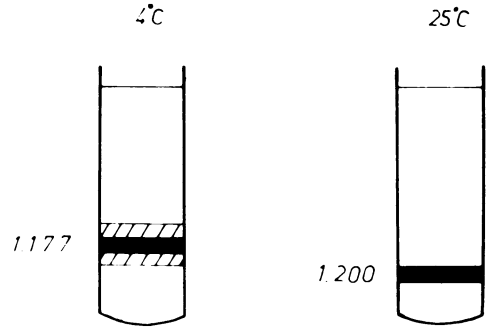

FIG. 9. The distribution of membranes from isolated starch granules on a linear, continuous density gradient. The membranes were isolated from starch granules obtained from tubers stored at 4 or 25 C for 36 days. Similar results were obtained after 54 days of storage. For experimental details see "Materials and Methods" and "Results." The numbers near the bands indicate the calculated average density of the bands.

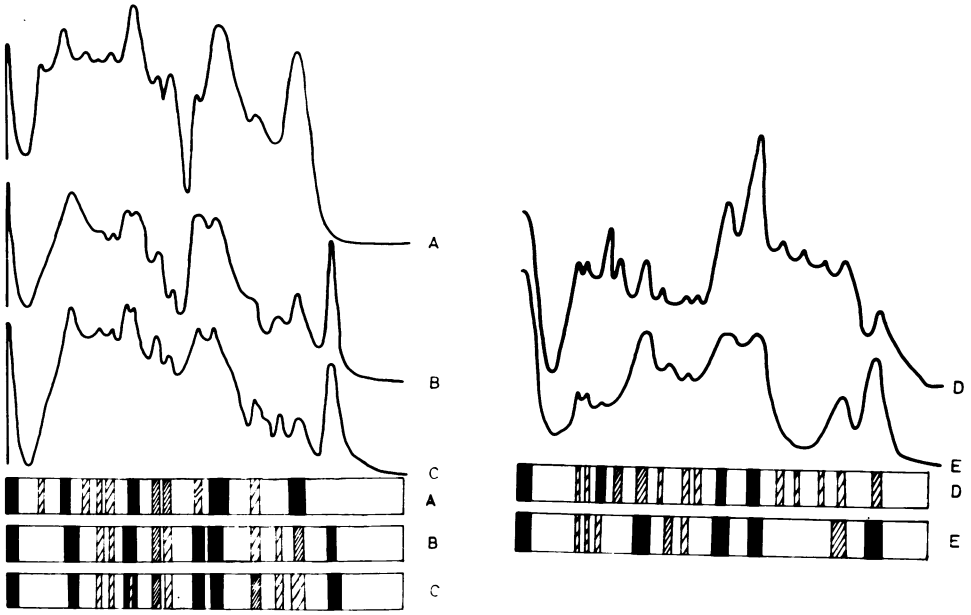

FIG. 10. Gel electrophoretic pattern of proteins from isolated membrane fractions. A, B, C: The total membrane fraction of potato tubers; D, E: membrane fraction of isolated starch granules. Changes in the electrophoretic pattern of the proteins from the total membrane fraction are induced as a function of storage time (compare A with B), but not as a function of temperature storage (compare B with C). On the other hand, changes in protein electrophoretic pattern induced by storage temperatures were observed in the membrane fraction of isolated starch granules (compare D with E). A: Samples from tubers stored for 21 days at 25 C; B: samples from tubers stored for 75 days at 25 C; C: samples from tubers stored for 75 days at 4 C; D: samples from tubers stored for 90 days at 4 C; E: samples from tubers stored for 90 days at 25 C. Storage conditions, preparation of membrane fractions, isolation of starch granules, and the procedure for gel electrophoresis were described in "Materials and Methods."

age temperature effects were observed (Fig. 10). The proteins obtained from the membranes of the isolated starch granules were markedly different from the proteins of the whole membranous fraction of the cell (Fig. 10). Differences were observed in the electrophoretic pattern of the proteins from the membranes of isolated starch granules of tubers stored at 4 C as compared to those stored at 25 C, after 91 days of storage (Fig. 10).

