HGCA

PROJECT REPORT No. 23

ABSOLUTE EVALUATION OF BARLEY FOR MALTING

AUGUST 1990

PRICE £10.00

HGCA PROJECT REPORT No. 23

Absolute evaluation of barley for malting

by

Head of section: **Dr. E. DENISE BAXTER**

Principal investigators: **Dr. M. O. PROUDLOVE**
Dr. N. L. DAVIES

Final report of a three year project at the Brewing Research Foundation (Malt and Wort Production Section), Nutfield, Surrey RH1 4HY. The work commenced in May 1987 and was supported by a grant of £138,003 from the Home-Grown Cereals Authority (Project No. 0031/1/87).

OVERALL SUMMARY OF PROJECT 0031/1/87

The overall objective of this project has been:

1. To define more closely the mixture of physiological and biochemical parameters which can affect the malting quality of barley, and which can currently only be assessed in their entirety by micro-malting.

and

2. having identified those factors which are most likely to be limiting in commercial barley varieties, to develop suitable small-scale tests which could be used for assessment; particularly under conditions when it is not feasible to carry out extensive micro-malting trials.

Areas of Investigation

Many aspects of grain physiology relevant to malting have been extensively investigated. For example, a considerable body of research indicates that even feed varieties are capable of producing ample quantities of hydrolytic enzymes, if given the correct stimulus. Other important parameters, such as endosperm structure, have also been examined, and suitable analytical techniques, for instance the milling energy test, have been developed.

Four aspects of grain physiology which are essential for germination were identified. In each case little was known concerning the range of variation likely to be encountered within

commercial varieties. In the course of this project each of these parameters has been evaluated for its likely contribution to malting quality.

The four aspects of grain physiology examined were:-

1. Distribution of water from embryo to endosperm.
2. Key respiratory enzymes.
3. Production and migration of gibberellic acid.
4. Response of aleurone cells to gibberellic acid.

The key findings in each area of investigation are summarised below:

1. **Distribution of water.** A technique has been developed whereby the extent of hydration of specific areas of the starchy endosperm can be measured from their reaction with iodine. The resulting purple colouration can then be assessed visually or measured very precisely by x-ray micro-analysis. The amount of water in the ventral endosperm close to the scutellum has been shown to relate to malting quality, with high grade barleys transporting more water into the endosperm from the scutellum than lower grade cultivars. This test requires only small amounts of seed, and could provide a useful technique for screening potential malting cultivars.

2. **Key respiratory enzymes.** Glucose-6-phosphate dehydrogenase

was chosen for investigation since this enzyme occupies a key role in controlling flux through the pentose phosphate pathway. Development of this enzyme in barley embryos during malting was found to vary between cultivars, but the differences appeared unrelated to malting quality.

3. **Production and migration of gibberellic acid.** Antibodies which react with natural gibberellins have been prepared. These antibodies can be used to locate endogenous gibberellins in tissue slices and to trace their movement and distribution during germination. The antibodies can also be used to measure levels of gibberellins in extracts of barley and malt. There is no strong correlation between malting quality and the level of gibberellins in the raw barley; there was a tendency for good quality varieties to contain lower levels of gibberellins than did feed varieties.

4. **The response of isolated aleurone layers to added gibberellic acid.** This was measured in terms of the production of alpha-amylase. Although feed varieties produced more alpha-amylase than malting varieties at high levels of added gibberellic acid, they were more sensitive to hormone concentration than were the malting varieties. Thus at lower levels of gibberellic acid addition (closer to likely endogenous levels) malting varieties produced more amylase than feed varieties.

The findings summarised above strongly suggest that the single most important physiological factor for discriminating between varieties of different malting quality is the flux of water from embryo into endosperm. A technique, utilising X-ray micro-analysis, has been developed whereby this flux can be readily quantified, and utilised in the assessment of new barley varieties.

TERMINAL REPORT

1. OBJECTIVES

The overall objectives of this project have been to investigate some of the physiological and biochemical parameters which might affect the malting quality of barley and to develop rapid, small-scale tests which could be used for assessing these parameters. Such tests could be used in conjunction with micro-malting, to give additional information, or as screening techniques in situations where micro-malting is not feasible; for example, in the earlier stages of barley breeding.

2. PRACTICAL APPROACHES

Attention has been paid to 4 specific aspects of grain physiology which might be expected to influence malting quality.

2.1 Water uptake during steeping.

2.2 Production of gibberellins.

2.3 Response of aleurone to gibberellins.

2.4 Activity of key respiratory enzymes.

3. MATERIALS AND METHODS

3.1 Measurement of Hydration by Scanning Electron Microscopy

Methods have been developed by which the hydration of specific areas of the barley grain can be measured, using either iodine vapour or manganese chloride as a tracer ion.

3.1.1 Material and Steeping Schedule

Barley grains (Hordeum distichon) were sieved and those within the size range 2.5 mm - 2.8 mm (in width), were selected. Grains were steeped in deionised water in glass jars at 17°C (250g barley plus 500 ml water) for various time periods. The water was then poured away and the grains maintained in air at 17°C in the same containers, which were covered with muslin to reduce evaporation (air rest period).

3.1.2 Localisation and detection of water

Two methods were used to locate the areas of hydration:

3.1.2.1 Iodine vapour method

Steeped grains were blotted dry and sectioned. The cut surfaces were exposed to saturated iodine vapour in a closed petri

dish for 60s. Only the hydrated areas reacted with iodine and stained purple.

X-ray microanalysis (XRMA) was used to detect quantitative differences in the amount of iodine present using a LINK KEVEX 290 analyser on a Philips PSEM 505 scanning electron microscope (SEM) operating at 25kV.

The sectioned grains were freeze-dried, attached to aluminium stubs, then carbon coated. Alternatively, to avoid the possible concentration effects of freeze drying, sectioned grains were attached to 3mm thick aluminium stubs using an equal volume mixture of Tissue Tek and colloidal graphite (Tissue Tek OCT compound, Mill Laboratories, USA), then plunge frozen in liquid nitrogen slush. Samples, maintained at -185°C to -180°C, were observed on an Oxford Instruments CT1000A cryosystem attached to the SEM.

X-ray peaks corresponding to iodine (L = 3.04keV, L = 4.22keV) were selected and, using the continuum method of quantification, the amount of iodine was determined.

3.1.2.2 Tracer ion method

To detect water in both the non-starchy embryo tissues and the endosperm a tracer ion was introduced with the steep liquor. A wide range of transition metal salts were tried (eg salts containing Cr, Mn, Fe, Co, Ni, Cu, Zu) since these have x-ray emission energies that do not overlap those of the normal biological elements. Only salts of manganese, copper, nickel and zinc penetrated into the endosperm. Manganese was chosen for its low toxicity compared to the others. It was introduced into the steep liquor at 10% (w/v) and the location and amount of water in the grain was detected by changes in the manganese peaks at 5.89keV and 6.50keV. Quantification was as described for the previous method. The units of hydration were expressed as P (counts in the area of the manganese peak) minus B (the background counts) divided by W (The counts in an area of part of the x-ray profile that corresponds to the volume analysed).

3.1.3 Standards for Quantitative x-ray microanalysis

Semi-quantitative analysis of the amounts of iodine or manganese in sectioned grains was

made by reference to resin standards. Salts of KI or $MnCl_2$ were added at concentrations of 50 to 200 mM to either a macrocyclic polyether complex in Taab resin or an aminoplastic complex.

Whilst both types of standards were useful for this study the aminoplastic complex was preferred since it had much better stability under the electron beam and had a background matrix (G factor, ($^{-2}$/A)) similar to biological material.

3.1.4 Specimen beam stability

To test specimen beam stability and the effect of beam exposure time on x-ray analysis profiles, the specimen beam current was measured for up to 1000 seconds with the SEM operating at 25kV and normal spot size. The experiment was conducted with specimens at the normal SEM stage temperature of 27°C and also with the cryosystem operating at -185°C. Measurements were averaged for longitudinal sections of five grains at the same site.

3.2 Extraction of gibberellin and estimation by HPLC

Gibberellins were extracted from barley flour as an

acidic-ethyl acetate fraction, as outlined in Figure 1. Barleys were milled with dry ice using a Buhler-Miag disc mill, setting 5. Inclusion of a Polyclar AT slurry at pH 7.0 improved subsequent partitioning between aqueous and organic layers, leading to less carry over of acid in the final residue and minimising the levels of oxidised polyphenols.

For detection by HPLC the residue was sonicated for 3 x 10 min in 1ml tetrahydrofuran, containing 1mg alpha-bromo-2'-acetonaphthone, 1mg 18-crown-6 and 2mg K_2CO_3, to form a gibberellin-acetonaphthone derivative which absorbed at 254nm. The tetrahydrofuran was evaporated under vacuum at 37°C and the final residue dissolved in a small, known volume of methanol. Separation of deriviatives was performed on a Nova-Pak C18 column (4mm spherical particles) using isocratic elution, in 80% (v/v) methanol, at 2ml min^{-1}.

3.3 Estimation of gibbrellin by ELISA

An ELISA (enzyme linked immunosorbant assay) techique relies on the specific reaction between an antibody and the compound to which it has been raised. Antibodies (Ab) were raised in rabbits against a GA_3-thyroglobulin conjugate and gibberellins detected by competitive ELISA (Fig. 2). A GA_3-Bovine Serum Albumin (GA-BSA) conjugate was bound to the wells of a polystyrene

microtitre plate. Concomitantly, a 1:1000 dilution of antibody-containing serum was incubated with a series of gibberellins or extract (acidic-ethyl acetate fraction) dilutions. The wells were blocked by unconjugated BSA (0.1% w/v) and the antibody-antigen mixtures pipetted into specific wells. The primary antibody was tagged with biotinylated goat-anti-rabbit secondary antibody which in turn was tagged with a streptavidin-horseradish peroxidase conjugate. Binding was determined from the catalysed oxidiation of o-phenylenediamine, using 4M H_2SO_4 to terminate the reaction, then measuring the absorbtion at 492nm (A_{492}).

The antibody reacted with pure gibberellins and gibberelllins extracted from Halcyon and Igri barleys. Control assays indicated the presence of an interfering factor which gave anomalous results at low dilutions. This was still evident with the varieties Triumph and Magie (Fig. 3) which do not contain high levels of anthocyanins in their aleurone cells. This interference has now been eliminated with the use of a Polyclar AT slurry stage in the extraction (see 3.2) and a linear response over a wide range of dilutions has been obtained. The accuracy of measurement has been further improved by including small, known amounts of GA_3 in each well of the ELISA plate. At the greatest dilutions, the absorption at 492nm tended

towards maximum absorption making calculations from these samples less accurate. When 2.5ug of GA_3 was added to each reaction well, the entire curve was shifted away from Bo (the A_{492} in the wells minus any gibberellin) and more reproducible measurements were possible (Figs. 4,5).

3.4 Immunolabellling of gibberellin in tissue slices

To identify the location of gibberellins in barley grains the secondary, goat-anti-rabbit, antibody was conjugated to latex spheres which could be visualised under the SEM.

Goat-anti-rabbit immunoglobulin G (100 $\mu g\ ml^{-1}$) was mixed with 225nm diameter latex beads (100 $\mu g\ ml^{-1}$) in 2ml of 10mM phosphate buffer, pH 7.0. Glutaraldehyde, 1% (v/v) in 10mM phosphate buffer, pH 7.0, was added dropwise to a final concentration of 0.1% (v/v) and the mixture stirred for 2h at 18°C. The reaction was quenched by adding 2.2ml of 0.2M glycine and dialysed overnight against 100mM phosphate buffer, pH 7.4, at 4°C. The conjugate was collected by contrifuging at 10,000g for 30 min and resuspended in 1ml phosphate buffered saline (PBS: 137mM NaCl, 27mM KCl, 10mM phosphate buffer, pH 7.4). Any aggregates which formed on storage were removed by centrifuging at 2000g for 10 min. To immunolabel the grain, samples

were taken of raw barley or green malt, freeze-dried at day 2 of germination. They were sectioned longtitudinally, washed, and non-specific sites blocked with 0.1M phosphate buffer, pH 7.0, containing 0.05% (w/v) BSA, by 3 x 5 min incubations. Sections were incubated in a 1500 fold dilution in PBS of primary antibody (15 min), washed with blocking buffer, PBS containing 0.05% (w/v) BSA, incubated with the goat-anti-rabbit antibody latex spheres conjugate (15 min) and stored in PBS for up to 30 min. Samples were then plunged into nitrogen slush (-196°C) and transferred to the SEM. After surface water had been etched off at -70°C, samples were gold coated and observed at -185°C.

3.5 Preparation and Incubation of Aleurone Layers

Aleurone layers were prepared from barley samples which were initially dehusked in 50% (v/v) sulphuric acid for 1.5-2h. The husk was removed by gently rubbing under running water and the grain was dried in air. Damage to the testa-pericarp-aleurone was assessed by immersing dehusked seeds in a solution of 24mM potassium iodide, containing 2mM iodine; those which showed characteristic blue-black staining with starch were rejected. Unstained seeds were rinsed, air dried, and stored in sealed glass jars until used.

Embryo and distal ends were cut off the dehusked seeds, and the remaining slices of endosperm were sterilised in 0.1% (w/v) sodium hypochlorite (0.01% (w/v) free chlorine), rinsed three times with sterile water and soaked in sterile water for 3 days at 20°C. Aleurone layers were isolated by splitting the rings and scraping off the starch, then incubated, in batches of ten, in buffer (0.5mM acetate, pH 5.7; 10mM $CaCl_2$) containing 0.5% (w/v) chloramphenicol, 0.5% (w/v) ampicillin and the desired concentration of GA_3.

