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A COMPARISON OF THE PROTEIN AND AMINO ACID COMPOSITION OF OLD AND RECENT BARLEY GRAIN

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Summary

The amino acid and protein contents of barley grain from archaeological sites dated between approximately 1000 and 3000 years B.C. have been compared with those of grain grown at Rothamsted between 1852 and 1977. The relative amino acid composition of the archaeological samples was remarkably similar to the recent grain, the main differences being decreased relative amounts of lysine and methionine. We were unable, however, to demonstrate the presence of salt-soluble proteins by electrophoresis or of hordein by electrophoresis or immunoassay. Salt-soluble protein and hordein fractions extracted from the oldest Rothamsted samples gave less clearly resolved patterns on electrophoresis and isoelectric focusing than fractions from recent grain, indicating some protein degradation. The rate of degradation was apparently faster for 'C' hordein than 'B' hordein polypeptides.

Scanning electron microscopy showed little difference in the relative amounts of large and small starch grains in the different samples. Transmission electron microscopy showed that although the cells of the starchy endosperm of the 1000 B.C. grain were partially disorganized, they did contain structures which resembled the protein bodies present in similar sections of recent grain.

Introduction

Recently discovered archaeological remains indicate that barley was used as a food source in the Egyptian Late Palaeolithic, some 17000 to 18000 years ago (Wendorf *et al.*, 1979). Although it is not known whether this grain was from wild or cultivated plants, it is by far the oldest recorded use of a cereal grain for human nutrition. What is more certain is that barley, together with wheat (emmer and einkorn) and a number of legumes, was cultivated in the near East (Iran, Syria, Palestine, Turkey) between 8000 and 9000 years ago (Harlan, 1976). Over the next few thousand years cultivation spread across Europe, Africa and Asia. Despite this widespread cultivation, however, archaeological finds of uncarbonized grain are comparatively rare.

Chemical analysis of such samples of barley and other cereal grain are also rare and the results contradictory. Zeven, Doekes and Kislev (1975) estimated that the maximum age for the demonstration of most types of protein in wheat seeds was between 125 and 175 years, but Derbyshire *et al.* (1977) reported the extraction and separation of protein fractions from maize grain dating from at least 700 years ago. The only chemical analysis reported for barley is that of Barton-Wright, Booth and Pringle (1944) who demonstrated the presence of riboflavin and nicotinic acid in carbonized grain from Tutankhamen's tomb (about 3300 years ago). So the suggestion by Derbyshire *et al.* (1977) that protein analysis might be a useful

criterion for archaeological studies and investigations of plant origin and evolution may sometimes be true.

In the present paper we report analyses of barley grain from Egyptian sites dated at approximately 1000 to 3000 B.C. and from British grain grown between approximately 1850 and the present day. These data suggest that although protein analysis can be used on relatively recent samples it is of little value for the study of early material.

METHODS

Sources of seed

Three samples of non-carbonized barley seed from Egyptian archaeological sites were supplied by Mr T. G. H. James and Dr A. J. Spencer, Department of Egyptian Antiquities, The British Museum. These were from sites dated at approximately 1000, 1900 and 3000 B.C. The 1900 B.C. sample was from a model granary while the 3000 B.C. sample was mixed with carbonized grain. Additional seed of barley from Egyptian and Nubian sites of the first and fourth millenia B.C. were supplied by Ms A. Ghaemi, The Oriental Institute, University of Chicago.

More recent barley seed was obtained from the Rothamsted Hoosfield Continuous Barley Experiment (1852 to present) while further samples dating from 1848 to 1889 were supplied by Ms R. Angel, Museum Division, Royal Botanic Gardens, Kew. The latter was mostly material which had been exhibited in nineteenth century agricultural exhibitions.

A control sample of barley cv. Julia was grown on Rothamsted Experimental Farm in 1977.

Extraction and separation of proteins

A modified Osborne procedure was used in which 1 g milled grain (0·2 g 1000 B.C. barley) was stirred for 3 × 30 min with 15 ml volumes of 0·5 M NaCl to extract salt-soluble protein (albumins and globulins) followed by 3 × 15 ml of 50% propan-1-ol containing 2% 2-mercaptoethanol to extract hordein (storage protein) (Shewry, Field *et al.*, 1980). Both extractions were at room temperature. The supernatants were bulked, dialysed against distilled water and lyophilized. Aliquots of the fractions were reduced and pyridylethylated (Cavins and Friedman, 1968).

Hordein was also extracted directly from single seeds, reduced and pyridylethylated as described previously (Shewry, Pratt and Miflin, 1978).

Proteins were separated either by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 17·5% acrylamide gels at pH 8·9 or by isoelectric focusing (IEF) in the pH range 3·5 to 10 in slab gels containing 5% polyacrylamide (Shewry, Ellis *et al.*, 1978).

