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Structural Changes in Beef Muscle During Ageing

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The ageing of beef muscle set in *rigor mortis* while restrained at twice its resting, excised length has been examined. Ageing leads to opening of gaps between the *A* and *I* bands of the sarcomere. With the shrinkage of both bands during cooking, the gaps become the major feature of the sarcomere and develop within the tenderising time-scale. As such they represent the most distinct structural change yet encountered to explain the phenomenon of ageing. A possible reconciling of the contradictory views of ageing—splitting at the *A–I* junctions and decay of *Z* lines—is discussed.

1. Introduction

Studies of meat ageing have, over the years been the subject of three comprehensive reviews.[1–3] The common belief is that ageing occurs in the myofibrils. Many past studies were concerned with the interaction of actin and myosin in the region of thick and thin filament overlap, the view being that if rigor linkages between the two sets of proteins were weakened meat would become more tender.[4–9] An alternative view is that ageing is due to disintegration of the *Z* lines of the sarcomeres leading to a loss in the tensile strength of the myobrils and in cooked meat, to increased tenderness.[10–15] Despite these intensive studies a direct correlation of structural and tenderness changes has yet to be established. In fact, compared with tenderising, *Z*-line decay to the point of disappearance is much slower. The assumption has been that *Z* lines first undergo an unobservable weakening affecting mechanical strength and that further decay is merely an extension into the observable range.[12]

In this study muscle has been set in *rigor mortis* while stretched to twice its resting, excised length. This is the limit to extension without tearing[12] and should in theory pull the thin filaments free from their locations within the thick-filament array of the *A* bands.[16] If the stretched meat tenderises on storage, a weakening of the association of actin and myosin cannot reasonably be invoked to explain ageing. The meat was also cooked while restrained in a fully stretched state. Through the prevention of cooking shortening[17] stresses should build up, leading to a possible snapping of structural elements at their weakest points; these would presumably represent the ageing sites.

2. Experimental

2.1. Meat

Sternomandibularis muscles (neck muscles) of Angus bulls (4–6 years of age) were obtained within 30 min after slaughter from the normal kill of the local abattoir and were trimmed of surrounding tissue. Only muscles of cross-section in the range 15–20 cm^2 and with normally low ultimate pH values (< 5.7) were used for further study. Each pair of neck muscles was cut into strips 10–12 cm along the fibre and 3–4 cm^2 in cross section. The strips were laid out at rest length (l_0) and two ink marks 5 cm apart stamped on the surface of each in the line of the fibres. Half were held at l_0 to enter rigor while the other half were stretched to 2 l_0 in the following way. Thick adhesive tape was bound around each strip approximately 1 cm from its ends. The strips were held by the taped ends with laboratory clamps supported on metal rods and stretched to extend the 5 cm marked

length to 10 cm (variation, 0.4 cm). Both the stretched and free strips were sprayed with chloramphenicol (20 parts/10^6) in ethanol and sealed in plastic bags flushed with nitrogen. They were held at 15°C for 24 h to set in *rigor mortis*, the free samples shortening by less than 5%. The strips were again sprayed with antibiotic, resealed in the bags and held at 25°C to age. With hygienic handling and the two sprayings, surface counts of bacterial aerobes remained at less than 50/cm^2 while the meat 0.2 cm in from the surfaces remained sterile throughout the experimentation.

A small sample (3 cm along the fibre × 1 cm × 1 cm) of one of the pre-rigor muscle strips was also used for measuring *rigor mortis* onset. It was loaded with 50 g and mounted within 90 min *post mortem* on a rigorometer operating at 15°C in moist nitrogen. The extensibility change during rigor onset was measured.[18]

2.2. Estimation of pH

Ultimate pH was determined on meat pieces left over from cutting the strips. They were held at 15°C for 24 h and 2°C for 12 h under nitrogen, and then samples (~2 g) were homogenised in 10 ml sodium iodoacetate (2 mM, pH 7.0).

2.3. Cooking and tenderness evaluation

At intervals commencing from 90 min *post mortem* strips were cooked and their tenderness assessed. The free samples in plastic bags and the stretched samples, still clamped to their rods and wrapped in thin polyethylene film, were cooked at 80°C for 40 min in a water bath. Tenderness was measured as a shearing force (SF) using the MIRINZ Tenderometer.[19] The SF values corrected to a sample cross section of 1 cm^2 are given in arbitrary units,[20] and are approximately nine times those given by the Warner Bratzler tenderometer in kg/cm^2 (1.27 cm cores).

2.4. Microscopy

Raw and cooked strips of stretched muscle were sampled for electron microscopy, after 24 h storage at 15°C (unaged) and after a further 70 h at 25°C (aged).