At the specified times, the medium was decanted and the aleurone layers homogenised in 2ml buffer (100mM NaCl; 10mM $CaCl_2$; 50mM acetate, pH 5.7) using a pestle and mortar. The incubation medium and the supernatant produced by centrifuging the homogenised aleurones at 2000g for 5 min were assayed for alpha-amylase using beta-limit dextrin as substrate and 24mM KI, containing 2mM I, to develop colour and terminate the reaction. Decline in A_{607} was used to calculate enzyme activity.

3.6 Extraction and assay of glucose-6-phosphate dehydrogenase (G6P-dh)

Glucose-6-phosphate dehydrogenase activity was monitored from the reduction of $NADP^+$ (oxidised nicotinamide adenine dinucleotide) at 340nm. Grain samples from steeps, normally 50 seeds, were frozen in

liquid N_2 and stored at -20°C. To extract the enzyme, tissue was ground with cardice in an electric coffee mill and homogenized in 5ml 0.1M Tris buffer, pH 7.5, using an Ultra-Turrax blender at 24,000 rpm (3 x 5s separated by 5s rest periods). The homogenate was centrifuged at 500g for 5 min, the supernatant recentrifuged at 11,600g for 3 min and the final supernatant used as a source of the enzyme.

Respiration rate was measured as oxygen consumption by 20 seeds, from 4ml of air saturated deionised water, using a Clarke-type oxygen electrode (Rank Bros., Cambridge) linked to a chart recorder. The concentration of oxygen in steep water was measured at 16°C using an Orbisphere 2609 oxygen indicator and a flow-through cell coupled to a peristaltic pump working at 100ml min^{-1}.

3.7 Barleys

All barley samples used were screened over a 2.2mm sieve, except those used for the SEM, where only corns between 2.5 and 2.8mm were selected. Measurement of whole grain moisture, total nitrogen, viability and germination was carried out according to IOB Recommended Methods, and only those samples with good germination characteristics were selected.

Wherever possible, samples from NIAB variety trials were used for the direct comparison of varieties, in order to eliminate environmental effects which might have otherwise obscured true genetic differences.

4. RESULTS

4.1 Water uptake during steeping

Methods based on iodine vapour staining and manganese tracer ion uptake have been developed to establish the route by which water enters the starchy endosperm of barley grains. They have been used to assess and quantify differences between barley varieties in the rate of entry of water into the endosperm.

4.1.1 Route of water entry

Results have shown that water enters the grain mainly through the embryo but also with some distal entry (Fig. 6). This pattern of hydration was not due to water uptake along the ventral furrow (Fig. 7). Grains were steeped in 10% (w/v) $McCl_2$ for 6 hours and it was shown that the levels of hydration diminished steadily from the scutellum junction towards the distal end. The high level of hydration at the distal tip was considered to be due to direct entry of water. These data strongly suggested that water does not

enter via the ventral furrow, since hydration would in that case would have been similar at all parts along the ventral-distal transect.

4.1.2 Varietal differences in water entry into the endosperm

Initial measurements with three barley varieties indicated that there could be a relationship between malting quality and endosperm hydration (results not shown). The work was therefore extended to cover a wide range of varieties from a number of sites in England and Scotland. Grains were steeped in $MnCl_2$ at 20°C for 6 hours, then rested in air for 18 hours at the same temperature before being analysed for endosperm hydration. A point in the ventral endosperm close to the scutellar junction was chosen, as it was considered that the level of hydration at this point would reflect only the flux through the scutellum. It would therefore be influenced less by the endosperm structure (for example, by the amount of nitrogen in the grain) than areas deeper within the endosperm itself. The results shown in Fig. 8 indicated that good malting varieties, with a NIAB malting grade of 9, redistributed significantly more water from the embryo into the endosperm than did the varieties with lower

malting grades.

In order to eliminate possible environmental effects, and to establish the extent to which grain nitrogen might influence the observed trends, two sets of barleys were selected from the 1988 NIAB variety trials. This enabled a comparison to be made between several varieties grown at the same site which had fairly similar nitrogen contents, and the same set of varieties grown at a different site, where all the nitrogen contents were substantially higher (see Table 1). As before, grains were steeped for 6 hours at 20°C in $MnCl_2$, air rested for 18 hours, then the same position in the ventral endosperm close to the scutellar junction was analysed for each sample. Fig.9 shows the hydration of the ventral endosperm (measured as described above) plotted against the malting grade for both sets of barley. There is clearly a straight line relationship between hydration level and NIAB malting grade which does not appear to be affected by the barley nitrogen. This has been confirmed in a second series of experiments where several samples of a single variety, Magie, grown at a single site (Rothamsted Experimental Station) in one year, but with widely differing nitrogen contents, were selected. In these experiments the barleys were steeped twice to allow more water to penetrate into the

endosperm, so that hydration could be assessed further into the endosperm as well as adjacent to the scutellum. The results, shown in Fig. 10, indicated that hydration of the ventral endosperm close to the scutellum was not affected to any significant extent by the level of barley nitrogen. The hydration of the endosperm just 1mm away from the scutellum was noticeably lower, as was expected, and was considerably more variable, but again there was no noticeable effect due to the nitrogen content.

These results strongly suggest that measurement of hydration of the ventral endosperm could be used to assess the malting quality of new barley varieties. Provided that the position analysed is close to the scutellar junction, differences in hydration appear largely unaffected by environmental influences such as the amount of nitrogen in the grain. It may be predicted, however, that hydration of other parts of the endosperm further away from the scutellum could be profoundly affected by factors such as nitrogen content.

The data shown in Figures 8 and 9 indicated that the test could be used to differentiate between varieties of substantially different malting

grades. Analyses of three new barley varieties, all provisionally considered by NIAB to have good malting quality, further suggested that the technique could be used to discriminate between varieties with similar, high malting grades. Thus Fig. 11 shows that differences in hydration could be detected in samples of Alexis, Nomad, Clarine, and a control sample of Triumph grown on the same site. These differences also corresponded broadly to differences in Hot Water Extract.

4.2 Production of Gibberellins

The application of exogenous gibberellic acid during malting is known to enhance modification for many barley varieties. Possible reasons for this are:

- the embryo may not produce enough endogenous gibberellin

- transport of gibberellins from embryo to aleurone may be limiting

- the response of the aleurone to stimulation by gibberellins may vary between varieties.

4.2.1 Extraction and estimation of gibberellin

Gibberellins from barleys and malts were extracted into an acidic-ethyl acetate fraction. Details of the method, including homogenising in methanol, aqueous/non-acqueous separations at high and low pH and the inclusion of a Polyclar AT slurry stage, are given in the Materials and Methods Section, 3.2. This resulted in clean extracts, containing little oxidised polyphenolic material, and gave a single peak on HPLC.

Data for the amount of gibberellin extracted from a range of Spring barleys, and estimated by HPLC and by ELISA, is shown in Figs. 12 and 13.

A significant difference was observed between the actual values obtained by the two methods, with the ELISA results being one order of magnitude lower. This may reflect the greater specificity of ELISA compared to HPLC. Values quoted in the literature come from a range of techniques, including bioassay, ELISA and GC-MS, which gave different endogenous levels. The data presented here may reflect a broad specificity for the polyclonal antibodies used in this study. They could have also recognised, and reacted with, gibberellin precursors and breakdown products and

hence have given higher results than would have been expected for endogenous gibberellins alone.

HPLC has been used by some workers as a clean-up procedure prior to final identification and quantification using GC-MS. This process is very time consuming but offers, in all probability, the most accurate method of estimating gibberellins by chemical means.

Although the actual values differed, both HPLC and ELISA gave a broadly similar ranking for the varieties in terms of gibberellin content. There was no obvious correlation between malting grade and level of endogenous gibberellins. With the exception of Blenheim, however, the better malting varieties appeared to contain less gibberellin than did the feed varieties. This is an interesting observation and will be discussed in more detail in the section dealing with the response of aleurones to gibberellic acid.

4.2.2 Detection of gibberellin in tissue slices using immunolatex labelling

Gibberellin bound to a plastic strip can be visualised under the SEM by conjugating latex beads of a known size to a secondary antibody

(results not shown). These methods have now been used to investigate the distribution of gibberellins in barley grains during malting.

In dry grains, antibody was found to be associated only with the scutellar epithelium (Fig. 14). There was no evidence of any binding in any other part of the grain. With grain which had been steeped and germinated for 2 days, however, antibody could clearly be seen binding to sections of the aleurone cells (Fig. 15) in addition to the scutellum and the scutellar epithelium.

4.3 Response of aleurone cells to added gibberellic acid

Aleurone layers were isolated from a number of Spring barleys and incubated in gibberellic acid (GA_3). The alpha-amylase activity was then measured in both the aleurone layers and the incubation medium.

Total alpha-amylase produced after incubation in 10^{-5}M gibberellic acid is shown in Fig. 16. As expected there was an initial lag period (24 hours) when little enzyme was produced, followed by large time-dependant increases in enzyme activity. The lag period represents the response time of the aleurones, and involves both RNA and protein synthesis (Jones, R.L. & MacMillan, J. (1984). Gibberellins. In "Advanced Plant Physiology", (Wilkins, M.B. ed.) pp 21-52.

Pitman, London). With this concentration of gibberellic acid the malting varieties, Blenheim and Triumph, produced significantly less alpha-amylase than did the feed varieties Digger and Klaxon.

Two varieties, Digger (Malting Grade 1) and Blenheim (Malting Grade 9) were then incubated in gibberellic acid at a range of concentrations. The results shown in Fig. 17 indicate that synthesis of alpha-amylase by Digger aleurones decreased markedly at lower concentrations of gibberellic acid. At 10^{-8}M and 10^{-9}M GA_3, virtually no enzyme was produced even after 96 hours incubation. With Blenheim, however, the production of alpha amylase was greater at low GA_3 concentrations, suggesting that higher levels are supra optimal, and may inhibit production of enzyme in this variety (Fig 18). This is supported by the general tendency of better malting varieties to produce less gibberellin (measured by both HPLC and ELISA) than feed varieties, although the high level of gibberellin detected in Blenheim by HPLC remains anomalous here.

4.4 Activity of Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6P-DH) was chosen for investigation because it is thought to control the flux of respiratory substrates between glycolysis and the pentose phosphate pathway.

Preliminary investigations into the activity of G6P-DH in winter barleys suggested that levels in the malting varieties Halcyon and Maris Otter were higher than in the feed varieties Igri and Panda. A wider range of spring varieties grown at the same site over three years, has been examined and the results are presented in Table 2. These showed that there was no correlation between malting grade and the level of G6P-DH in dry barley seeds.

Enzyme activity was also measured in samples withdrawn from steeping. Once again there was no apparent correlation between malting grade and enzyme activity. Figure 19 A and B shows the results obtained with a range of varieties from one site, steeped using a highly aerobic steeping schedule of 7h wet, 17h air rest, 7h wet, 17h air rest and 1h wet.

It is interesting, however, to compare the activity of G6P-DH with the availability of oxygen in the steep (Fig. 20). Decrease in available oxygen, which occurred more rapidly during the second steep, appeared to coincide with an initial decrease in G6P-DH activity, although levels later recovered. The increased availability of oxygen during the air rest was not associated with a permanent increase in G6P-DH activity. Results shown in Fig. 21 however (where a less aerobic schedule was used, and measurements were

also taken during the air rest periods) indicated that there was an initial increase in activity at the start of the air rest period.

Further investigations of G6P-DH and related respiratory enzymes may be of benefit in the context of optimising malting schedules. This enzyme does not, however, appear to play a role in determining malting quality.

5. DISCUSSION AND CONCLUSIONS OF OVERALL PROJECT

This project has studied the relationship between malting quality and four specific aspects of barley physiology. The results indicated that one in particular, the measurement of ventral endosperm hydration at the scutellar junction during steeping, could form the basis of a rapid test for malting quality. This could be used both to select potential malting varieties during breeding, and to discriminate between newly available varieties with similar high malting grades. Only very small quantities of grain are required, which is a particular advantage during breeding. The test appeared to be independent of barley nitrogen, provided that measurements were confined to the ventral endosperm close to the scutellum, as specified.

The technique could also find wide application in assessing the evenness of hydration across the whole endosperm, since

this factor is crucial for the production of good quality malt and can be affected both by malting schedules and by environmental influences. In the initial stages of steeping useful information can be gained by visual inspection of cut grains after exposure to iodine. This colour reaction is, however, soon saturated, and at the later stages of steeping some means of quantification is necessary. While the scanning electron microscope and x-ray micro-analysis used to develop the technique might be available to those plant breeders who are part of large international companies, such equipment would not be available or appropriate for commercial maltsters. It would therefore be desirable to transfer the technology to simpler instrumentation, such as a light microscope, possibly with a photo multiplier as a detector.

The development of a polyclonal antibody to gibberellic acid has allowed endogenous gibberellin to be located and visualised inside the barley grain. This should provide a valuable tool for fundamental studies of barley germination in relation to malting quality. Further work is required to determine whether it could form the basis of a test for malting quality. The antibody can also be used to measure the amount of natural gibberellin in barley or malt samples. The ELISA technique is relatively simple and does not require expensive instruments such as HPLC. The method does, however, still require a lengthy extraction step and is therefore unsuitable for routine use in its present form.

It may be possible to use the antibody directly with crude extracts but further studies would be necessary to develop this technique and to confirm the specificity of the antibody.

Aleurone layers isolated from a range of different barley cultivars all responded to the addition of gibberellin by producing alpha-amylase activity. Their patterns of alpha-amylase production, over a 96h incubation, were all similar; a rapid increase in activity was observed after 48h incubation. There did appear to be an inverse relationship between malting grade and alpha-amylase activity at 10^{-5}M GA, cultivars Blenheim and Triumph (NIAB grade 9*) producing less enzyme than Klaxon (4), Regatta (3) and Digger (1), respectively.