Amino acid analysis

Samples of milled or ground grain or protein fractions were hydrolysed under N_2 in 6 N HCl for 21 h at 110 °C (Kirkman, 1974). Amino acids were determined either by gas chromatography of their *N*-heptafluorobutyryl *n*-propylesters or by ion exchange chromatography using a Technicon TSM-1 amino acid analyzer.

Immunological methods

Immunodiffusion was carried out in 1% agar in 0·01 M sodium phosphate buffer, pH 7, containing 0·85% NaCl (phosphate buffered saline, PBS). Hordein

samples were dissolved in 6 M urea–PBS (1 mg ml^{-1}) and tested against antiserum to total Risø 1508 hordein prepared as described by Festenstein and Hay (in preparation); immunodiffusion was allowed to proceed for 1 to 2 days at room temperature.

Electron microscopy

For scanning electron microscopy, dry barley seeds were nicked with a razor blade, fractured transversely and mounted on circular (9 mm diameter) specimen stubs. The samples were coated with a 20 to 30 nm layer of gold in a vacuum evaporator and viewed in a Cambridge Stereoscan IIA scanning electron microscope (SEM) at 5 to 10 kV.

For transmission electron microscopy seeds were imbibed in distilled water for 30 min at room temperature and then fixed for 3 h at room temperature with 2·5 % glutaraldehyde in 0·05 M piperazine-*N*-*N*′-bis(2-ethanesulphonic acid)–HCl buffer, pH 6·8. After post-fixing for 2 h in 1 % osmium tetroxide, the seeds were dehydrated through a graded acetone series and embedded in Epon. Ultrathin sections were cut using a Reichert Ultramicrotome OMU2, and stained in uranyl acetate and alcoholic lead citrate for viewing in a Phillips transmission electron microscope.

Results

Sources of samples

Although we tried a number of sources, we were only able to obtain old barley seed of two types. The first was from Egyptian and Nubian archaeological sites dated between approximately 1000 and 3000 B.C., the second from British agricultural exhibitions and experiments dating from approximately 1850 to the present day. Uncarbonized grain from the intervening period are very rarely found.

Three archaeological samples were selected for comparative analysis. All were Egyptian barleys from the British Museum, and they were dated at approximately 1000, 1900 and 3000 B.C. respectively. These were dark brown in colour and readily crumbled to a fine powder. They were compared with two samples from the Rothamsted Hoosfield continuous barley (cv. Chevallier from 1852 and cv. Halletts Pedigree Chevallier from 1902) and a control sample of cv. Julia grown at Rothamsted in 1977.

Amino acid composition of the grain

Amino acid analysis of the six samples (Table 1) showed a great similarity in composition, even between the oldest and most recent samples. There were, however, some differences, notably low lysine in the three Egyptian barleys and low methionine in the two oldest samples. These results agree with those of Derbyshire *et al.* (1977) who analyzed maize grain dating from at least 700 years ago. We also showed, however, that there were reduced proportions of arginine and glycine in the two oldest samples only.

The two samples from the Hoosfield experiment were similar in amino acid composition to the control sample, the only differences being slightly more arginine and less lysine in the 1852 sample. The significance of the former difference is not known, but the reduced lysine may represent the start of degradation of this amino acid. Thus it appears that under good storage conditions (the Hoosfield samples are stored in sealed jars at room temperature), amino acid

Table 1. *Amino acid analysis of old and recent barley grain*

	1977 A.D.	1902 A.D.	1852 A.D.	1000 B.C.	1900 B.C.	3000 B.C.
Asp*	6·0	6·1	5·8	6·8	4·6	4·1
Thr	4·3	4·3	4·2	5·0	5·3	4·2
Ser	6·4	6·0	5·8	5·7	5·7	4·1
Glu*	23·0	22·3	21·8	17·5	17·3	23·6
Pro	15·9	14·4	15·3	13·9	13·2	13·5
Gly	6·5	7·2	7·7	7·4	4·8	5·2
Ala	6·4	7·1	6·9	9·2	9·8	8·6
Val	6·6	7·1	6·6	8·3	9·6	10·1
Met	1·4	1·4	1·3	1·5	0·6	0·3
Ile	3·7	4·1	4·3	5·5	6·7	6·8
Leu	7·8	8·2	8·2	9·8	10·4	10·0
Tyr	2·1	1·9	1·9	2·0	2·6	2·2
Phe	4·8	4·8	4·7	4·8	5·8	5·1
Lys	2·6	2·7	2·1	1·0	1·4	1·4
Arg	2·6	2·4	3·5	1·6	2·0	0·8

Amino acids were determined by gas chromatography, His, Trp and Cys were not determined. Results are expressed as percentage mol. and are the mean of duplicate hydrolyses and analyses for the 1977, 1902 and 1852 samples. The results for the 1000, 1900 and 3000 B.C. samples are single analyses.