A surface layer (~0.5 cm in depth) was discarded from each strip close to its centre and drops of 2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.0) were placed on the freshly exposed surface for a period of 4 h. Samples of fixed meat (~0.1 cm thick) were removed from the strips and fixed for a further 4 h in fresh glutaraldehyde. Washing for 12 h in cacodylate buffer was followed by embedding and staining.[12]

3. Results

The post-mortem time courses of rigor development and of tenderness changes in the strips are shown in Figure 1 and are typical of six such experiments. The extensibility change (Curve I) was characteristic of muscle in its progress into *rigor mortis*. In this example the delay phase lasted 16–18 h while the rapid phase was completed by ~24 h.

From the death of the animal SF values of free samples (Curve II) rose to reach a broad maximum (SF 50) over the period of the rapid phase. From there tenderness increased through ageing to approach the maximal achievable in ~70 h with a halving of SF values to 25. In stretched muscle strips (Curve III), initial values (SF 53) were two to three times higher than their unrestrained counterparts. They rose by about 10% through the early, pre-rigor phase to reach their maximum (SF 57) again over the rapid phase, falling during ageing to approach their lowest values (SF 39) in 60–70 h. Although the rates and extents of tenderising from the free and stretched muscle strips were somewhat different, commencement of distinct tenderising at the end of the rapid phase was the same.

Ultrastructural changes due to ageing in unrestrained muscle in the raw and cooked states have been described.[12,21] Figure 2 is therefore solely concerned with identifying the changes of ageing in stretched meat. The typical, highly ordered appearance of raw muscle at 2 l_0 is shown in Figure 2(a). The sarcomere length in this case was 4.2 μm, in theory ~10% greater than the length at

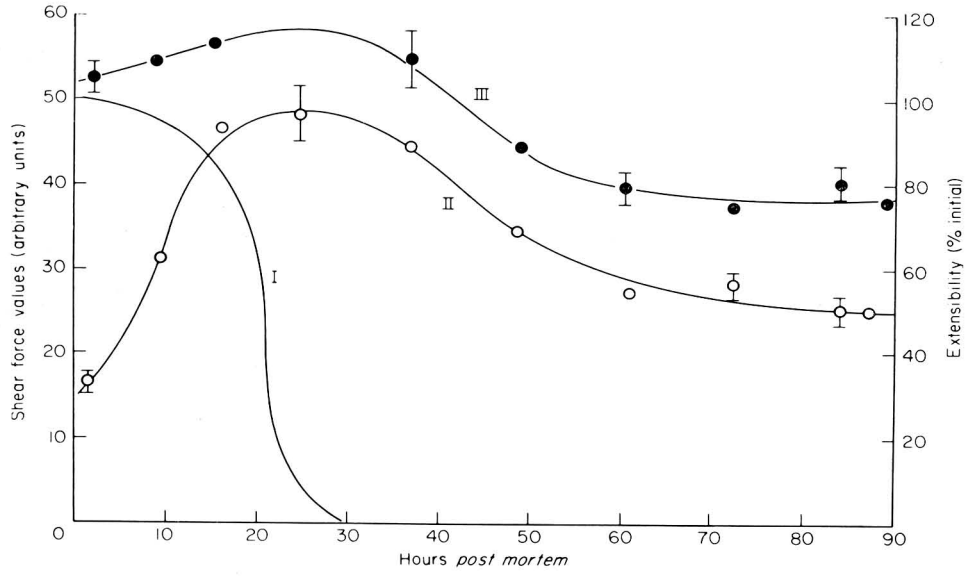

Figure 1. Changes in the extensibility and shear-force (SF) values of beef neck muscle as it progresses into *rigor mortis* at 15°C over 24 h, and is then aged at 25°C. Curve I: Extensibility as percentage of that at 90 min *post mortem*. Curve II: The time-course of change in SF values for unrestrained muscle free to shorten during cooking. Curve III: The time-course of change in SF values for muscle restrained at 2 l_0 during cooking. The SF values are given in arbitrary units, and are approximately nine times those given by the Warner Bratzler tenderometer in kg/cm^2 (1.27 cm cores). Each SF value is the mean of 6–8 determinations on each sample. Standard deviations (vertical lines through means). For clarity these are not all shown.

which the thick and thin filaments in beef just part from their overlap.[16] The length of the *A* bands (~1.5 μm) was difficult to determine through lack of clearly defined edges from which to measure. The *I*-band region, measured from the edges of the *A* bands of adjacent sarcomeres and including the central *Z* line was 2.7 μm. A zone (~0.1 μm) of relatively low staining, but still spanned with filaments was often seen at the junction of the *A* and *I* bands.

Cooking markedly affected the structure. Although the strips were held in the stretched state, *A* bands shortened to 1.1 μm (Figure 2(b)) as has been found for unrestrained muscle,[21] whereas the sarcomere lengths at 4.3 μm were virtually unchanged. Gaps which opened between the ends of the *A* and *I* bands were bridged with filaments often in pairs. The *I* filaments had undergone side-by-side aggregation into coarser strands.