When alpha-amylase activity was measured in the two cultivars Digger and Blenheim with decreasing levels of gibberellin in the incubation, different patterns were observed. Production of alpha-amylase by Digger was proportional to the gibberellin concentration, being greater at 10^{-5}M than at 10^{-9}M, but, for Blenheim, enzyme production was greater at lower gibberellin levels. This may have been caused by inhibition of alpha-amylase production at 10^{-5}M gibberellin. These results can be used to explain why malting grade barleys do not need exogenous gibberellin in order to produce hydrolytic enzymes, represented here by alpha-amylase, and so modify the endosperm. If endogenous

levels are below the optimum for feed grade barleys, addition of extra gibberellin might be expected to ennance modification. This is indeed the case. Future work in this area could extend these studies to the affect of gibberellin concentrations on other GA-linked hydrolytic enzymes, for example beta-glucanase.

The activity of the enzyme G6P-DH does not reflect the malting quality of barley either in the dry seed or during steeping. It may, however, be a useful parameter to monitor when designing steeping schedules, particularly its variation in activity during immersion periods.

Figure Legends

Table 1 Total nitrogen (TN) and moisture levels in a variety of winter barleys grown at two different sites and harvested in 1988. Bridgets was deemed the 'low nitrogen' site and Sutton Bonnington the 'high nitrogen' site.

Table 2. Levels of G6P-DH activity in spring barley varieties over three consecutive harvests. Grain was from the same growing site in 1986 and 1988 (Headley Hall) and from Meyerscough in 1987.

Figure 1 Extraction of gibberellins:
Procedure for an acidic-ethyl acetate fraction from barley samples.

Figure 2 Scheme for competitive ELISA to determine GA. Low [GA] in step 2 means more Ab binds to well and more coloured product is formed. At higher [GA], less Ab binds and less colour is formed. Details of the methods are included in the text.

Figure 3 ELISA of barley extracts:
Microplate well absorbance values for binary dilutions of a standard GA_3 solution and acidic-ethyl acetate extracts from Magie and Triumph barleys.

Figure 4 Effect of extraction procedure on ELISA response for binary dilutions of GA_3.

Figure 5 Improved response from ELISA when GA_3 was added to wells including binary dilutions of a Triumph acidic-ethyl acetate extract.

Figure 6 Hydration of barley endosperm visualised by iodine staining. Dry barley (0h), steeped barley (6h) and steep and air-rested barley (6,18h) were cut and exposed to iodone vapour for 60s. Dark areas show hydrated starch with iodine.

Figure 7 Hydration pattern along barley ventral furrow. Barley was steeped for 6 hours.

Figure 8 Varietal differences in water distribution during steeping. Hydration at the ventral scutellum for a range of barley varieties. Seeds were sampled after 6h wet, 18h air-rest.

Figure 9 Relationship between water distribution at the ventral endosperm and NIAB malting grade.

Figure 10 Effect of sampling position and nitrogen content on the hydration of the endosperm in a single barley variety.

Figure 11 Ranking of new barley varieties by hydration of the ventral endosperm. Hot water extract (HWE) was determined independently and concurrently using the IOB Recommended Method.

Figure 12 Determination of endogenous gibberellin levels by HPLC.

Figure 13 Determination of endogenous gibberellin levels by ELISA.

Figure 14 Electron micrograph of barley scutellum showing immunolatex binding. Raw barley was labelled as described in the methods.

Figure 15 Electron micrograph of barley aleurone showing immunolatex binding. Barley steeped and germinated for 2 days was labelled as described in the Methods.

Figure 16 Aleurone response to GA_3 - different cultivars.

Figure 17 Aleurone response to different concentration of GA_3 - (Digger cultivar).

Figure 18 Aleurone response to different concentrations of GA_3 - (Blenheim cultivar).

Figure 19 Activity of G6P-DH during steeping of different spring barley cultivars. Steep regime: 7 h wet, 17h air-rest, 7h wet, 17h air rest, 1h wet. Samples taken from 1988 NIAB trials.

Figure 20 Oxygen levels and respiration rates during steeping of Blenheim barley. Steeping regime: as described for Figure 17.

Figure 21 Activity of G6P-DH during steeping of Blenheim and Digger cultivars. Steeping regime: 8h wet, 16h air-rest, 24h wet.

APPENDIX I

Publications and presentations

Presentation to MAGB Technical Committee, October 1988, 1989, 1990.

Report in BRF Quarterly, April 1989.

Papers on Barley hydration and Immunoassay of Gibberellins given at EBC Zurich, May 1989.

Lecture to Scientific and Technical Annual Meeting of Institute of Brewing, May 1989.

Lecture to Plant Biology Research Group, Sussex University, May 1989.

Posters at Farming Open Day, Semundo Plant Breeders, June 1989.

Lectures to BRF Autumn Course, October 1989, 1990.

Lectures to H-GCA, MAGB, IOB, RASE at Stoneleigh, January, 1990. Later published in Ferment, June 1990.

Poster at Cereals '90, Stoneleigh, June 1990.

Report in BRF Quarterly, June 1990.

Presentation to Bass plc, R & D, July 1990.

Paper entitled 'Use of X-ray microanalysis to study hydration patterns in barley', submitted to J. Cereal Sci., June 1990.

Paper entitled 'Water uptake and respiration during steeping: effects of barley variety and steeping conditions', in preparation.

Paper entitled 'Prediction of malting quality by endosperm hydration', in preparation.

Table 1: Total Nitrogen and Moisture Levels in a variety of Winter Barleys

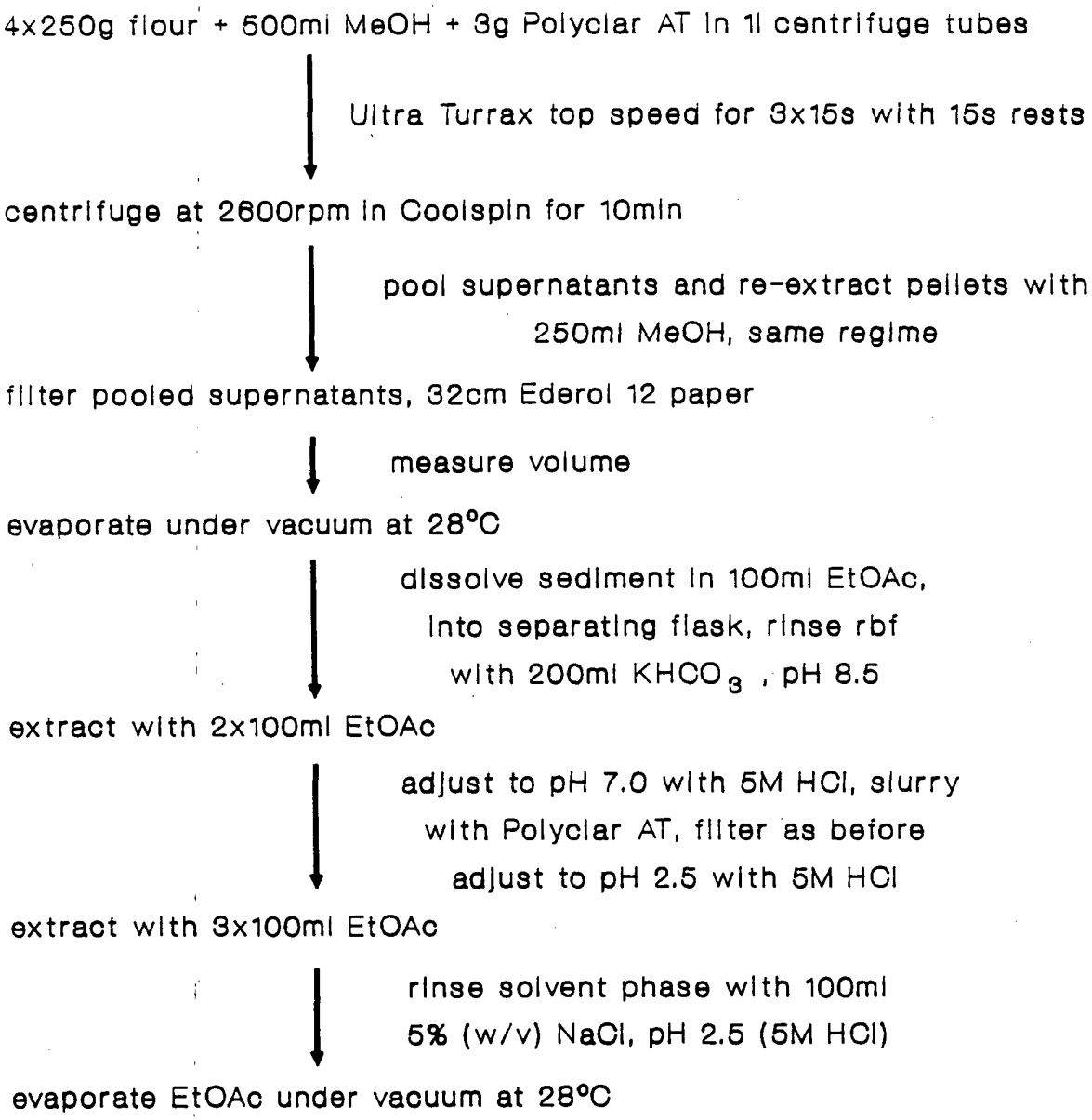
Variety	Sutton B'ton		Bridgets	
	TN (%)	Moisture (%)	TN (%)	Moisture (%)
Finesse	2.32	12.0	1.80	12.7
Halcyon	2.18	12.0	1.82	12.1
Kaskade	2.34	11.3	2.00	11.9
Pipkin	2.24	11.8	1.80	12.1
Waveney	2.49	11.4	1.89	11.5
Magie	2.18	11.5	1.99	12.2
Marinka	2.31	11.4	1.80	12.1
Torrent	2.41	11.5	1.87	11.7
Masto	2.16	11.7	1.78	12.2
Concert	2.23	12.5	1.92	12.0
Kira	2.16	11.9	1.82	12.0
Nevada	2.17	11.7	1.92	11.4
Igri	2.00	11.9	2.02	12.4
Mimosa	2.27	11.2	1.71	12.1

Table 2: Glucose-6-Phosphate Dehydrogenase Activity In Spring Barley Samples

Cultivar	Malting Grade	Activity (nmol NADPH produced/mim/g dry wt) 1986	1987	1988
Corniche	9	662	530	638
Natasha	9	572	ns	543
Triumph	9	533	428	493
Prisma	9	ns	430	461
Doublet	9	422	418	430
Blenheim	9	442	394	303
Atem	5	428	ns	447
Kym	5	415	ns	ns
Regatta	4	589	470	483
Klaxon	3	551	444	469
Cameo	3	436	397	ns
Digger	1	444	493	428
Joline	1	ns	392	418