* Includes amides.

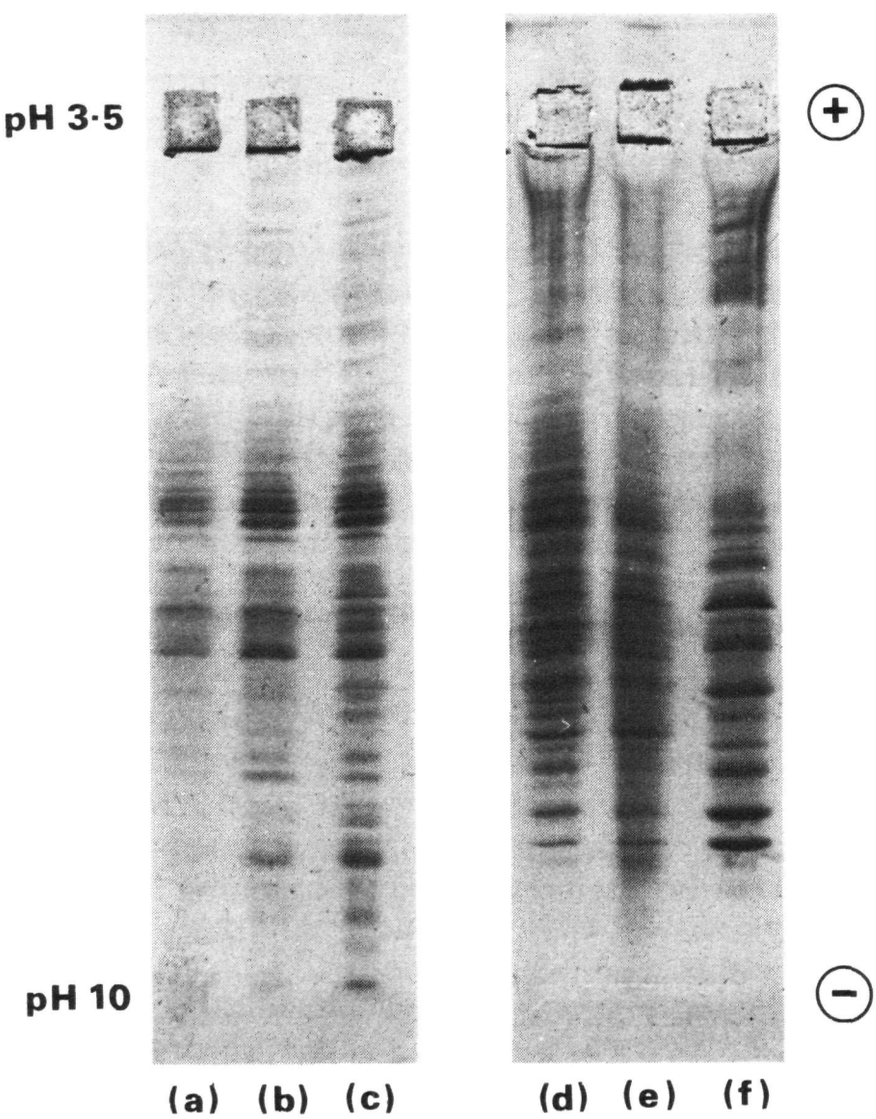


Fig. 1. Isoelectric focusing (pH range 3·5 to 10) of reduced and pyridylethylated protein fractions. (a) to (c) Salt-soluble proteins from grain harvested in 1852 (cv. Chevallier), 1902 (cv. Halletts Pedigree Chevallier) and 1977 (cv. Julia) respectively; (d) to (f), hordein fractions from grain harvested in 1852, 1902 and 1977 respectively.

degradation is very slow. The extent of degradation over longer time periods varies between amino acids, but even after approximately 5000 years the relative amino acid composition of the grain is still recognizable as that of barley. This is, to our knowledge, the oldest cereal seed sample which has been analyzed.

Extraction and analysis of grain proteins

The proteins of cereal seeds are usually classified into groups, termed Osborne fractions, on the basis of their extraction in a series of solvents. Such a procedure was used to extract protein fractions from the 1977, 1902, 1852 and 1000 B.C. samples. The first fraction, extracted with aqueous sodium chloride, is called salt-soluble proteins or albumins and globulins. This fraction from barley grain contains mainly metabolic proteins. The second fraction, which is extracted with aqueous propan-1-ol plus a reducing agent, contains storage proteins which are called hordein. Hordein is a mixture of polypeptides which vary in number, molecular weight and isoelectric point between genotypes (Shewry, Ellis *et al.*, 1978). These are usually classified into two groups, termed 'B' and 'C' hordein, and genetic analysis has shown that each group is coded by a single and probably multigenic locus (Shewry, Faulks, Pickering *et al.*, 1980; Jensen *et al.*, 1980). The hordein fraction also contains low mol wt polypeptides, often called 'A' hordein, which are probably not storage proteins. The residue after the extraction of salt-soluble proteins and hordein also contains proteins, commonly called glutelin, but in barley these are mainly structural and metabolic protein.