Morphological Changes. Intact membranes around the starch granules were discernible in fresh, mature potato tubers, when thin sections were examined with the electron microscope (Fig. 11a). When tubers were stored at 25 C during 30 days, most of the membranes around the starch granules remained intact (Fig. 11b). On the other hand, the starch granule membranes were fragmented and disappeared after storage at 4 C (Fig. 11c). The same effect was observed in tubers stored under nitrogen or 5% CO_2 in air at 4 C.

DISCUSSION

Of the procedures for the accurate determination of starch based on the isolation of pure starch, that of Hassid and Neufeld (14) is widely utilized. However, although accurate, this method necessitates many quantitative transfers of the material to be analyzed to different containers and is not too suitable when many analyses have to be performed within a short period of time. Methods in which starch is enzymatically solubilized (8) are more suitable for our purpose, since the specificity of the test is preserved while the number of steps necessary for the quantitative isolation of the material are considerably reduced. In our modification, carrying out the whole set of treatments in the same test tube avoids repeated transfers and inaccuracy due to loss of material. In addition, use of the heat-stable enzyme enabled us to perform both solubilization and degradation of the starch in a single test tube, thus avoiding the difficulties encountered when cooling of a concentrated starch solution might cause gelation and prevent accurate sampling and enzymatic degradation.

Although general methods for preparation of plant material for electron microscopy are well described and widely used (*cf.* 26), difficulties were encountered when we tried to prepare thin sections from potato tubers or isolated starch granules. The main difficulties lie in the fact that the potato tuber consists of a mixture of highly unhydrated dense material (cellulosic cell walls, starch granules) contained within a frame of highly hydrated cells with big water-filled vacuoles. The preparation of this tissue poses several problems: (*a*) penetration of the fixative and nonpolymerized embedding material within the cell wall and especially the starch granules; (*b*) preservation of the cytoplasmic structure and vacuole structure from mechanical damage due to the dislocation of the heavy and dense starch granule within the "empty" cell; (*c*) filling of the intracellular space with an embedding material of a hardness comparable to that of the starch granule in order to prevent chattering during the sectioning of the tissue blocks.

The fixation and embedding of isolated starch granules present a major problem: that of proper infiltration of the fixed granule with the unpolymerized plastic material. After trials of several variations of the basic methods we have chosen the method of fixation and embedding described in this work. This method so far has given acceptable results which, however, deserve further improvements.

The results presented in this work indicate that the starch granule at all stages of its development in the potato tuber is contained within the plastid membrane. The granules also continue to grow after the plastid has left the cytoplasm and entered the cell vacuole. The plastid matrix surrounding the starch granule, which is clearly seen at the early stages of development, is less evident in the mature tubers. It is possible