ns = no samples available

Figure 1: Extraction of Gibberellins: Modified Extraction Procedure



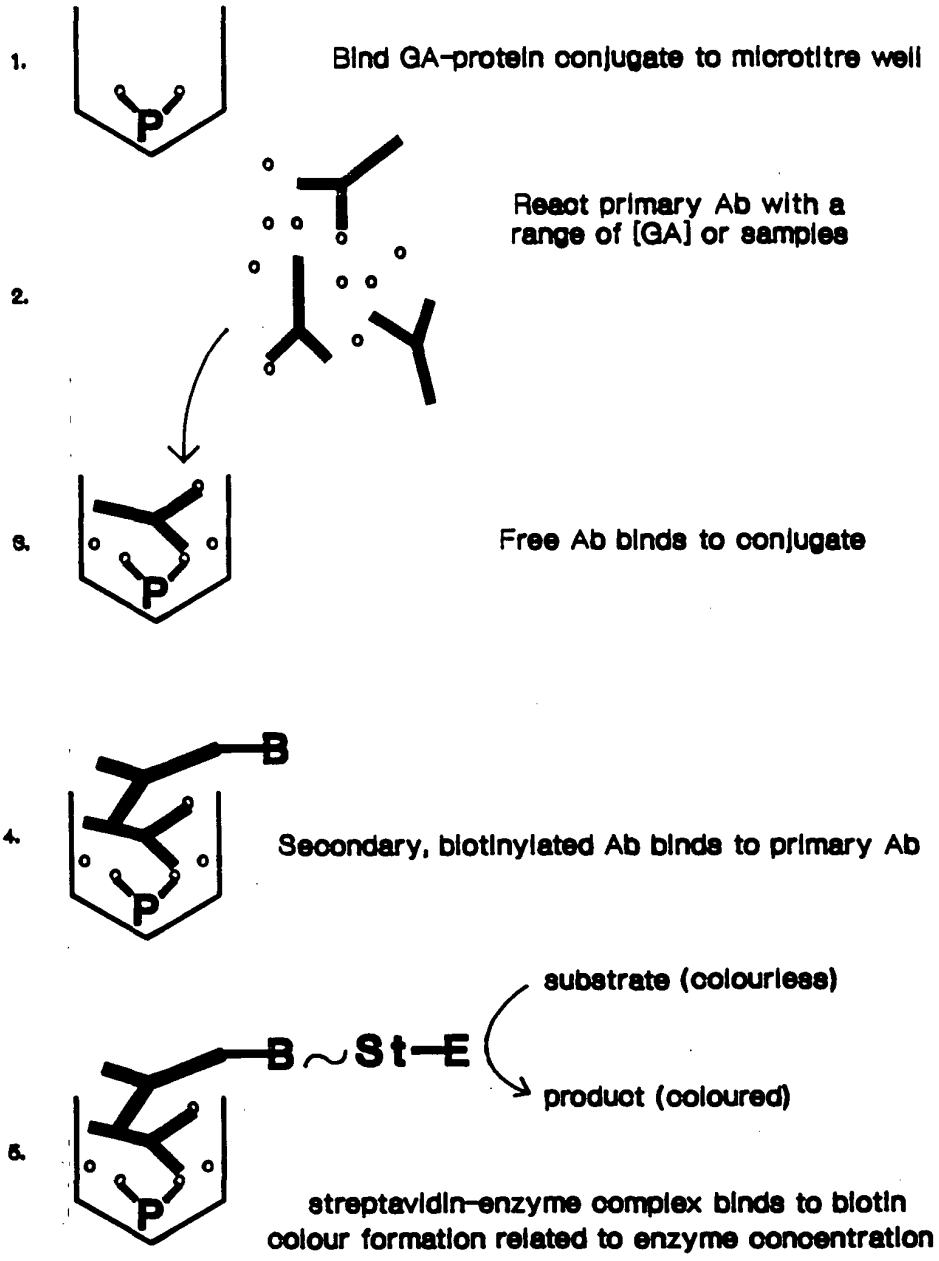


Fig 2: Scheme for competetive ELISA

Fig 3: ELISA of barley extracts Triumph and Magie

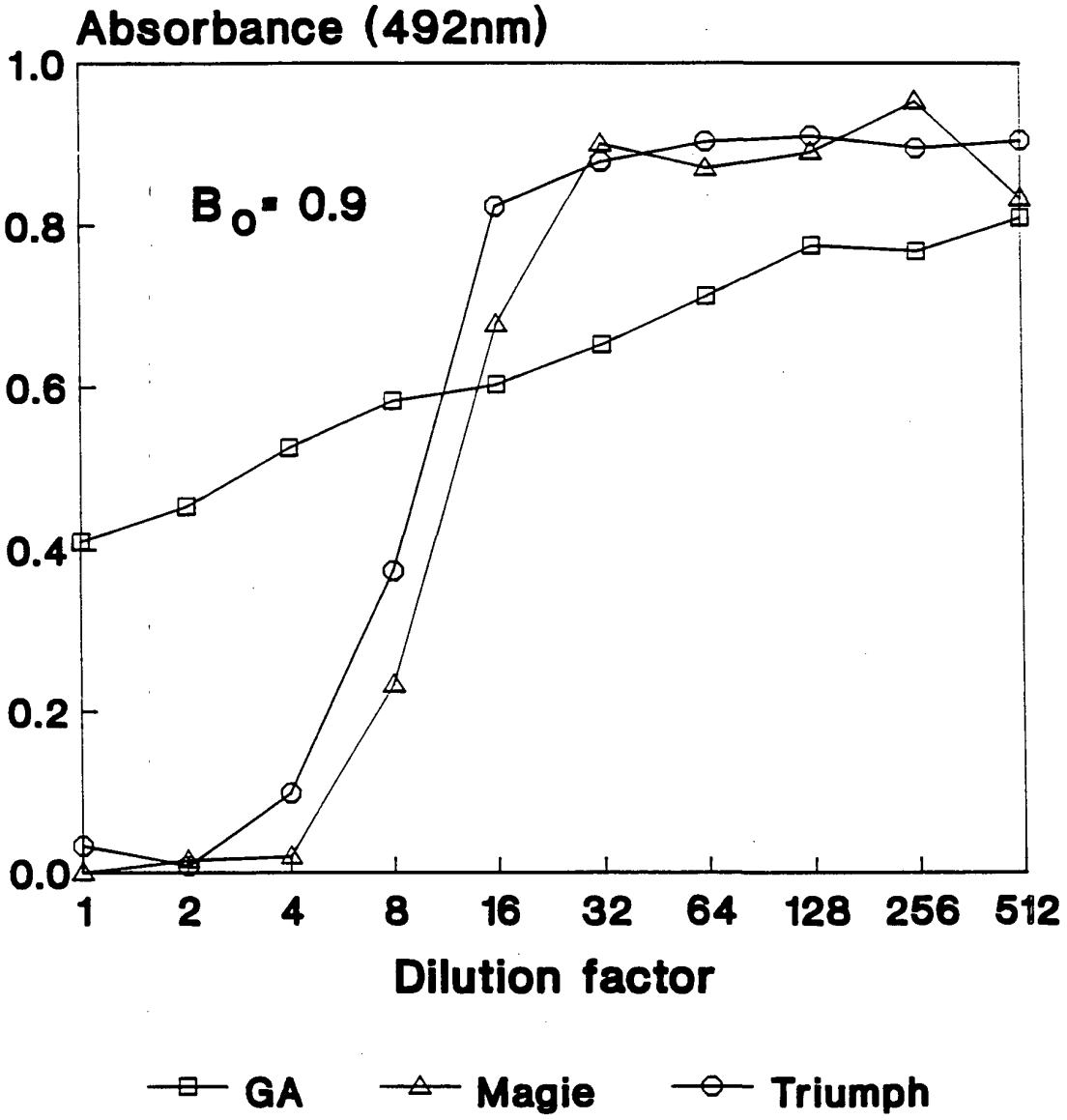


Fig 4: Effect of Extraction Procedure on ELISA response

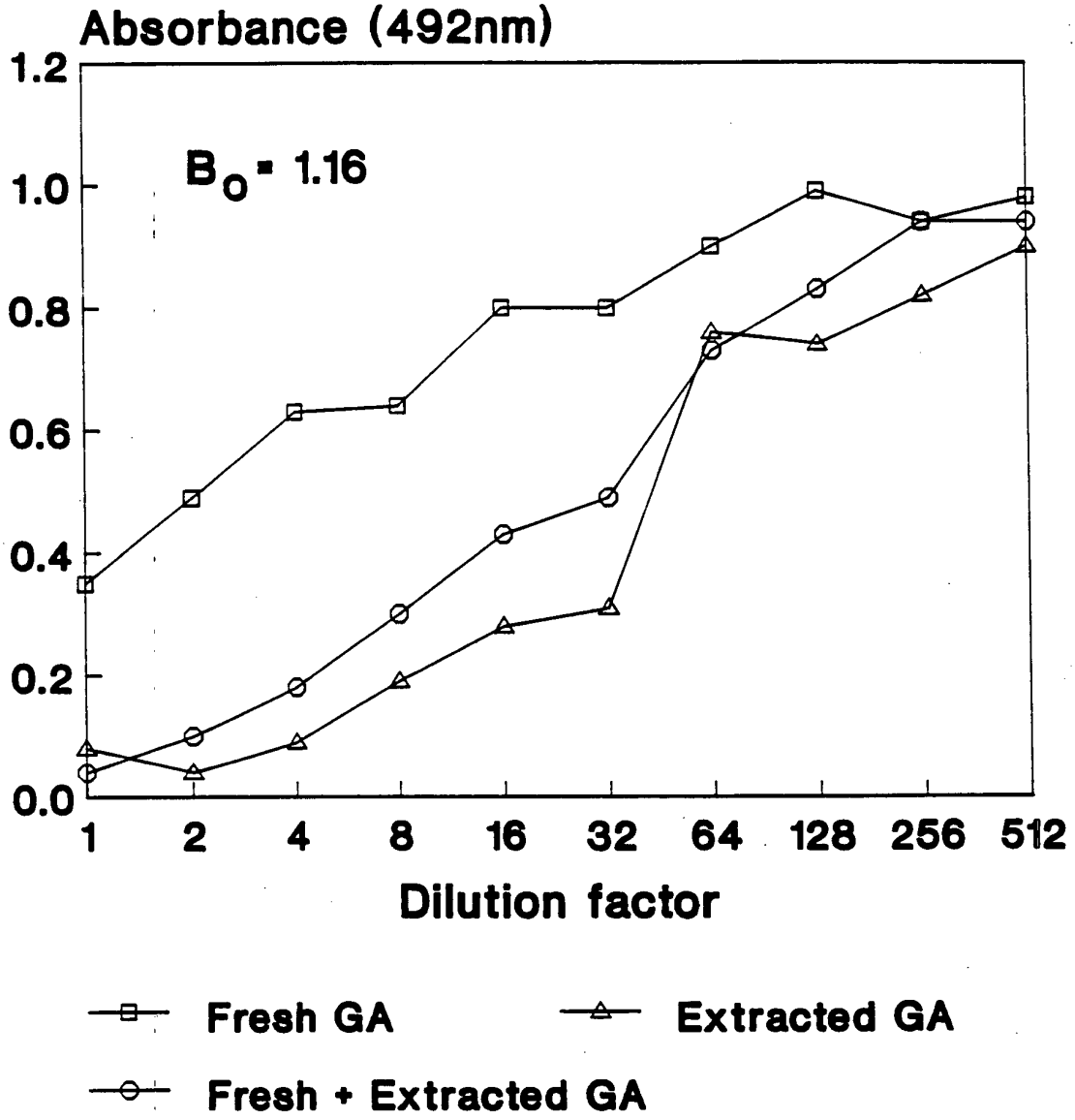


Fig 5: Improved Response from ELISA with added GA_3

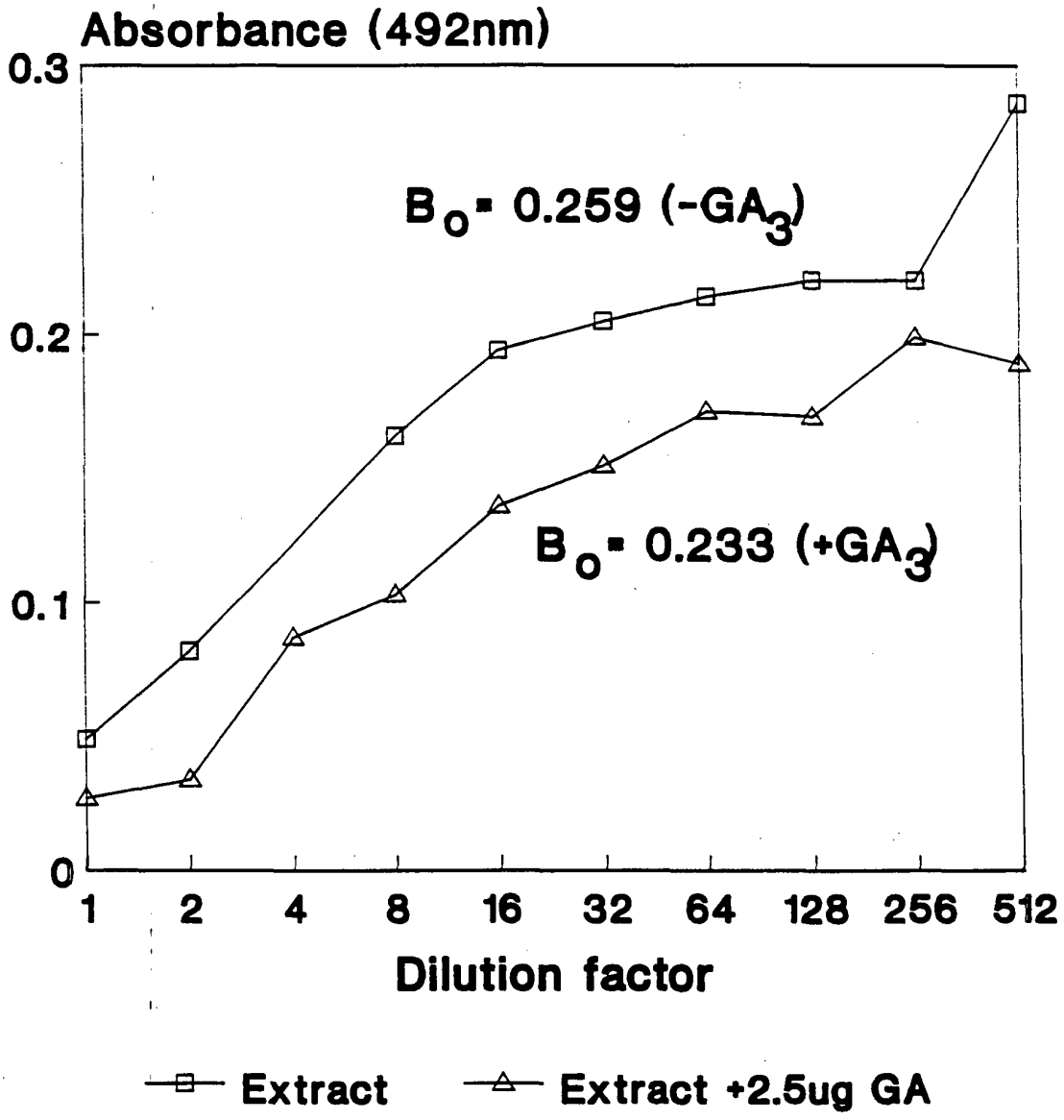


Figure 6: Iodine staining of steeped barley

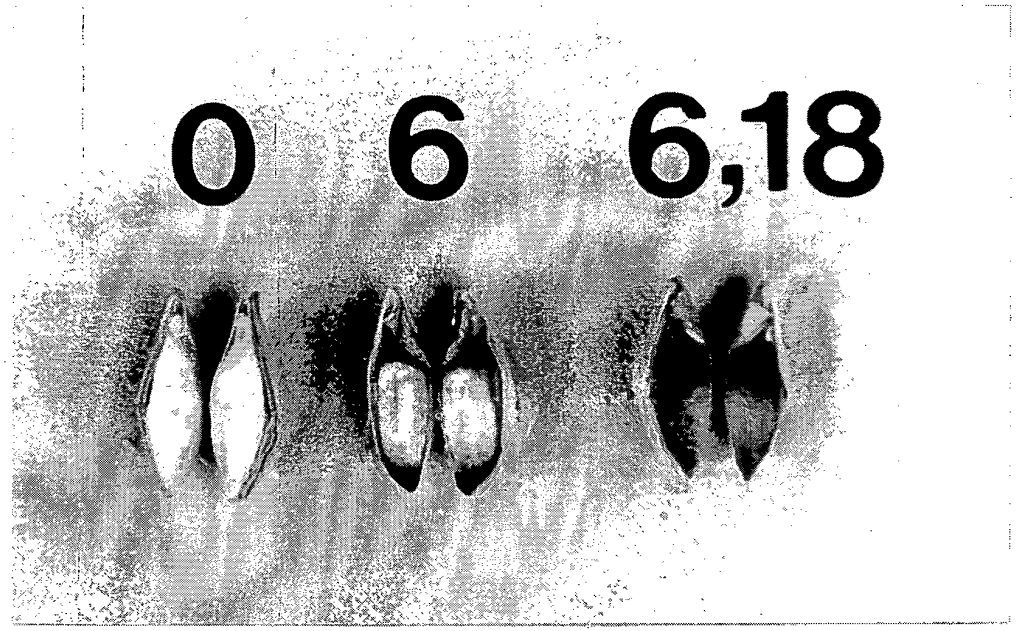