Reduction and alkylation of the protein sulphydryl groups often gives sharper band patterns on electrophoresis, probably due to the elimination of inter- and intramolecular disulphide bonds. This is especially important to obtain good separations of hordein. We therefore reduced and pyridylethylated the protein fractions and separated them by IEF in the pH range 3·5 to 10 (Fig. 1). With both types of protein the samples from the 1977 grain gave sharper patterns than did those from the 1852 and 1902 grain. The salt-soluble protein fractions from the three samples showed some differences in band pattern, notably a decreased relative amount of alkaline bands in the samples from the old grain. This may have been due to degradation of the basic amino acids in these proteins, or to decreased solubility on ageing. There were also differences in the bands of intermediate and low pI, which were probably due to differences in genotype (Shewry, Faulks and Miflin, 1980).

Hordein, however, shows greater genetic variability in polypeptide pattern and this was apparent in the fractions from the 1977, 1902 and 1852 samples. As well as being more diffuse, the fractions from the older grain had relatively less acidic polypeptides which corresponded to 'C' hordein.

No bands could be detected when the fractions from the 1000 B.C. grain were separated in the same way. Amino acid analysis of these fractions indicated that they were not comparable to those extracted from recent grain (Table 2). Thus the salt-soluble fraction had higher glutamate, proline and phenylalanine, low lysine and methionine, and resembled more closely the hordein fraction from the recent grain. The hordein fraction from the old grain appeared intermediate in composition between the salt-soluble and hordein fractions of the recent grain, with lower glutamate and proline and more lysine and methionine than the recent hordein. It is probable that partial degradation of these groups of proteins had resulted in changes in their solubility properties. In contrast, the residue after the extraction of the salt-soluble and hordein fractions had a similar composition in

all the grain, the main difference being reduced relative amounts of basic amino acids. This may indicate that these proteins are more resistant to degradation, which is in contrast to the results of Derbyshire *et al.* (1977).

We also extracted hordein fractions from single seeds of these and other samples using a procedure developed for the varietal identification of barley (Shewry, Pratt and Miflin, 1978). The reduced and pyridylethylated samples were separated by

Table 2. *The amino acid compositions of protein fractions from old (1000 B.C.) and recent (1977) barley grain*

	Salt-soluble protein		Hordein		Residual	
	Old	Recent	Old	Recent	Old	Recent
Asp*	6·2	9·2	7·1	2·4	9·3	9·6
Thr	4·0	4·9	4·3	3·2	5·3	4·6
Ser	4·4	5·8	5·1	4·7	5·5	5·8
Glu*	25·8	12·7	20·8	29·6	13·4	10·3
Pro	18·5	7·9	11·3	21·6	5·5	5·7
Gly	7·9	10·8	7·1	4·3	9·4	9·9
Ala	5·9	9·6	7·4	3·7	11·4	10·7
Val	5·5	6·4	8·0	5·8	7·9	7·2
Met	0·6	1·4	1·2	0·8	1·6	1·3
Ile	3·7	3·8	4·9	3·8	5·2	4·7
Leu	5·6	7·5	9·3	6·6	10·1	9·1
Tyr	3·2	3·1	3·0	3·0	2·7	2·7
Phe	4·6	2·7	3·9	4·8	4·6	4·1
His	1·0	2·2	1·6	1·9	1·4	2·4
Lys	1·0	5·4	1·6	1·0	2·7	6·4
Arg	2·0	6·4	3·3	2·6	3·9	5·5

Amino acids were determined by ion exchange chromatography. Cys and Trp were not determined. Results are expressed as percentage mol. and are the mean of duplicate extractions and determinations.

* Includes amides.

SDS-PAGE at pH 8·9. Figure 2 shows separations of fractions from Hoosfield barley from 1852, 1877, 1902, 1927 and 1952. In all these samples the 'C' hordein bands were less intense than those in the 1977 sample, and these bands became increasingly diffuse with age. This is consistent with the decrease in acidic bands in the IEF separations (Fig. 1). Although the N content of the seed may affect the ratio of B:C hordein (Kirkman, Shewry and Miflin, 1982), this did not vary greatly in the samples studied (for example the 1877, 1952 and 1977 samples all had approximately 13·6 mg N g^{-1} dry wt). Consequently the decrease in intensity and sharpness of the 'C' bands probably represents more rapid degradation or reduced solubility of this group of polypeptides. In contrast, the 'B' hordein group of polypeptides were still quite well resolved in the sample from 1852. The differences in the band pattern in this region were again due to genotype (see legend to Fig. 2). There was also an increasing proportion of unidentified material which did not migrate into the gel. Similar results were obtained with a number of other samples of grain from the Hoosfield experiment and from Agricultural exhibitions held between 1848 and 1899 (results not shown).