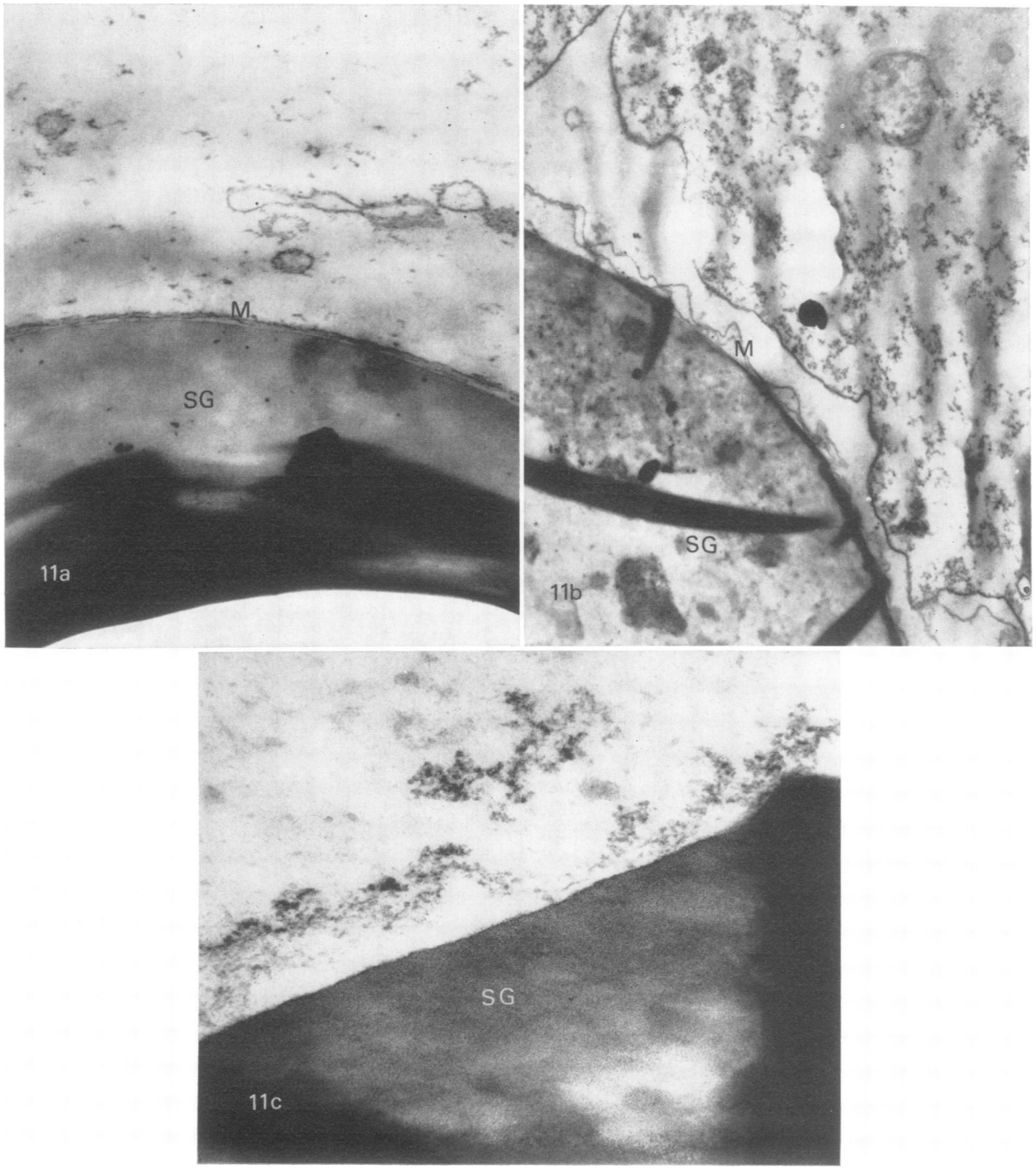

FIG. 11. Sections through cells of mature potato tubers. Changes in the morphology of the starch granule membranes as a function of storage conditions. a: Potato tuber 1 day after harvesting. The membranes (m) around the starch granule (sg) apparently intact. × 35,000. b. Same material as part a, but after 32 days of storage at 25 C. The membranes (m) around the starch granule (sg) are apparently intact, although they are slightly removed from the granule. × 11,300. c: Same material as part a, but after 12 days of storage at 4 C. Notice the disintegration of the membranes around the starch granule (sg). × 43,500.

that this apparent disappearance is concerned with the spreading of a constant amount of the matrix material around the increasing body of the starch granule.

Since the starch granule continues to grow inside the amyloplast (either in the cytoplasmic region or in the vacuolar space), one might expect that the synthesizing enzymes are contained at all times within the amyloplast membranes, which should be permeable at least to some of the substrates involved in starch synthesis, such as glucose, glucose-1-P, ADP-glucose, ATP, and pyrophosphate. The presence of the synthesizing enzymes within the amyloplast or in close association with the starch granule itself is not improbable in view of the fact that ADP-glucose transglucosylase was repeatedly reported to be associated with the starch granules (3, 6, 12, 13, 34). On the other hand, one might assume that the hydrolyzing and phosphorylyzing enzymes such as phosphorylase will be exterior to the starch granule (3). This possibility is again supported by the finding that phosphorylase in potato tubers was found as a soluble enzyme and is always recovered from the supernatant fraction of meshed tubers (17).