Figure 7: Hydration pattern along Barley Ventral Furrow

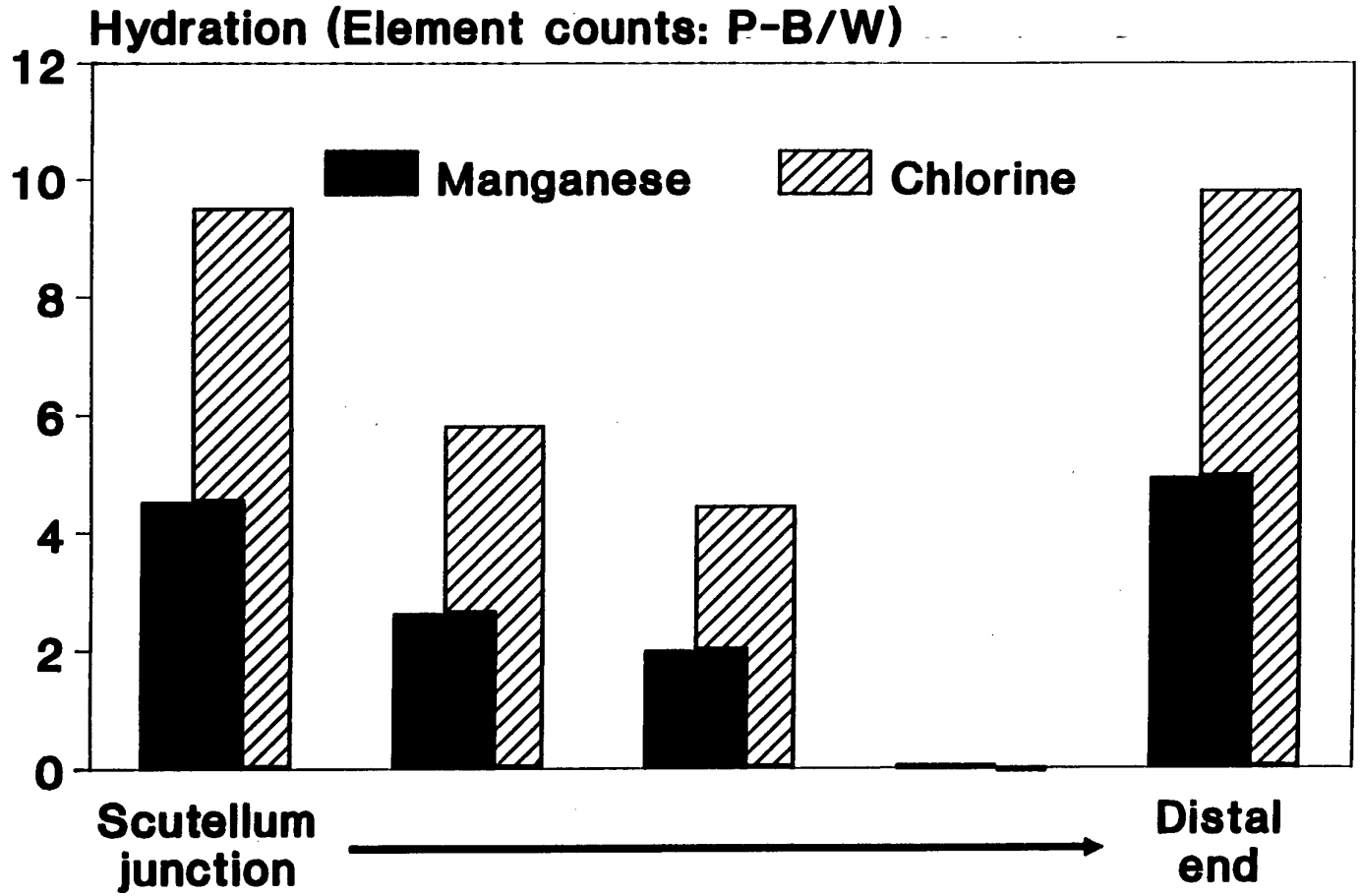


Figure 8: Varietal Differences in Water Distribution During Steeping

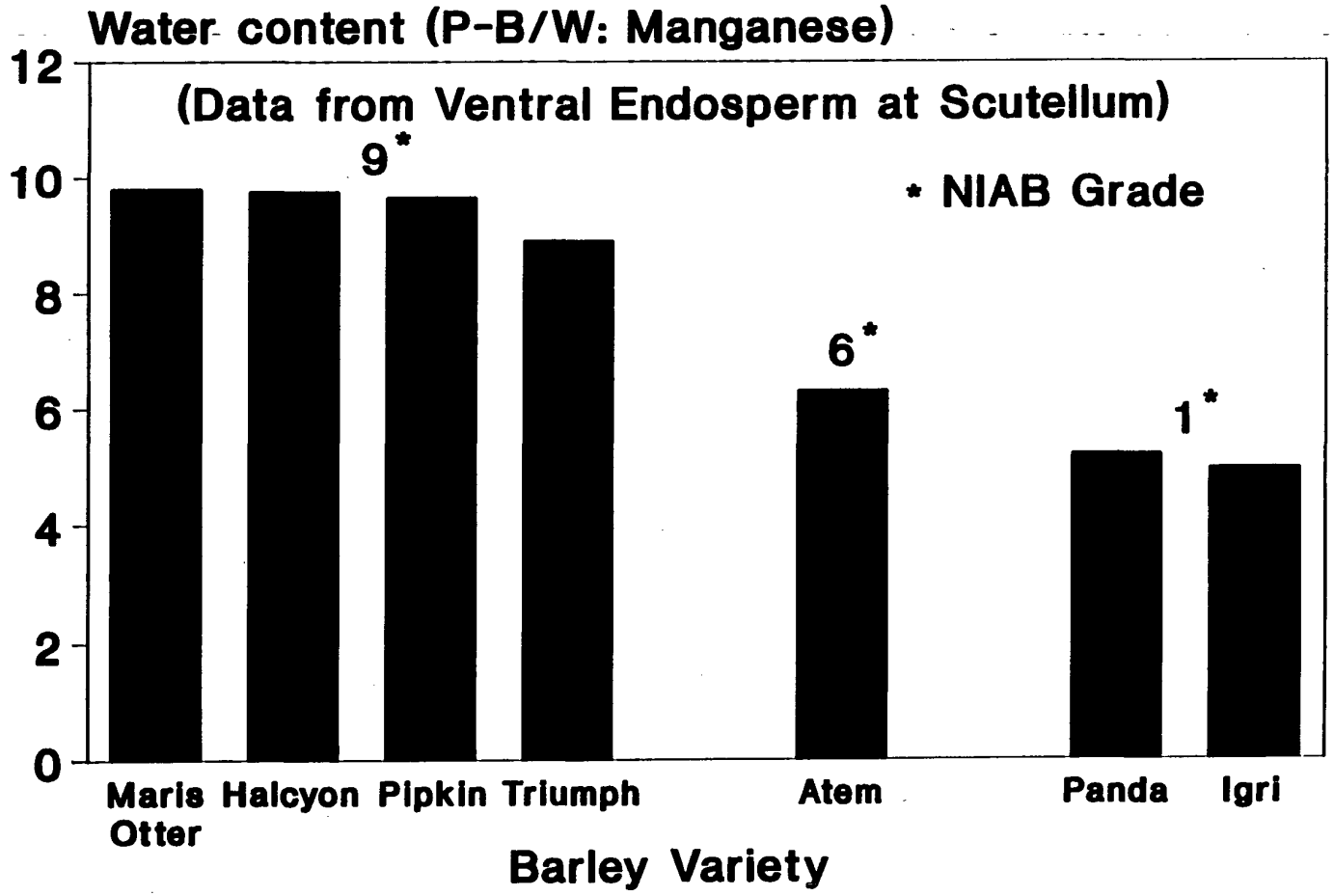
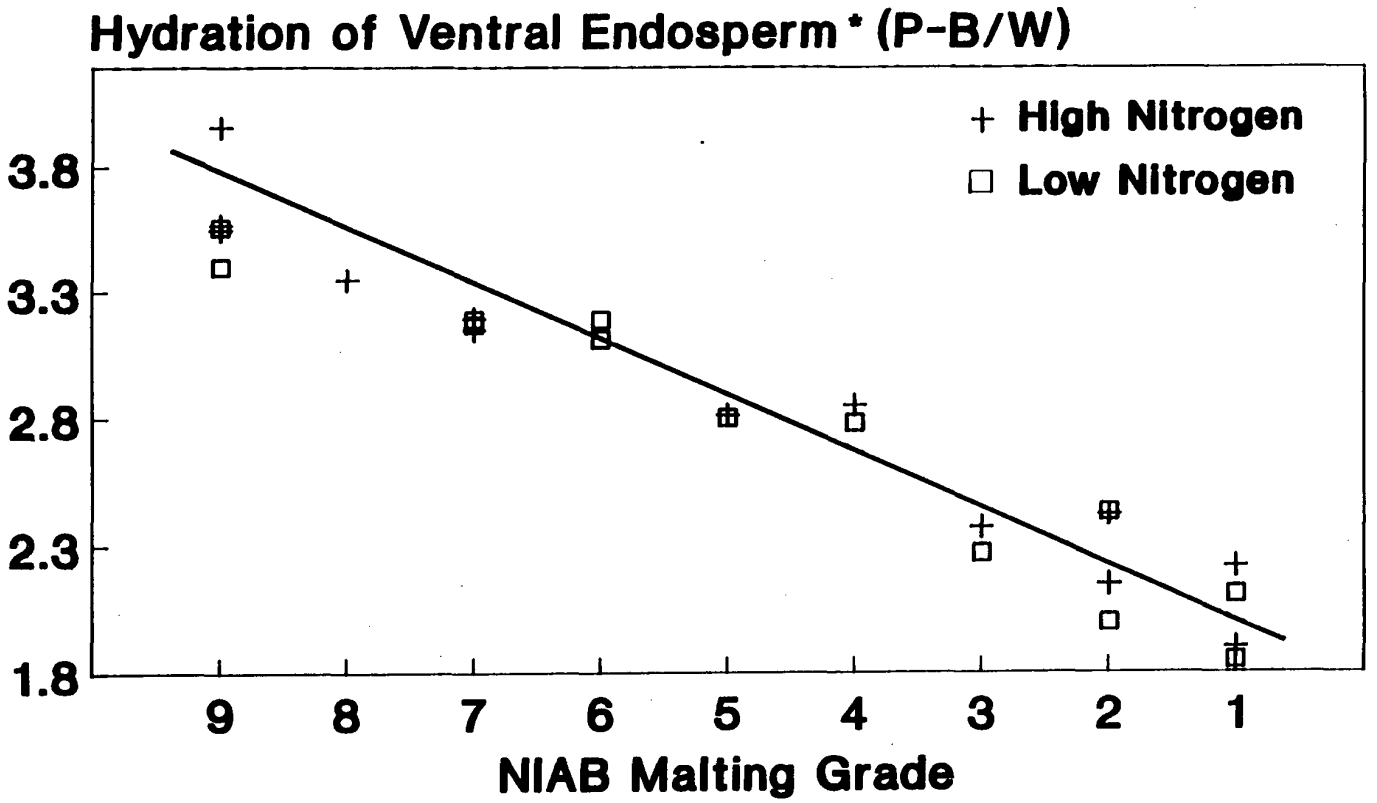


Fig 9: Relationship between Water Distribution at the Ventral Endosperm and NIAB Malting Grade



(After 6h Steep, 18h air rest at 20 °C)

* Adjacent to Scutellum

Fig 10: Effect of Sampling Position and Nitrogen Content on Hydration of the Ventral Endosperm