Separation of fractions from the 1000, 1900 and 3000 B.C. samples showed no distinct bands, instead stain-absorbing material appeared to be distributed throughout the gel with more in the low mol. wt region and a high proportion

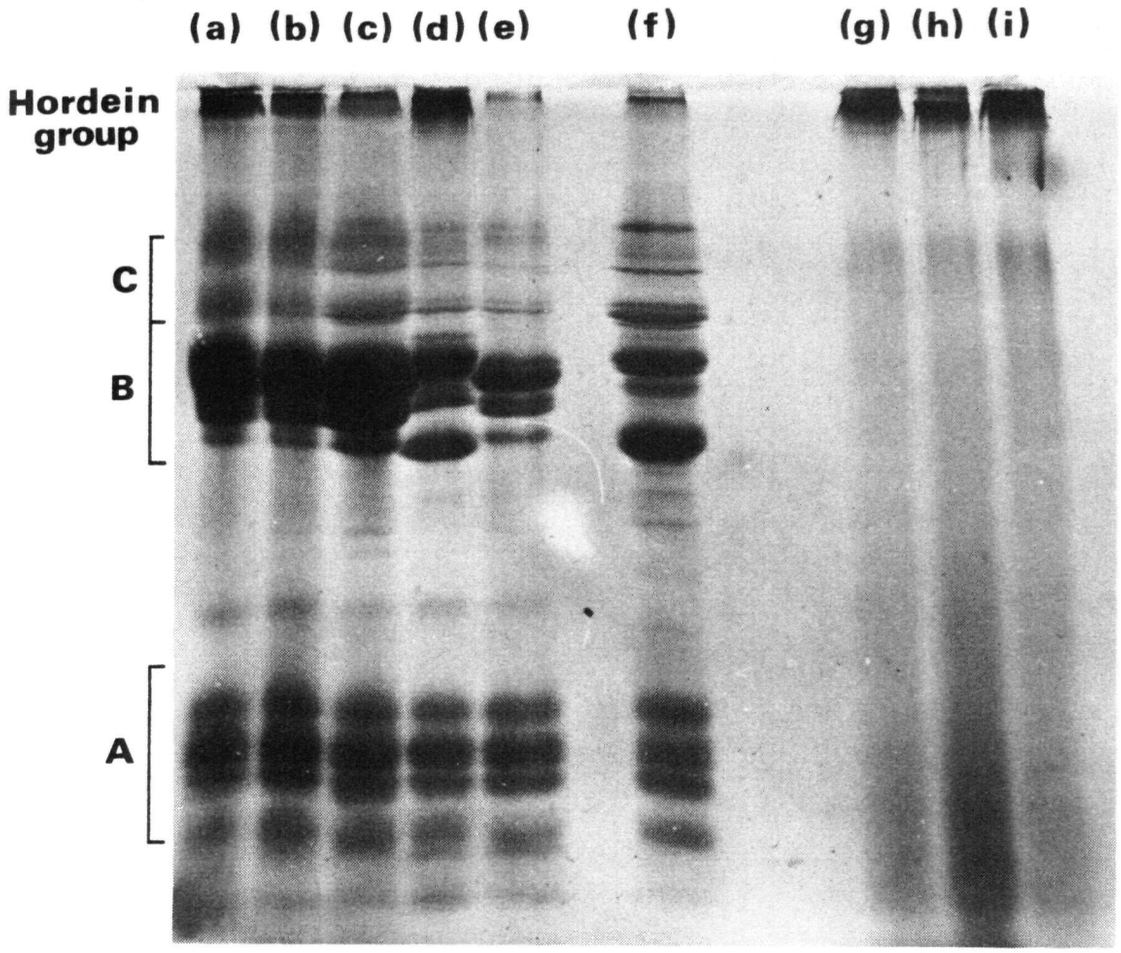


Fig. 2. SDS-PAGE of reduced and pyridylethylated hordein fractions extracted from single seeds of barley. (a) to (e) Seed from the Hoosfield continuous barley experiment harvested in (a) 1852 (cv. Chevallier), (b) 1877 (cv. Chevallier), (c) 1902 (cv. Halletts Pedigree Chevallier), (d) 1927 (cv. Spratt Archer), (e) 1952 (cv. Plumage Archer). (f) Seed of cv. Julia harvested 1977. (g) to (i), Barley grain from Egyptian archaeological sites and dated approximately 3000, 1900 and 1000 B.C. respectively.