Storage at 4 C induced changes in the amyloplast membranes which appeared as changes in structural continuity as well as buoyant density and electrophoretic pattern of proteins. The structural damage which was evident after several weeks of storage in the cold and the changes in the sugar and starch content were found in both developing and mature tubers. Thus during storage of mature potato tubers at 4 C or at 25 C a correlation was found between reduction of the starch content, elevation of the free sugar level, changes in the properties and composition of membranes of the whole tuber and of the starch granules, and morphological changes of these membranes. Disappearance of membranes surrounding the starch granules during storage at 4 C might indicate that they have some role in the preservation and maintenance of the starch granule, possibly by preventing the contact between the enzymes involved in starch degradation and their respective substrates. The kinetics of these changes have not been studied yet. It is possible that structural damage will be detected after shorter periods of storage. However, it should be mentioned that absence of early structural damage during cold storage will not exclude the possibilty that permeability changes have already occurred before structural damage is visible in the electron microscope. Thus, our working hypothesis that permeability changes in the amyloplast membrane play a role in the cold-induced degradation of starch seems to be supported, so far at least, by circumstantial evidence presented in this work. One should also mention that this hypothesis implies that the cold-induced damage to the amyloplast membrane will show an "all or none" rather than "gradual" effect on the degradation of any particular starch granule. This situation will be averaged at the level of chemical determinations of starch and free sugar but will cause a certain degree of heterogeneity in the morphology of starch granules and their surrounding membranes when observed in electron microscopy.

Thus, in the same cell one could expect to find granules with an advanced degree of damage as well as intact amyloplast. The same will be true for tubers stored at 25 C, the difference being the change in the ratios of damaged to intact membranes.

It is not clear yet whether transfer of cold-stored tubers in which damage to the starch granule membranes has been already initiated to 25 C will allow the membranes to be repaired. If this were to occur, we should expect a cessation of further degradation of starch whereas degradation should continue at an increased rate if the cold-induced damage is not reversible. Data shown in this work (Table I) indicate that after such a transfer starch degradation continues but at a lower rate. In addition, a slight reduction in the relative content of free sugar is also observed which might be partially due to utilization through respiration.

Measurement of changes in the total lipid content of the whole tuber showed little variation for all the samples. Similar results were obtained when the composition was analyzed by thin layer chromatography. This result indicates that if there are changes in the lipid content they are expected to occur only in a fraction of the total membrane content of the tuber. In order to establish the role of the starch granule membrane in the process of formation and preservation of starch, it is proposed in the future to analyze in greater detail its structural, chemical and enzymatic properties and the kinetics of changes induced in these properties by different storage conditions.