Distinct changes in sarcomere patterns occurred on ageing. In the raw meat (Figure 2(c)) the highly ordered arrangement of the sarcomere was maintained, and its length unaltered. The most prominent change was the development of a relatively clear gap (~0.3 μm) between the *A* and *I* bands with a few bridging filaments. The *A* bands, 1.5 μm in length, had clearly defined but rather knobby ends. The *I* bands (2.3 μm) had coarsened through thin-filament aggregation while the *Z* lines were somewhat thickened. A further distinct change occurred on cooking (Figure 2(d)) with the widening of the gaps between the ends of the *A* and *I* bands. Except for very occasional bridging filaments, and a number of featureless organelles, the gaps were free of structure. The *A* bands had again shortened longitudinally to 1.1 μm. Although there was a loss of structure on cooking, individual *A* filaments could be seen with knobby ends. The *I* bands shrank by 30% to 1.5 μm and, linked sideways across the myofibrils seemed to float between the *A* bands also in close side-by-side linkage. Judging from a wide survey of samples the gaps revealed on cooking occurred universally throughout the muscle structure. In view of past observations on the disappearance of *Z* lines, it is significant that these remained prominent throughout the ageing period.

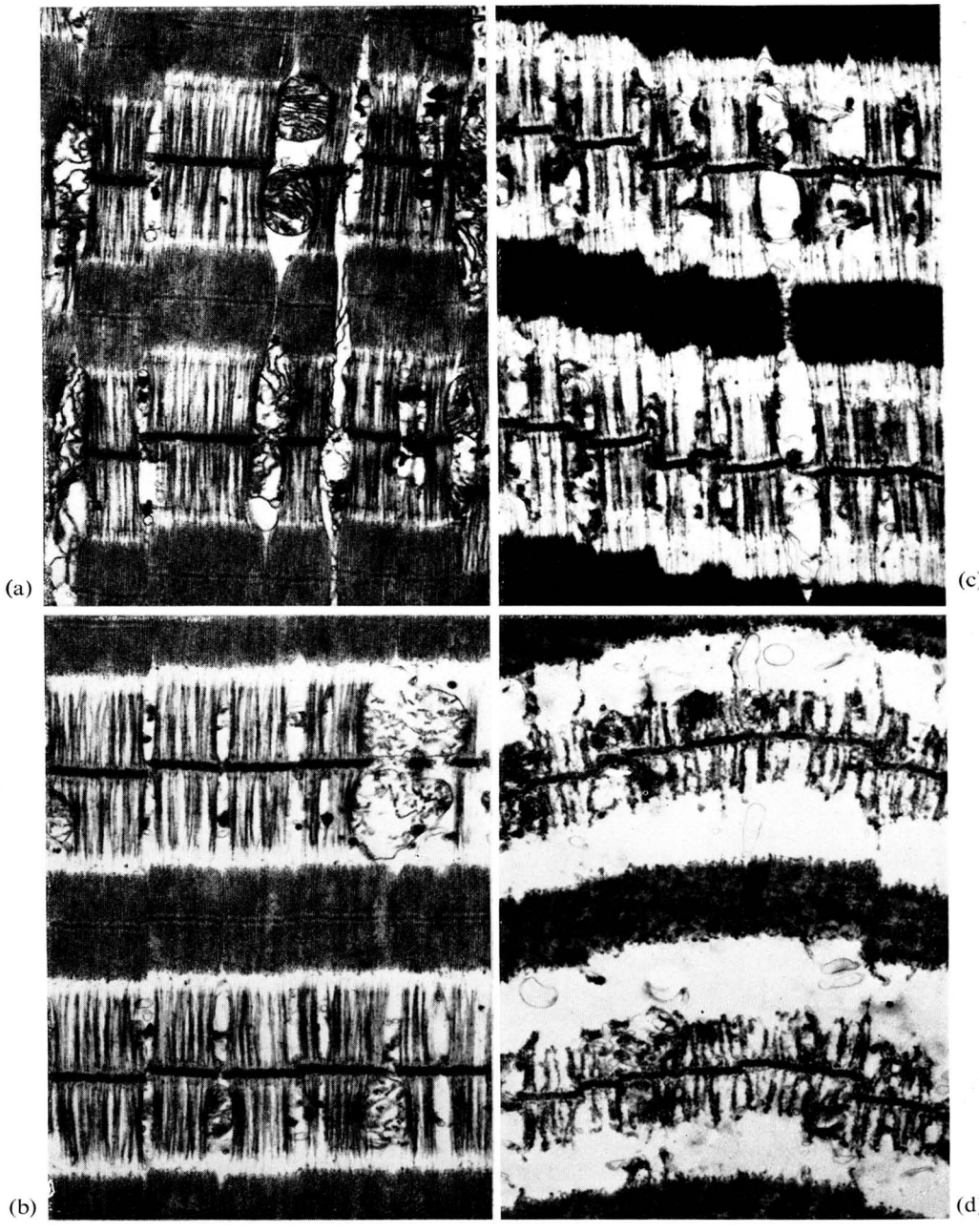

Figure 2. Electron micrographs of muscle stretched to 2 l_0 and set in *rigor mortis* at 15°C. (a) Unaged (raw) (b) Aged 70 h at 25°C (raw). (c) Unaged (cooked). (d) Aged, 70 h at 25°C (cooked). Magnifications, × 10 000.

Progress of structural changes are difficult to quantify by electron microscopy. Using the disappearance of bridging filaments in cooked meat as a rough guide to ageing, numbers diminished progressively to approach zero in 3 days at 25°C. In contrast at 15°C they took about twice as long to disappear.

4. Discussion

The present study has produced useful information on structural changes during ageing. Despite the muscles having been stretched to the point at which thick and thin filament overlap was supposedly non-existent, the meat showed the same capacity to tenderise as unstretched meat. This strongly tells against a progressive weakening or breaking of rigor bonds between the filaments as the mechanism of ageing. There remains a possibility that vestiges of overlap exist in the stretched muscles. Rigor links from such overlap would be so tenuous and meagre that their weakening would hardly produce the distinct and very considerable tenderising shown to occur. The point, however, needs proving.

The present study differs from a previous histological examination of ageing[12] in which muscles were either set in rigor at l_0 or at a slightly extended length but were not aged under tension. It was realised that prolonged ageing might be necessary to reveal sites of weakening within the sarcomere claimed to occur at the *Z* lines. This was supported by the fact that aged meat stretches readily at quite low loads causing breakages at the *I–Z* junctions. The present results, demonstrating specific breaking at the *A–I* junctions would, on first sight contradict this view of ageing. A simple reconciling of the seemingly conflicting observations is possible. There is quickening interest in the rather elusive filaments that are claimed to form a structural skeleton to the sarcomere.