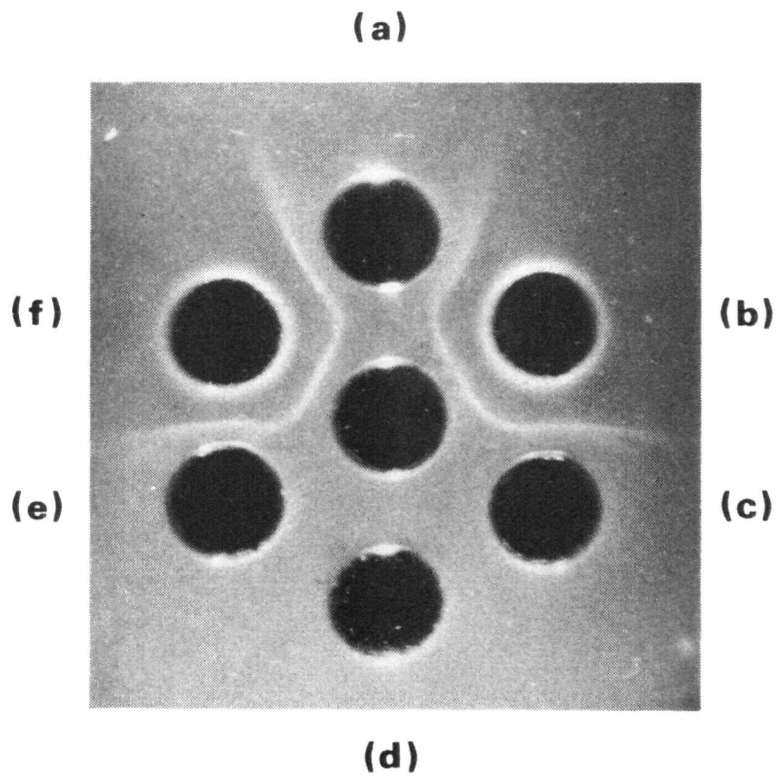
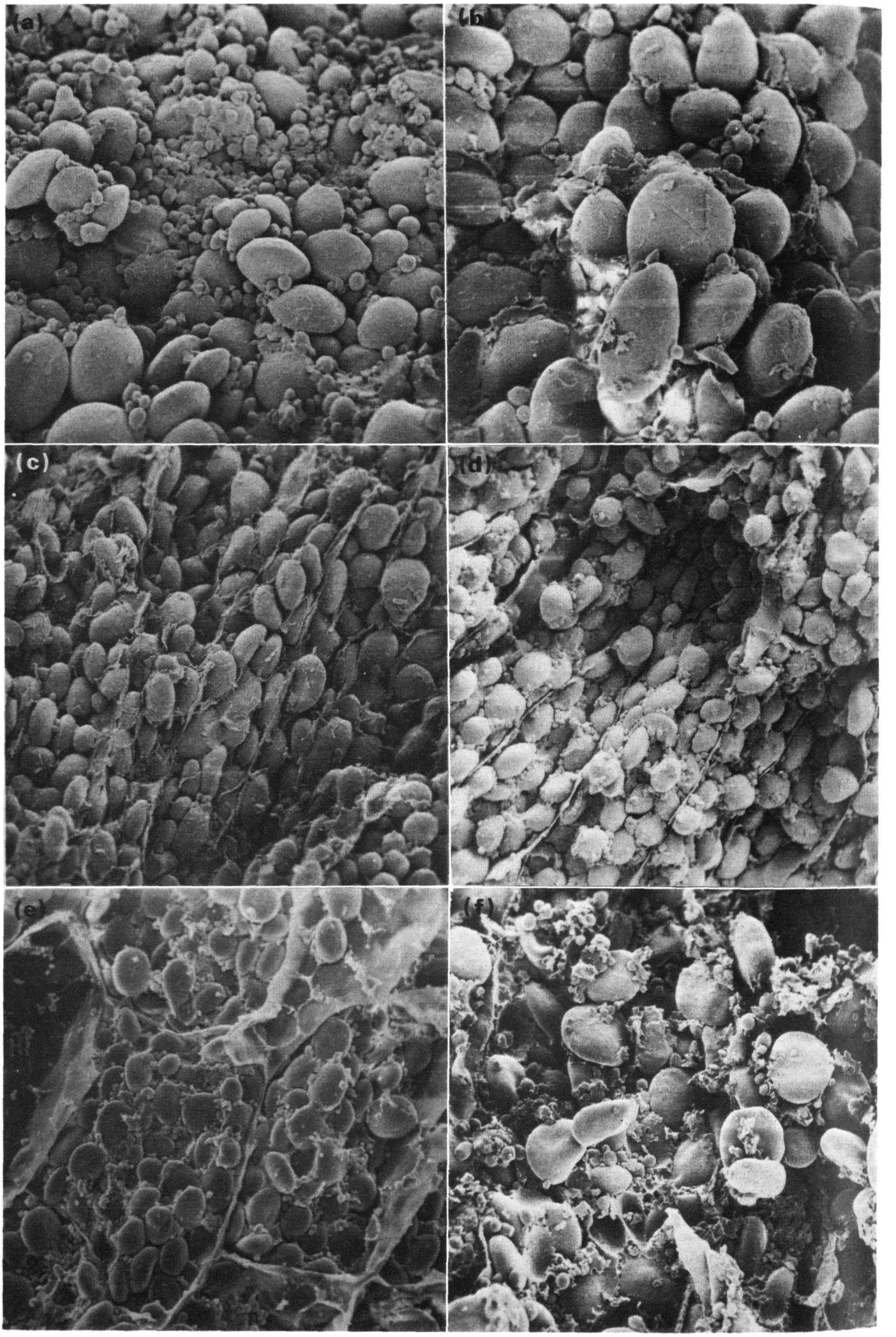