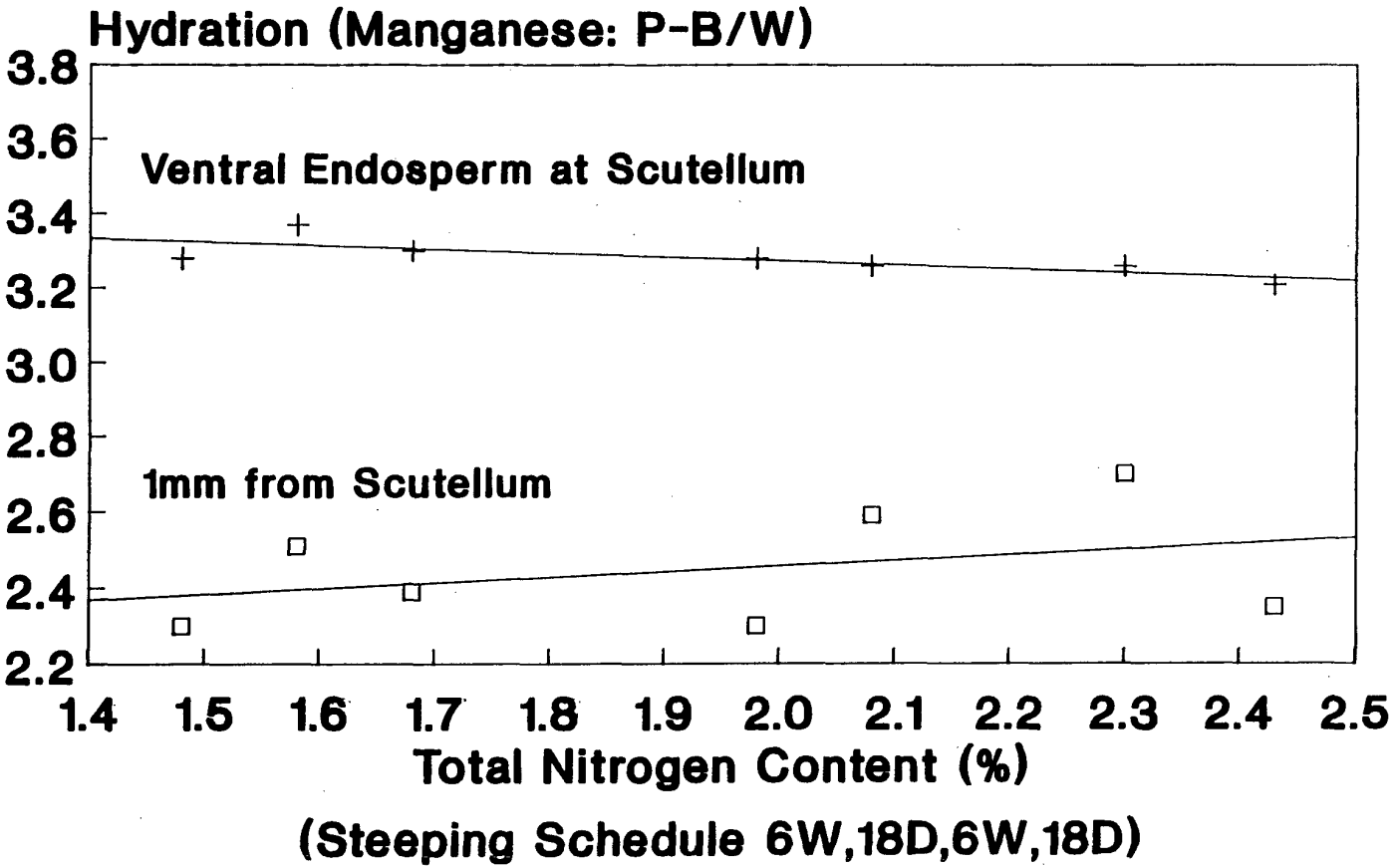
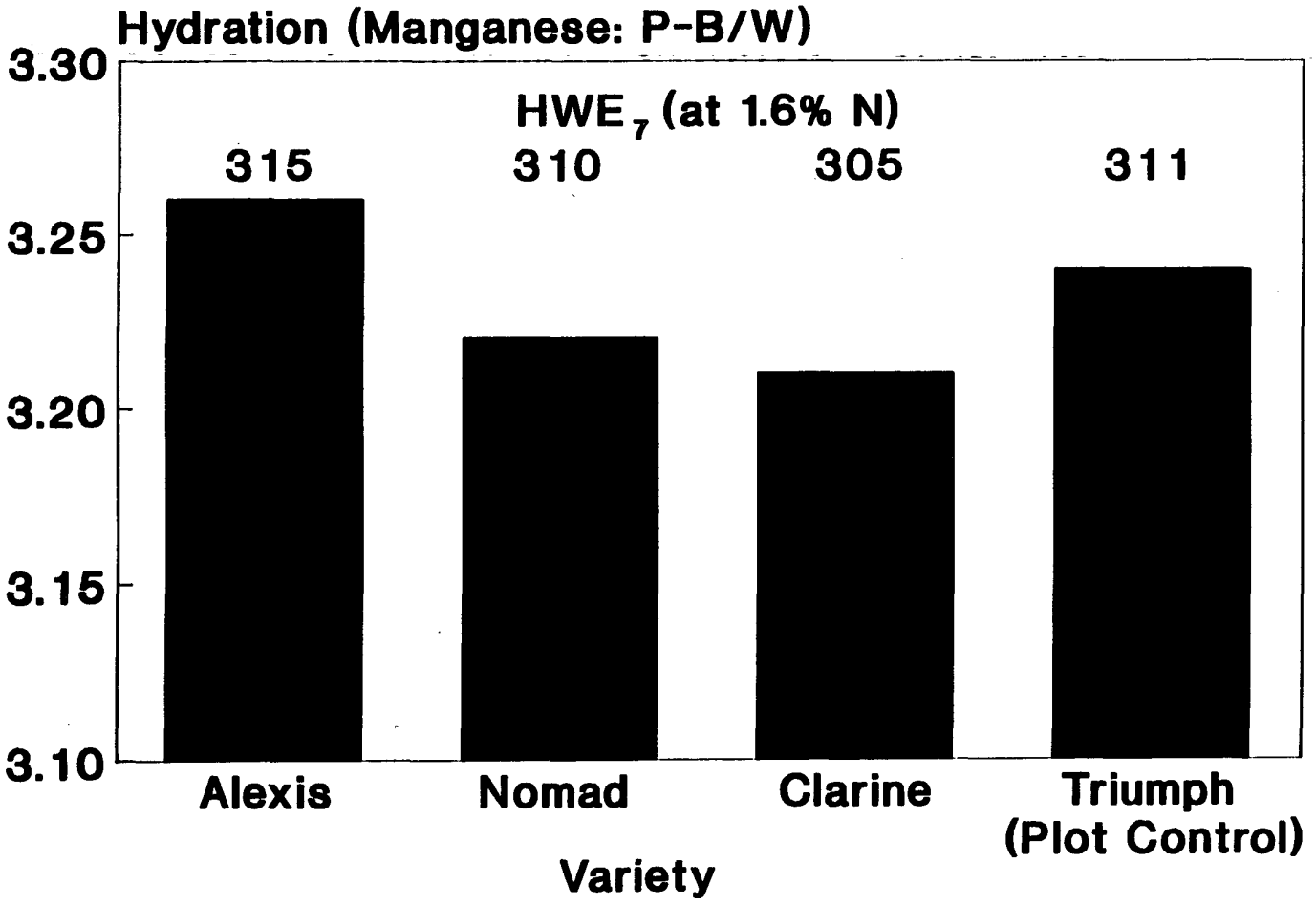


Fig 11: Ranking of New Barley Varieties by Hydration of the Ventral Endosperm



Flg 12: Determination of Endogenous Gibberellin levels by HPLC

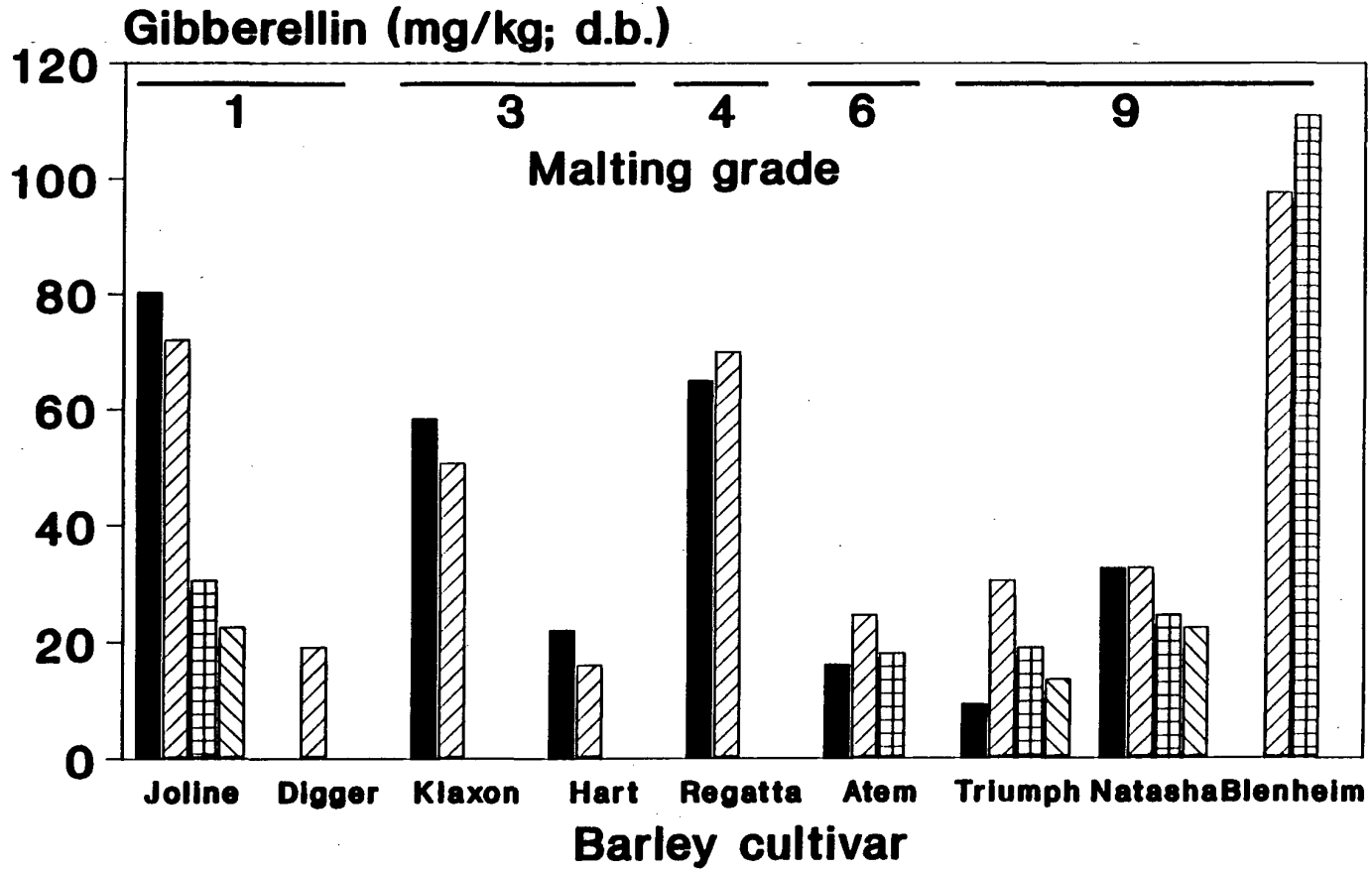


Fig 13: Determination of Endogenous Gibberellin levels by ELISA

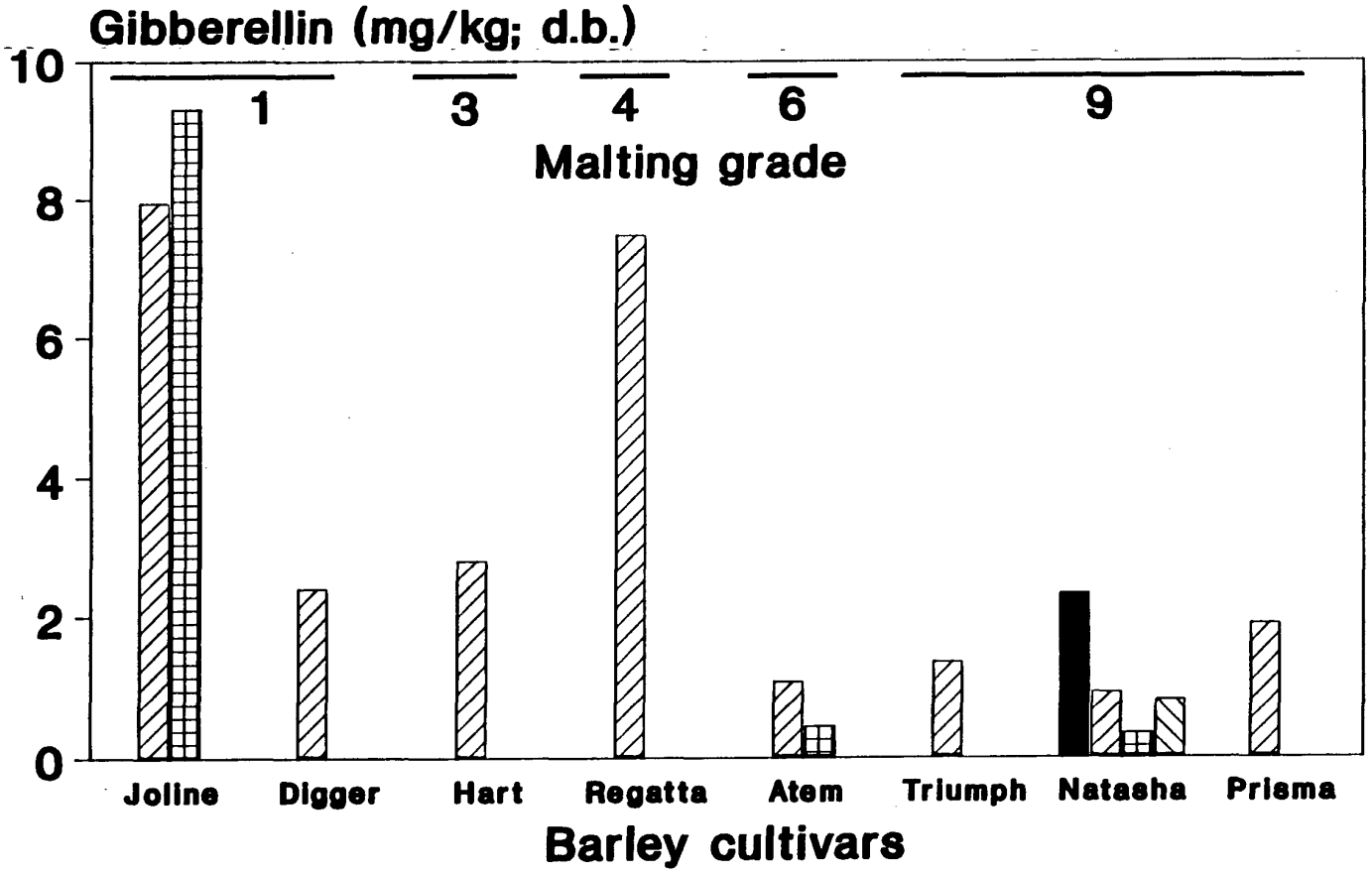
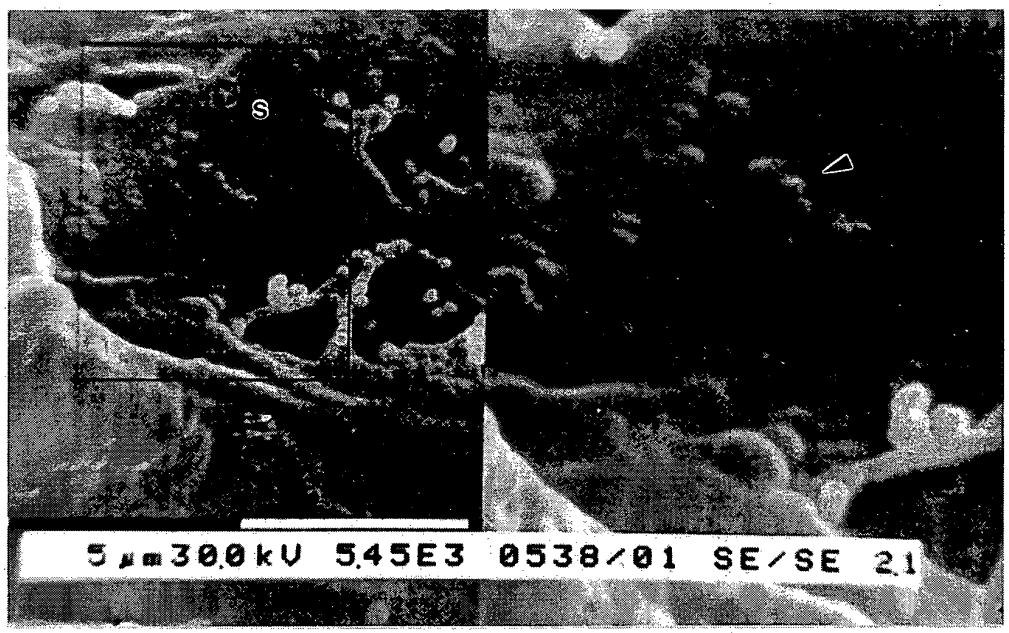