[22–25] A recent suggestion[25,26] is that they reside in the thick filaments as a core and are attached to or pass through the *Z* lines giving a greater continuity to the structure of the sarcomere than do the *A* and *I* filaments alone. It can be imagined that with *Z*-line decay through ageing, attachment of the filaments to the *Z* lines is weakened or lost. In stretched meat they may pull away to lodge against the *A* bands and leave the empty gaps we have identified. Alternatively the filaments themselves may weaken during ageing causing meat to become more tender.

Whatever the case the behaviour of the gap filaments on ageing and cooking in the abnormal condition of highly stretched muscle, gives a clue to what may be occurring during ageing at normal sarcomere lengths.

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Batch Dry Rendering: the Influence of Controlled Processing Conditions on the Quality of Meat Meal Prepared from Sheep Stomachs

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A series of meat meals was prepared from the one type of offal material, sheep stomachs, by batch dry rendering in a model cooker. The meals, designated normal cook (NC), overcooked (OC), rapidly cooked (RC), normal cook with added glucose (GC) and spoilt offal cook (SC), were evaluated as protein supplements by chick growth tests in rations based on wheat and skim milk powder. A control ration (CR) and one incorporating a commercial meat and bone meal (MR), were also included for comparison. No significant differences in weight gain were evident within replicate batches or between treatments NC, GC, OC and RC. Feed conversion ratio (FCR) did not differ significantly within replicate batches NC, OC and RC. A low weight gain and high FCR was given by SC. Weight gain did not vary significantly with feed intake.

A significantly higher weight gain and lower FCR was given by CR whilst the lowest weight gain and highest FCR were given by MR, whose results were comparable only with meal from spoilt offal. Available lysine values (ALV) did not show significant decrease in the OC batches, but were lower in the GC batches. These results show that the nutritional value of meat meal is not adversely affected by varying the time of cooking from 1 to 2¼ h, by severe overprocessing or by addition of glucose, but that gross spoilage will decrease the nutritive value.

1. Introduction

The rendering industry is of considerable importance to the meat industry as a whole. Of the two main products, meat meal and tallow, the former is extensively utilised in pig and poultry rations as a protein supplement in grain based rations.

The by-products industry and the feedstuffs industry are therefore concerned that production techniques do not impair the feeding value. That variation occurs in nutritive value of meat meals is well established.[1–4] Surveys of large numbers of meals have been conducted on commercial products for which the ingredient and process history are for the most part unknown. These surveys, which tested the meals by a variety of chemical and biological procedures,[4–8] yielded much valuable information but did not resolve the relative influences of processing as against ingredient composition.