Fig. 3. Immunodiffusion of hordein fractions from old and recent barley grain. Centre well and wells (a), (c) and (e), antiserum against total hordein from Risø 1508; (b) hordein from 1852 grain; (d) hordein from 1000 B.C. grain; (f) hordein from 1977 grain.


(a)
(b)
(c)
(d)
(e)
(f)

of material which only just entered the gel. It is suggested that the proteins in these fractions were highly modified to give a continuous range of mol. wt. Similar results (not shown) were obtained with seed of comparable age from other Egyptian and Nubian sites.

Immunological determination of hordein

Although the hordein fractions from the 1977 and 1852 grain gave clear precipitin lines in diffusion tests with antisera raised against hordein polypeptides, no precipitation was observed in the hordein fraction from the 1000 B.C. grain (Fig. 3).

Fine structure of the grain

The structure of the central parts of the endosperms of the old and recent barley grain were compared by scanning electron microscopy (Fig. 4). This showed very little difference between the distribution and relative abundance of large and small starch grains in the different samples. It is not possible, however, to readily observe protein bodies using the SEM (Burgess *et al.*, 1982). We therefore prepared sections of 1000 B.C. and 1977 seed for examination by transmission electron microscopy.

Mature barley grains are very hard and dry so it is necessary to soak them in distilled water for a short period to facilitate penetration of the fixative. Even so the structures are generally less well preserved than in tissues with a higher content of water. Examination of the recent (1977) grain [Fig. 5(a)] showed the presence of numerous large and small starch grains between which were irregularly-shaped bodies which probably represented protein deposits. Examination of a similar section of 1000 B.C. grain [Fig. 5(b)] showed some disruption, notably of the cytoplasm and the cell wall, and also the presence of numerous small air-spaces between the starch grains. There were, however, some areas which resembled the protein bodies of the recent grain.

Discussion

Our results are in agreement with those of Zeven *et al.* (1975) who showed that both salt-soluble proteins and gliadins (the storage protein homologous to hordein) could be extracted and separated from grain of *Triticum* up to 125 years old. They also showed that although salt-soluble proteins could not be demonstrated after this time, gliadin-like proteins appeared to survive up to 175 years. We were, unfortunately, unable to obtain old barley grain to confirm this observation. In contrast Derbyshire *et al.* (1977) reported the presence of zein (storage protein) and glutelin-type proteins in maize grain dating from before 1300 A.D. It is possible that in this case the proteins were preserved for an unusually long period by the conditions of storage.

We were not able to detect proteins in the archaeological grain by electrophoretic

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Fig. 4. Scanning electron microscopy of the central endosperm of barley grain. (a) to (c) Grain from Egyptian archaeological sites and dated approximately 3000, 1900 and 1000 B.C. respectively. (d) to (e) Grain from the Hoosfield continuous barley experiment harvested 1852 and 1902 respectively. (f) Grain of cv. Julia harvested 1977. The large and small bodies are large and small starch grains (see Burgess *et al.*, 1981). Magnifications are (a) ×490, (b) ×975, (c) ×525, (d) ×490, (e) ×490, (f) ×450.