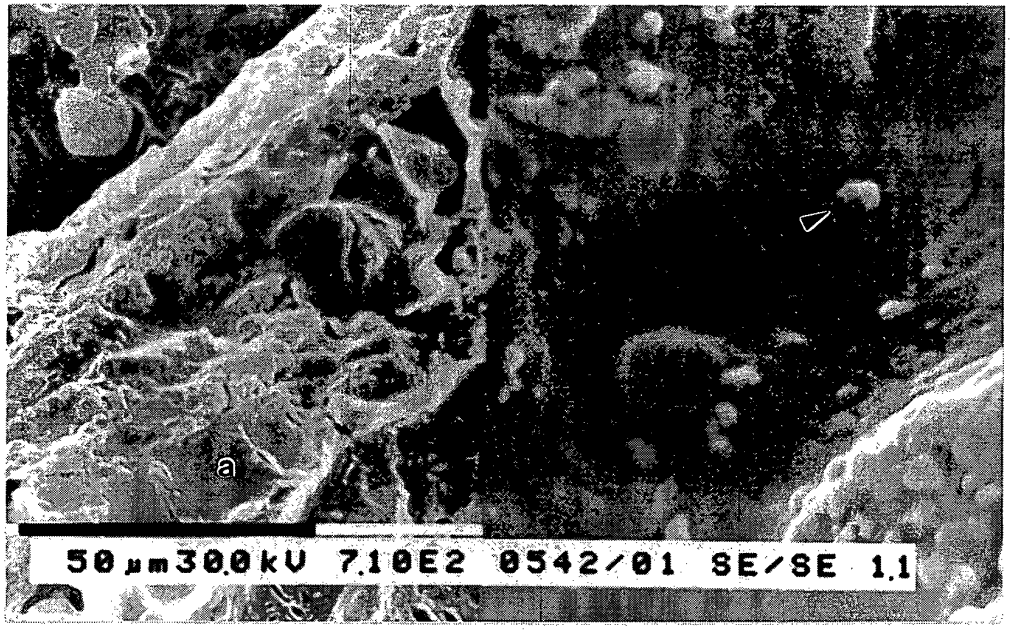
Fig 14: Electronmicrograph of immunolabelled barley grain. Raw barley showing scutellum.



s - scutellum

› - immunolatex label

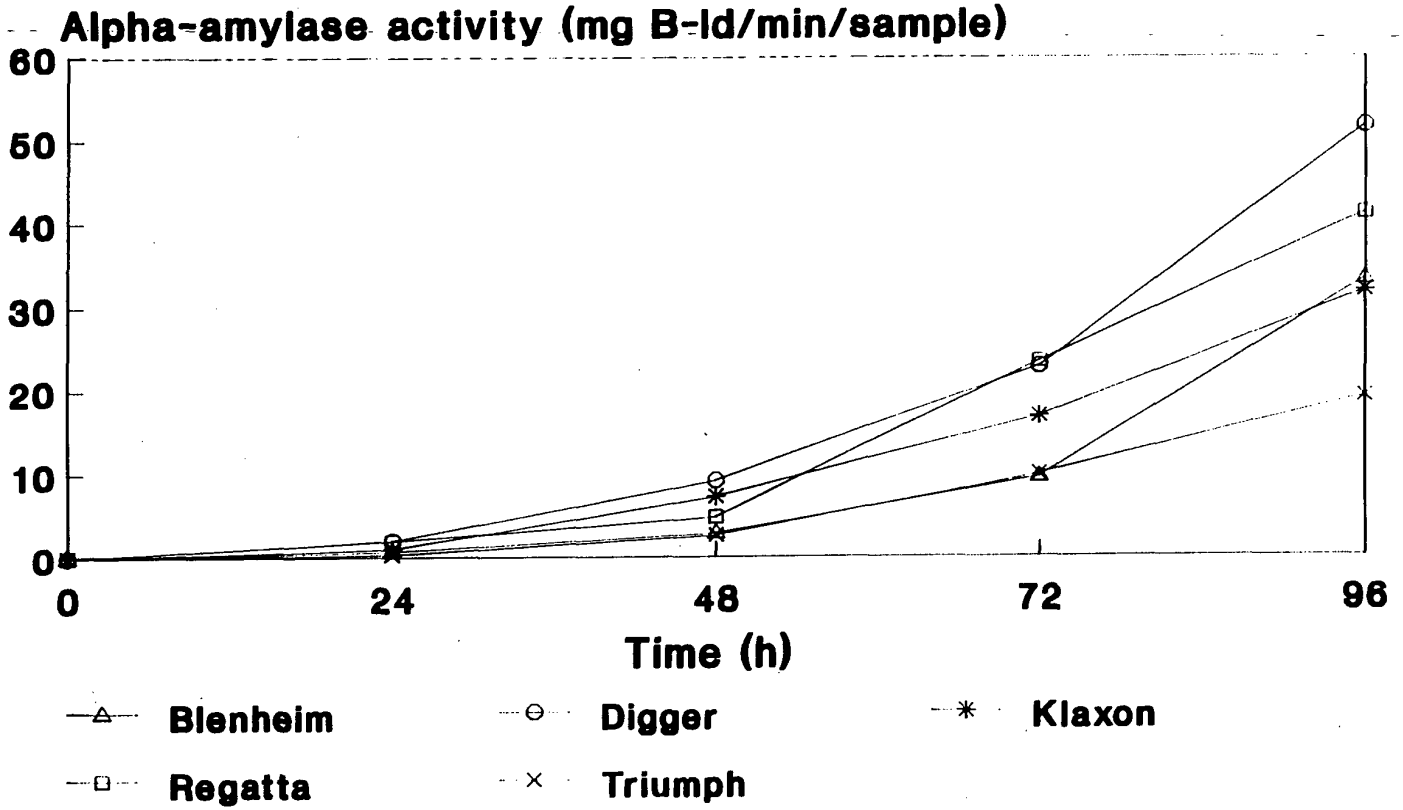
Fig 15: Electronmicrograph of immunolabelled barley grain. Day 2 germination showing the aleurone layer.



a - aleurone layer

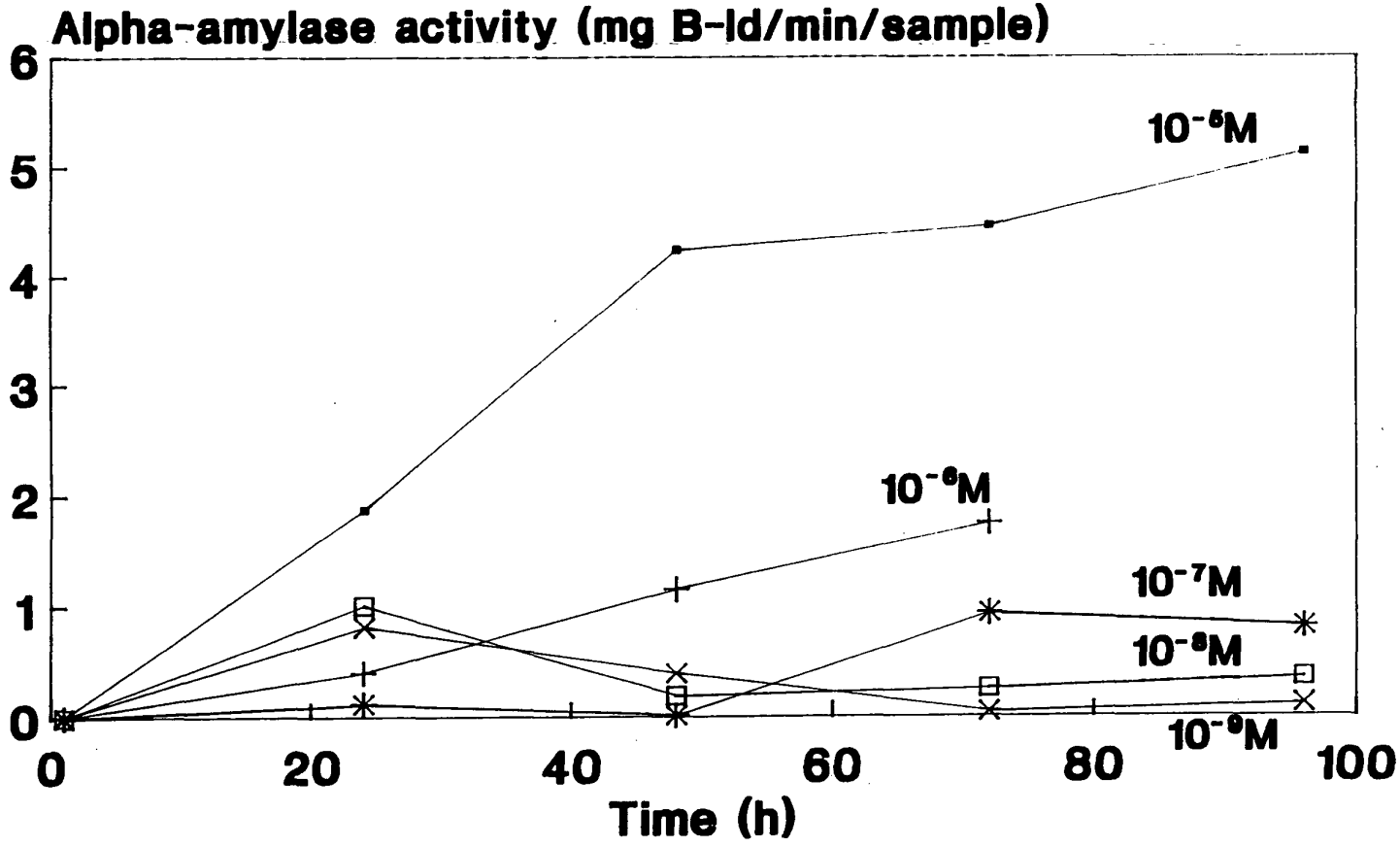
› - immunolatex label

Fig 16: Aleurone Response of Different Cultivars to GA_3 (Alpha-amylase Activity; 10^{-5} M GA)