LITERATURE CITED

1. AMES, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *In:* E. F. Neufeld and V. Ginsburg, eds., Methods in Enzymology, Vol. VIII. Academic Press, New York. pp. 115–118.
2. AVIGAD, G. AND Y. MILNER. 1966. UDP-glucose:fructose transglucosylase from sugar beet roots. *In:* E. F. Neufeld and V. Ginsburg, eds., Methods in Enzymology. Vol. VIII. Academic Press, New York. pp. 341–345.
3. BADENHUIZEN, N. P. 1969. The Biogenesis of Starch Granules in Higher Plants. Appleton-Century-Crofts, New York.
4. BADENHUIZEN, N. P. 1965. Occurrence and development of starch in plants. *In:* R. L. Whistler and E. F. Paschall, eds., Starch: Chemistry and Technology. Academic Press, New York. pp. 65–104.
5. BREGOFF, H. M., E. ROBERTS, AND C. C. DELWICHE. 1953. Paper chromatography of quaternary ammonium bases and related compounds. J. Biol. Chem. 205: 565–574.
6. CARDINI, C. E. AND R. B. FRYDMAN. 1966. ADP-glucose:α-1,4-glucan glucosyltransferase (starch synthetase and related enzymes) from plants. *In:* E. F. Neufeld and V. Ginsburg, eds., Methods in Enzymology, Vol. VIII. Academic Press, New York. pp. 387–391.
7. DITTMER, J. C. AND R. L. LESTER. 1964. A simple specific spray for the detection of phospholipids on thin layer chromatography. J. Lipid Res. 5: 126–127.
8. DONALSON, J. R. AND W. T. YAMAZAKI. 1958. Enzymatic determination of starch in wheat fraction. Cereal Chem. 45: 177–182.
9. DUBOIS, M., K. GILLES, K. HAMILTON, P. REBES, AND E. SMITH. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.
10. FISKE, C. H. AND Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375–400.
11. FOLCH, J., M. LEES, AND G. H. SLOANE-STANLEY. 1957. A simple method for the isolation of total lipid from animal tissues. J. Biol. Chem. 226: 497–509.
12. FRYDMAN, R. B. 1963. Starch synthetase of potatoes and waxy maize. Arch. Biochem. Biophys. 102: 242–248.
13. FRYDMAN, R. B. AND C. E. CARDINI. 1967. Studies on the biosynthesis of starch. II. Some properties of the adenosine diphosphate glucose: starch glucosyltransferase bound to the starch granule. J. Biol. Chem. 242: 312–317.
14. HASSID, W. Z. AND E. F. NEUFELD. 1964. Quantitative determination of starch in plant tissues. *In:* R. L. Whistler, ed., Methods in Carbohydrate Chemistry, Vol. IV. Academic Press, New York. pp. 33–36.
15. HUGGETT, A.St.G. and D. A. NIXON. 1957. Enzymic determination of blood glucose. Biochem. J. 66: 12P.
16. KEILIN, D. AND E. F. HARTREE. 1948. The use of glucose oxidase (Notatin) for the determination of glucose in biological material and for the study of glucose producing systems by manometric methods. Biochem. J. 42: 230–238.
17. LEE, Y. P. 1966. Potato phosphorylase. *In:* E. F. Neufeld and V. Ginsburg, eds., Methods in Enzymology, Vol. VIII. Academic Press, New York. pp. 550–554.
18. LEPAGE, M. 1964. The separation and identification of plant phospholipids and glycolipids by two dimensional thin layer chromatography. J. Chromatgr. 13: 99–103.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
20. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biochem. Biophys. Cytol. 9: 409–414.
21. MANNERS, D. J. 1962. Enzymic synthesis and degradation of starch and glycogen. Advan. Carbohyd. Chem. 17: 371–430.
22. MANNERS, D. J. 1968. The biological synthesis of starch. *In:* J. A. Radley, ed., Starch and Its Derivatives. Chapman and Hall, Ltd., London. pp. 66–90.
23. MEJBAUM, W. 1939. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsaure. Z. Physiol. 258: 117–120.
24. NELSON, N. 1944. A photometric adaptation of the Somogyi method for determination of glucose. J. Biol. Chem. 153: 375–380.
25. PAZUR, J. H. 1965. Enzymes in synthesis and hydrolysis of starch. *In:* R. L. Whistler and E. F. Paschall, eds., Starch: Chemistry and Technology. Academic Press. New York. pp. 133–176.
26. PEASE, D. C. 1964. Histological Techniques for Electron Microscopy. Academic Press, New York.
27. RAZIN, S., Z. NE'EMAN, AND I. OHAD. 1969. Selective reaggregation of solubilized mycoplasma-membrane proteins and the kinetics of membrane reformation. Biochim. Biophys. Acta 193: 277–293.
28. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208–212.
29. ROE, J. H., J. H. EPSTEIN, AND H. P. GOLDSTEIN. 1949. Photometric method for the determination of inulin in plasma and urea. J. Biol. Chem. 178: 839–845.
30. SIMS, R. P. A. AND J. A. G. LAROSE. 1962. The use of iodine vapor as a general detection in the thin layer chromatography of lipids. J. Amer. Oil Chem. Soc. 39: 232.
31. SMITH, P. 1967. Effect of transit and storage conditions on potatoes. *In:* W. F. Talburt and O. Smith, eds., Potato Processing. Avi Publishing Co., Westport, Conn. pp. 167–217.
32. SOMOGYI, M. 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160: 61–68.
33. STERN, I. AND B. SHAPIRO. 1953. A rapid and simple method for the determination of esterified fatty acids and for total fatty acids in blood. J. Clin. Pathol. 6: 158–160.
34. TANAKA, Y. AND A. TAKASHI. 1968. Substrate specificity of the granule-bound and chloroplastic starch synthetase. Plant Cell Physiol. 9: 405–410.
35. TERZAKIS, J. A. 1968. Uranyl acetate, a stain and a fixative. J. Ultrastruct. Res. 22: 168–184.