Many workers have shown that it is possible to inflict such heat damage on food[9] and feedstuffs[10,11] that the nutritive value is impaired. Heat damage generally causes a decrease in digestibility which results in accumulation of less digestible peptides that compete for active transport sites thus slowing absorption from the intestine;[12,13] there is then time for the intestinal microflora to act on the products of protein digestion bringing about some deamination and so contributing to the overall loss in nutritive value of the protein. Evidence from chemical analyses has also shown

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that the generally low and variable nutritive value of meat meals may also be related to the intrinsically poor nutritional quality of many of the raw materials used in manufacture.[14–16] The effects of some laboratory drying and heat processes on a variety of animal tissues combined into meat meals have shown that both the nature of the material used and the severity of the heat treatment can alter nutritive value.[17,18]

The work of Herbert and Norgate[19] and Herbert and Mamers[20] has documented the sequence of events that occur during the dry rendering process. With this knowledge it has become possible to determine the effects on meat meal quality of ingredient composition[21] as opposed to processing variables.[25,26] Herbert *et al.*[25] showed that under commercial conditions prolonged overcooking of a mixed hard/soft offal charge did not detract from the quality of the product. Following this it was shown[21] that when different offal types are rendered, the result is a wide variation in the nutritive value of the meat meals produced.

The present study set out to extend these results by using a single offal (sheep stomachs) collected each time in the same way, and rendered under controlled conditions, simulating commercial practice, to yield a variety of meals whose full process history was known. The offal chosen had given meat meals of acceptable nutritive value in the previous study[21] and was taken to be a sensitive indicator of processing conditions. The project was thus undertaken to ascertain whether large variations that may occur in commercial rendering have any real bearing on the nutritive value of meat meal in practical terms.

2. Experimental

2.1. Raw materials

The sheeps stomachs were obtained in the rendering department of the local abattoir, directly after evisceration and inspection. They were washed free of contents and hashed by the normal commercial system and on a weight basis comprised mainly the rumen. The commercial meat and bone meal was obtained from a local supplier.

2.2. Process cooker and conditions

The cooker, a scaled down model of a large commercial cooker[19] 1.2 m long × 0.48 m diameter with a nominal capacity of 150 kg, has been fully described by Bremner.[22] The cooker was equipped with solid metal beaters to stir the contents and sweep the steam jacketed walls. The temperature of the contents was measured using thermocouples mounted in the end plates and the end point of each normal cook was taken to be when this temperature reached 122°C.

Preliminary experiments showed that the heat and mass transfer conditions established in a commercial cooker were applicable and that the total time to end point for the cook could be controlled by adjustment of the steam pressure (temperature) in the jacket. Process conditions for the tests could be set to simulate those used in commercial production. The typical charge weight was 140 kg and all cooks were done under "open vent" conditions, i.e. with a pressure above the contents of approx. 1 atm. Figure 1 shows the time/temperature history of the meals. The following conditions were used:

1. Normal cook (NC)—process time 2¼ h, discharged at 122°C end point.
2. Overcooked (OC)—produced as for NC but held in cooker at 122 to 125°C for a further 2 h after end point.
3. Rapidly cooked (RC)—process time 1 h, discharged at 122°C end point.
4. Added glucose cook (GC)—produced as for normal cook with 500 g D(+)-glucose added at the start of the cook, i.e. 0.36% on wet weight giving approx 2.5% on dry fat free meal.
5. Spoilt offal cook (SC)—produced by dividing a barrow load of offal into two parts. One part was rendered as for NC, immediately. The other part was stored for 24 h before rendering under the same conditions as for NC.

With the exception of SC the delay between slaughter and start of cooking of the offal was between 1 and 1½ h.

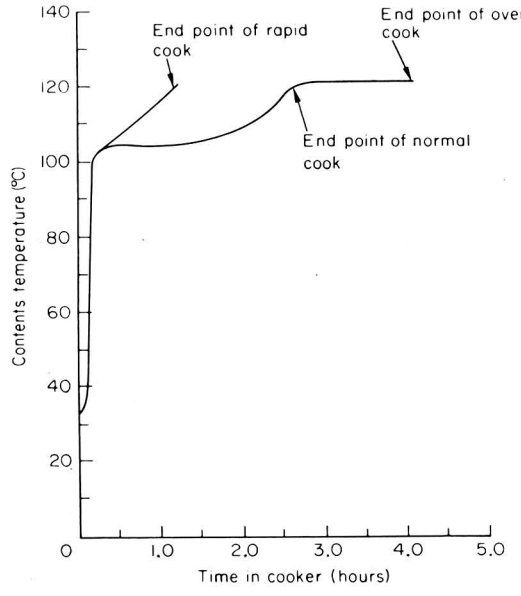

Figure 1. Time–temperature history of rapid cook (RC), normal cook (NC) and overcook (OC) meat meals.