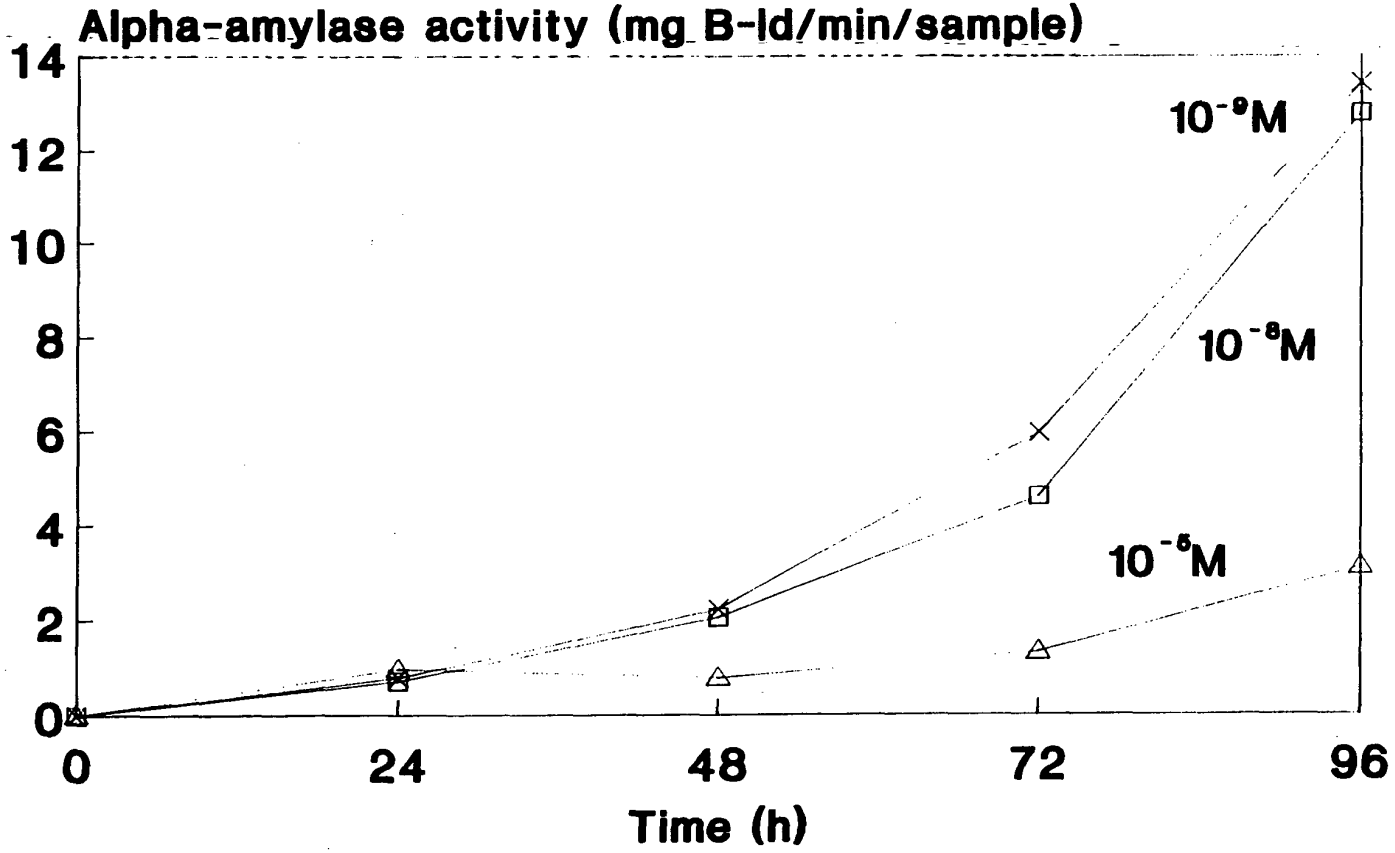
Values displayed are the means of three separate assays

Fig 17: Aleurone Response to Different Concentrations of GA_3
(Alpha-amylase activity)



Cultivar: Digger

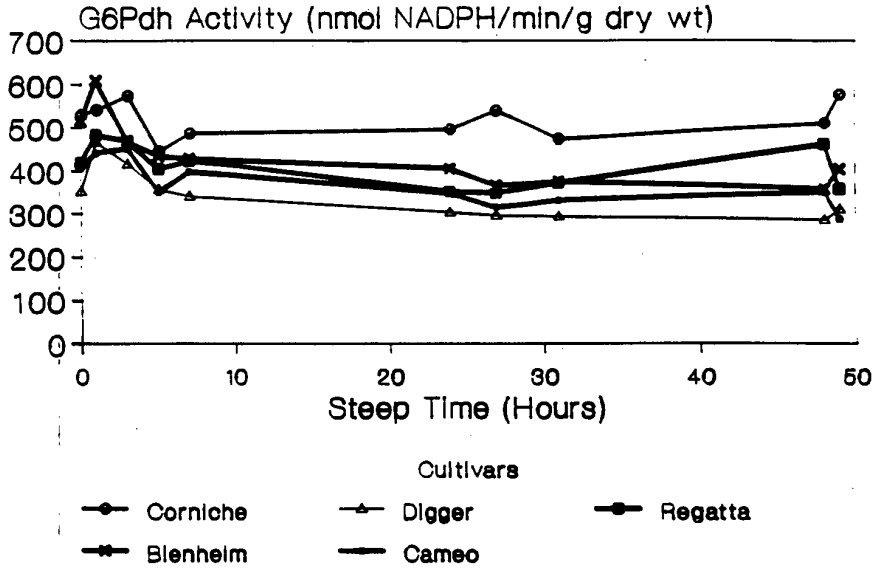
Fig 18: Aleurone Response to Different Concentrations of GA_3
(Alpha-amylase activity)



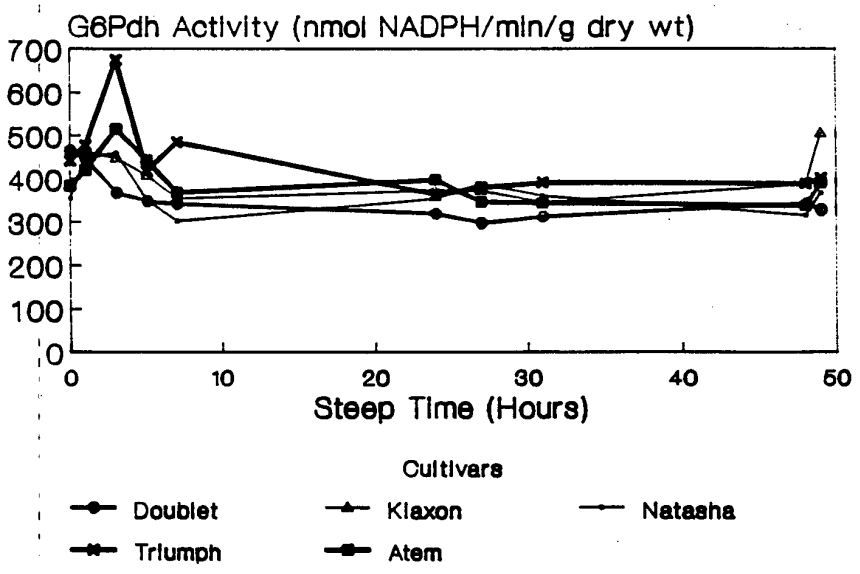
Cultivar: Blenheim

Fig 19: G6Pdh Activity in Different Spring Cultivars

A)



B)



(Steep 7h wet; 17h air rest; 7h wet; 17h air rest; 1h wet)

Fig 20: Oxygen Levels and Respiration Rates of Barley During Steeping

Cultivar: Digger

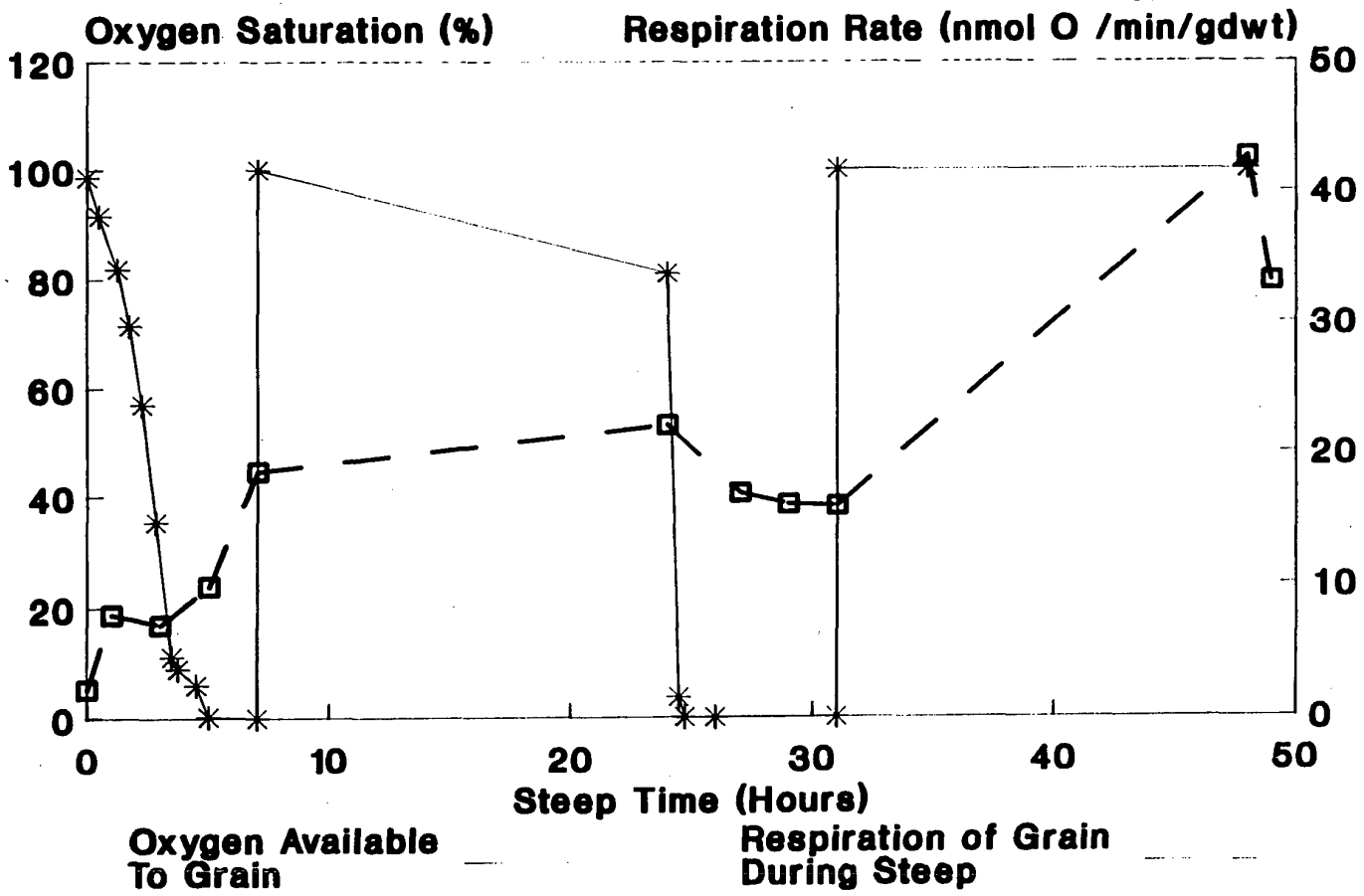
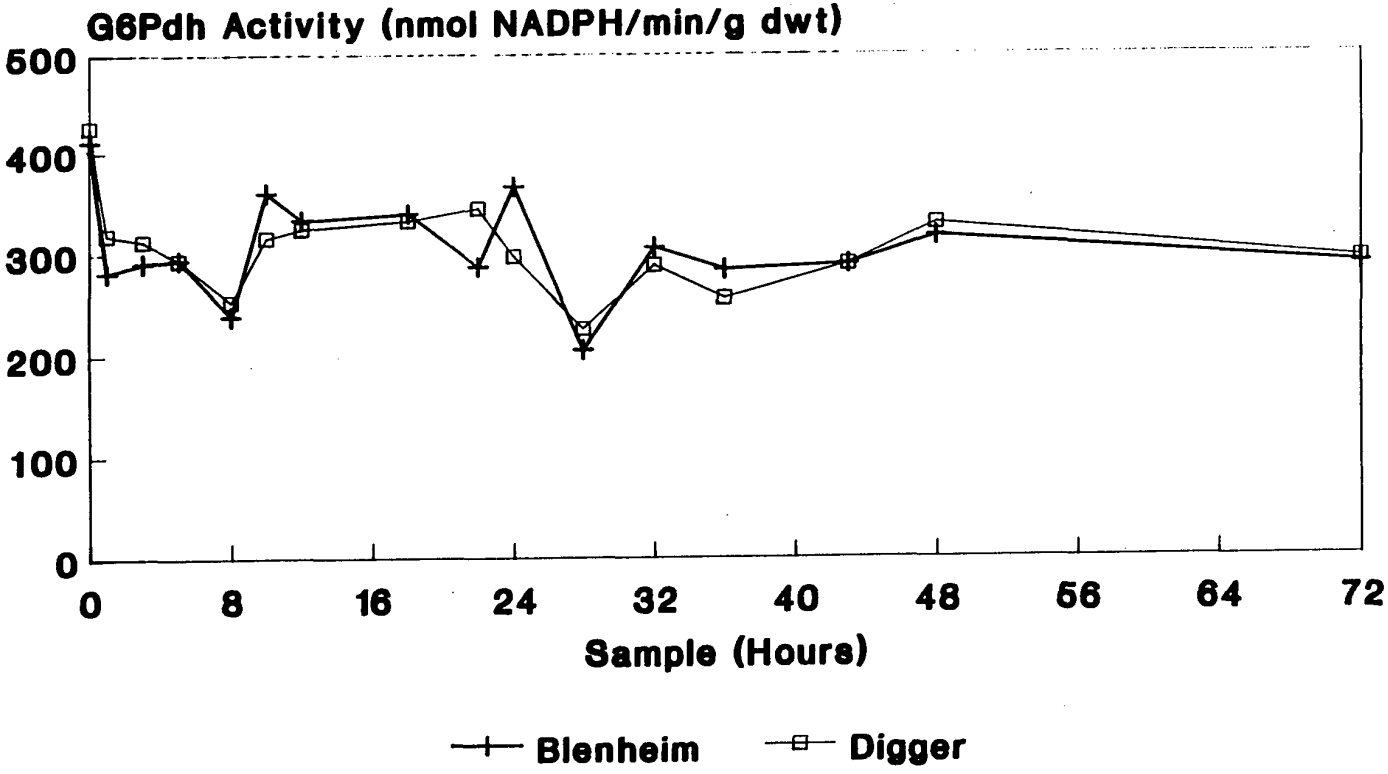


Fig 21: Activity of G6Pdh During Steeping of Blenheim and Digger Cultivars



(Steep Schedule: 8h wet; 16h air rest; 24h wet)