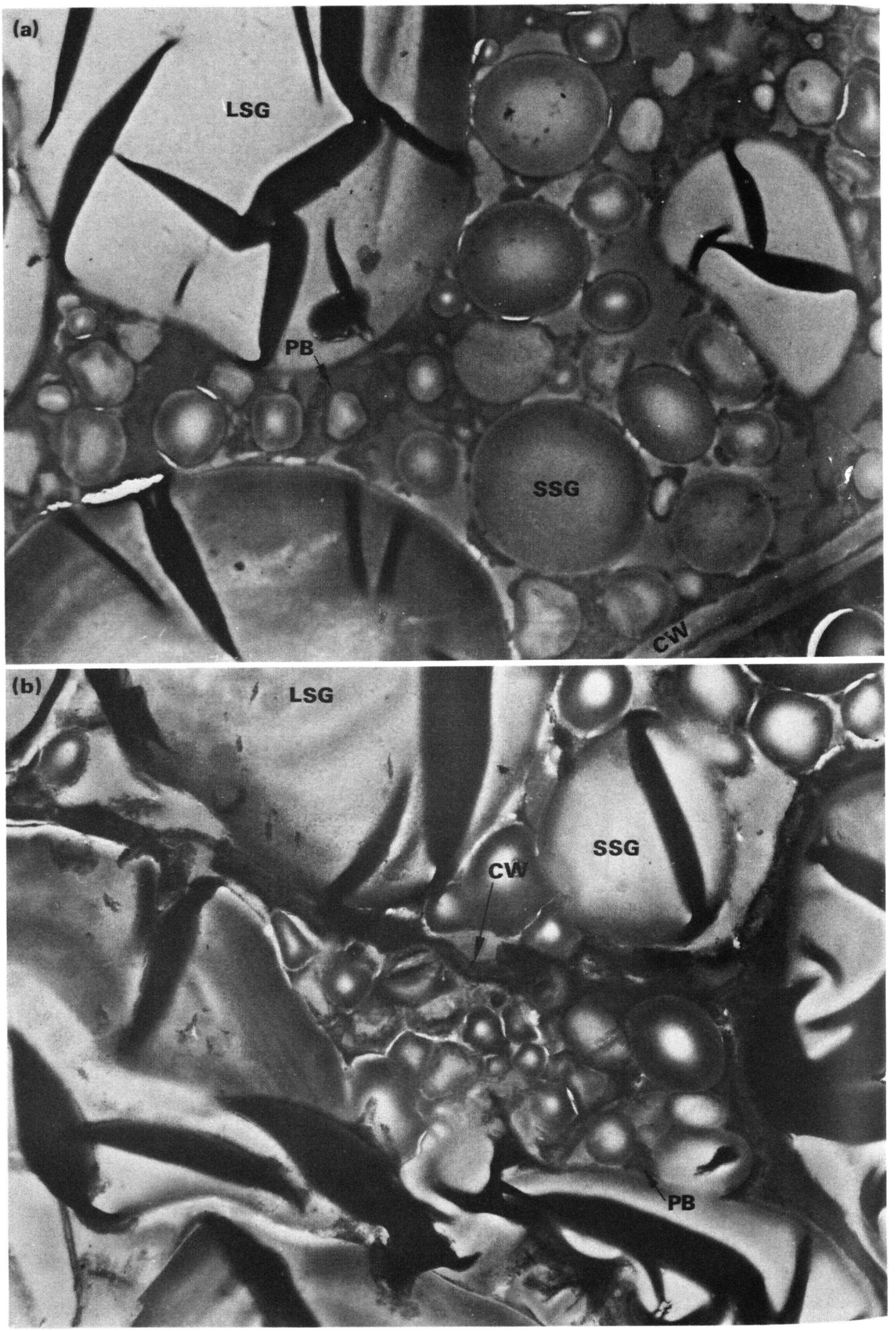

Fig. 5

analysis or, in the case of hordein, by immunoassay. The grain did, however, contain amino acids and the relative composition was remarkably similar to that of the recent grain. It is probable that they were present in partially degraded proteins. Such degradation would also account for their loss of antigenicity and their failure to give discrete bands on electrophoretic analysis and could also result in changes in solubility leading to the altered amino acid composition of the Osborne fractions. Palozzo and Jaffé (1965) made an immunological study of salt-soluble proteins in archaeological samples of *Phaseolus* and showed that antigenicity was only retained for approximately 1000 years, which is consistent with our results.

Transmission electron microscopy showed that although the subcellular structure of the 1000 B.C. grain was somewhat disorganized, it was still possible to recognize structures which resembled the protein bodies of the recent (1977) grain. The irregular shape of the bodies is characteristic of mature barley grain and results from their being squashed between the starch grain during the later stages of grain-filling and dehydration. Derbyshire *et al.* (1977) also reported the presence of numerous protein bodies in their old maize grain, although in this case they contained proteins recognizable as zein, the prolamin storage protein in this cereal. The protein bodies in our old grain probably contained a mixture of partially degraded proteins. Hallam (1973) reported a similar degree of preservation of the subcellular structure of embryos from wheat grain of a similar age to our barley, but he did not report similar studies of the endosperm cells.

Our results indicate that analysis of grain proteins is of limited value for identifying and dating archaeological samples, but further work on grain from the period between our older (1000 to 3000 B.C.) and recent (1852 to 1977) samples is necessary to confirm this.

Acknowledgements

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Fig. 5. Transmission electron microscopy of cells from the starchy endosperm of barley grain. (a) Grain of cv. Julia harvested 1977. (b) Grain from Egyptian archaeological site dated approximately 1000 B.C. Magnifications are ×4900. Abbreviations: SSG, small starch grain; LSG, large starch grain; CW, cell wall; PB, protein body.

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