After rendering, the meals were centrifuged free from the bulk of the tallow, spread to cool, bagged within 24 h and stored at ambient temperature. After 1 month the meals were ground through 3/16 in screen and sieved. They were then stored at 1 °C prior to despatch for testing.

2.3. Chemical analysis

Moisture, crude protein and crude fat were determined according to the methods of the AOAC.[23]

Available lysine (ALV) was determined by the chemical method of Carpenter.[10] Calcium was determined by atomic absorption spectrophotometry after wet ashing, and phosphorus by the method of Fiske and Subbarow.[24]

2.4. Feeding experiment

Day-old male broiler chicks were reared on a commercial broiler starter ration in an electrically heated, wire-floored, battery brooder unit with feed and water provided *ad lib.* After 7 days the chickens were individually weighed to an accuracy of (± 0.1 g) and were distributed at random within weight ranges to form groups of ten. Two replicates of ten chicks were fed the test diet *ad lib.* over the next 8 days and then were reweighed. A further set of two replicates of ten chicks each were fed the same diets (freshly made up) 4 weeks later. Total feed consumption per group was recorded and corrected for mortalities. Composition of the experimental diets is shown in Table 1.

The test diet used was a ration shown by Sathe and McClymont,[1] and subsequently by other workers[26,28] to be suitable for evaluating differences in meat meal quality. The test meat meals contributed one half of the protein of wheat-based diets containing 20% crude protein. With such a high proportion of meat meal protein in the diets, amino acid deficiencies of the diets reflect those of the test meals and not those of the cereal.[26] Since wheat-meat meal diets are usually known to be limiting in lysine and methionine[30,31] emphasis is placed on the meat meal as a source of these amino acids in the diet. Moreover, since lysine and methionine are the two amino acids most prone to heat damage,[10,32] this test system should be particularly sensitive to changes in their levels, e.g. due to over-processing. By employing a high quality offal containing no bone, it was

possible to keep the calcium and phosphorus level equalised at the optimum level for the growing chick.[27]

3. Results

3.1. Chemical analysis

The crude protein, moisture and fat determinations show little variation over the separate batches of meal (see Table 2). Though SC had a fairly high residual moisture content no spoilage was apparent on storage. The higher calcium levels for NC1 and NC2 are most probably due to the presence of some bony material in the offal. A typical analysis of the commercial meat and bone meal from the supplier is included in note *d* to Table 1.

Table 1. Composition of experimental rations

Ration[b,c]	Meat meal sample	% of variable ingredients		
		Wheat	Meat meal[d]	Limestone
A	CR		Control[a]	
B	NC1	77.15	14.00	1.10
C	NC2	77.65	13.40	1.20
D	NC3	77.95	12.90	1.40
E	GC1	77.55	13.40	1.30
F	GC2	77.45	13.40	1.40
G	OC1	78.25	12.60	1.40
H	OC2	77.95	13.00	1.30
I	RC1	77.35	13.50	1.40
J	RC2	76.65	14.30	1.30
K	SC1	77.45	13.40	1.40
L	MR	75.55	19.20	—

[a] The control ration contained the following ingredients, expressed as a percentage of the diet; wheat 74.05, fish meal 9.70, soyabean meal 5.00, skim milk powder 5.00, dripping 4.00, salt 0.25, calcium phosphates 1.30, limestone 0.70, methionine 0.10, and vitamin–mineral premix (see below).

[b] The following ingredients were constant for rations B to L (% of diet): skim milk powder 5.00, salt 0.25, and calcium phosphates 2.50.

[c] A vitamin and mineral supplement was added to all rations and provided per kg of diet: vitamin A, 15 000 i.u.; vitamin D_3, 1500 i.u.; vitamin E, 30 mg; thiamine, 4.5 mg; riboflavin, 8.0 mg; menadione dimethylpyrimidinol bisulphite, 2.0 mg; calcium pantothenate, 10.0 mg; pyridoxine, 4.0 mg; vitamin B_{12}, 20.0 μg; niacin, 40.0 mg; choline, 600.0 mg; folic acid, 1.0 mg; manganese, 60.0 mg; zinc 50.0 mg; cobalt, 1.0 mg; iodine, 1.1 mg; copper, 2.0 mg; ethoxyquin, 125.0 mg; and zinc bacitracin, 20.0 mg.

[d] A typical analysis of meat and bone meal from the commercial supplier is as follows (%): moisture 5.5, crude protein 44.6, crude fat 11.8, calcium 12.9, phosphorus 5.6.

The ALV results for NC, OC and RC (mean 5.90) show no consistent variation with processing but the mean (5.00) of the "added glucose" cooks, GC, is significantly lower ($P < 0.01$), indicating that reaction of the added glucose with some of the ϵ-amino groups of lysine has occurred during the processing. The SC meal had a high ALV indicating that there was no loss by bacterial degradation and also that advanced proteolysis had not occurred, sufficient to expose α-amino lysine groups which would result, in the analysis, as doubly dinitrophenylated molecules and thus not be determined as ALV.

3.2. Feeding experiment

Table 3 shows the weight gains and feed conversion ratios (FCR) of chicks fed the meat meal-based diets. The results for NC1, NC2 and NC3 give a measure of the reproducibility of the procedure

Table 2. Chemical analyses of meat meals

Sample no.	Crude protein (%)	Moisture (%)	Crude fat (%)	Ca (%)	P (%)	Available lysine (g/100 g crude protein)[a]
NC1	58.4	9.4	18.9	0.77	0.21	5.77
NC2	61.2	8.8	17.0	0.55	0.15	5.70
NC3	63.5	7.5	18.5	0.11	0.12	6.40
GC1	61.2	10.0	17.4	0.20	0.17	5.29
GC2	61.1	8.8	18.3	0.10	0.09	4.71
OC1	65.2	7.2	16.9	0.11	0.13	5.29
OC2	62.9	8.1	18.0	0.18	0.15	6.39
RC1	60.5	9.3	19.4	0.13	0.12	6.26
RC2	57.6	9.8	21.0	0.19	0.16	5.62
SC	61.3	10.7	19.0	0.14	0.09	6.24

[a] Means of duplicate analyses. Mean (difference between replicates) = 0.08, s.e. = 0.08.

Table 3. Mean weight gains, feed intake and feed conversion ratio (FCR) of two sets of two replicates, each of ten chicks, in feeding experiment analysed by analysis of variance

Ration	Meat meal	Mean weight gain in 8 days (g)	Feed intake (g)	Feed conversion ratio (FCR)*
A	CR	144.97^{a}	221.0	1.52^{a}
B	NC1	$118.97^{b,c}$	213.6	$1.80^{c,d,e,f}$
C	NC2	126.95^{b}	221.1	$1.74^{b,c,d,e}$
D	NC3	$125.95^{b,c}$	213.5	$1.70^{b,c}$
E	GC1	127.23^{b}	209.9	1.65^{b}
F	GC2	$118.75^{b,c,d}$	217.3	$1.83^{e,f,g}$
G	OC1	$115.28^{c,d}$	215.0	$1.87^{f,g}$
H	OC2	$120.59^{b,c}$	217.0	$1.81^{d,e,f}$
I	RC1	$119.30^{b,c}$	205.0	$1.72^{b,c,d}$
J	R	$123.14^{b,c}$	209.0	$1.70^{b,c}$
K	SC	$107.39^{d,e}$	201.5	$1.88^{f,g}$
L	MR	104.86^{e}	201.1	1.92^{g}
Standard error of treatment means (36 d.f.)		3.98	5.86	0.036

* Feed conversion ratio (FCR): Feed intake (g)/mean live weight gain (g).
^{a,b,c,d,e,f,g} Values followed by same superscript not significantly different at $P < 0.05$.

used to produce the test meals and form the basis of performance and variability against which the other meals are measured. They give quite close results.

The RC meals, which were produced in order to assess whether faster cooking time would inflict less heat damage, gave close duplicates and showed no nutritional improvement over the NC meals, either in weight gains or in FCR. The OC meals, produced by agitating the meals in the cooker for 2 h after the normal end point was reached, gave reasonable duplicates which showed no decrease in nutritive value. These results imply that, in practice, wide variations in processing conditions do not affect the nutritive value of the meal.

The spoilt meal, SC, did show a significant decrease in weight gain and a high FCR. Feed intake was also markedly lower. Microbial degradation of the raw materials used in the SC cook had obviously progressed to an advanced state, and the accompanying exothermic reactions maintained

heat in the offal overnight such that although the ambient temperature overnight was 20°C the offal temperature next morning was 34°C. Foul odours were generated during the cooking cycle and the resultant meal was straw yellow in colour and malodorous. It was noted that the tallow from this cook was dark brown and had a pungent odour and high free fatty acid content (approx. 40%).

Results of the chick growth test for the added glucose meals, GC1 and GC2, show no significant difference (5% level) for weight gain as compared with NC1, NC2 and NC3, but GC2 shows a higher FCR; this may reflect the well-known fact that where chemicals are added to foodstuffs the nature and extent of the reaction is not always consistent, particularly since foods contain a wide variety of complex substances of differing solubilities and particle sizes both of which criteria affect degree of reaction.

4. Discussion

Neither severe overprocessing nor addition of glucose had significant deleterious effects on the protein quality of the meat meals as assessed by chick growth assay. The results of Skurray and Herbert[21] demonstrate that a factor such as excess of calcium can lead to a reduction in the growth potential of the fish soybean control ration, holding it down to the level of the sheep stomach meal. It could be argued that some other unconsidered factor was holding the results for NC, OC, GC and RC together as a group. Whilst it is possible that diets could be formulated that would show up differences in the meals due to processing, such differences would scarcely be detectable in commercial use where other protein concentrates are incorporated in the diet and where meat meal would contribute a smaller proportion of the total protein. All experimental meals gave better weight gains than a single commercial meal but poorer results than a control ration where fish meal and soyabean meal replaced meat meal. The poorer performance of the commercial meal can be ascribed to its bone content.[14,29]

Putrefaction in the offal prior to cooking decreased the nutritive value of the resultant meal even though both crude protein and ALV remained high. Fish meals made from spoilt fish viscera have also shown lower nutritive value.[39] During holding of proteins, particularly intestinal protein, microbial and enzymic activities occur. These reactions may produce metabolites which are to some extent toxic. Olley[39,40] has discussed the production of histamine and other imidazole compounds and Mackie[42] has shown that proteolysis causes the destruction of histidine.

In this experiment, employing *ad lib.* feeding, the weight gains did not vary significantly with feed intake over the series of meals. However, the lower feed intakes of SC and MR have important commercial implications.

The present work agrees with that of Herbert *et al.*,[25] where batches of meal were "overcooked" on a commercial scale. Here the results are extended by having replicate batches of a meal of known high quality evaluated in a sensitive test. The results are in line with those of Kondos and McClymont[33] who found that temperatures up to 138°C did not affect biological availability of the amino acids of meat meal samples as measured by assay with *Tetrahymena pyriformis*, nor was their growth promoting ability for chickens affected.

The conclusions presented here also concur with those of Gartner[34] who obtained meat meals 5 min undercooked, normally cooked and 5 min overcooked from a commercial manufacturer and who concluded that for broiler chickens the nutritive value of a particular meat and bone meal was not significantly affected by the processing conditions.

The ALV results show no decrease ascribable to prolonged heating and no change in retention as a result of rapid cooking when compared to normal cooking, presumably for the reasons discussed by Skurray and Cumming,[26] that the Maillard reactions responsible for decreasing ALV have occurred before the end point is reached and that further processing has little effect.

The cookings with added glucose show lower ALV levels. The decrease was not the same in both cooks nor has it been reflected in significantly lower weight gains since GC1 exhibits the highest weight gains and low FCR whilst GC2 has a lower weight gain. Theoretical calculations based on the total lysine value reported for sheep stomach meal[21] indicate that with no loss of availability,

ALV should be 6.8 g/100 g crude protein. Smooth muscle, of which the stomachs are mainly comprised, generally contains between 0.7 and 1.1 mg/g, glucose.[35] Such an amount if reacting stoichiometrically would reduce ALV to 5.9–6.2 g/16 g N in good agreement with the values obtained (NC1, NC2, NC3, OC2, RC1, RC2, SM1). The 2.78 mol glucose added to GC1 and GC2 could therefore decrease ALV to between 3.9 and 3.8 g/16 g N if fully reacting with the ϵ-amino groups of lysine. The ALV results obtained for GC1 and GC2 are consistent with only about 50% of the added glucose reacting with the lysine, a figure which is in line with available data on fish muscle quoted by Jones.[41]

It is more probable that low ALV levels in meat meals reflect low total lysine in the initial raw materials used to produce the meal, with however, some reduction in ALV inevitably occurring even in the best rendering practice due to the inherent glucose plus glycogen content of the tissues.[26] Also, Atkinson and Carpenter[17] have shown not only that tendon and ossein have low levels of total lysine, but that their overall essential amino acid content is low;[3] gelatin, too, is deficient in nearly all essential amino acids.[36,37] Obviously where these materials comprise a large proportion of the raw materials, the product cannot be high in nutritive value[21] nor high in total lysine.

Carpenter[38] has pointed out that there are two types of heat damage which will affect the nutritional value of protein supplements: (a) that which occurs readily under mild conditions; and (b) that which occurs under more severe conditions. Whilst the former occurs as a matter of course in the dry rendering process,[26] this study indicates that even the severe conditions obtained during overprocessing in commercial dry rendering practice do not affect the usefulness of the product as a protein supplement in wheat-based commercial poultry rations.

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Detection and Assay of Single Cell Protein Products in Blends with Animal Feeds

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Electrophoretic patterns of protein extracts obtained with different procedures from the BP-yeast product named Toprina (a *Candida lipolytica* strain grown on n-alkanes) have been compared with protein electrophoretic patterns of a number of different microbial species. The gel electrophoretic pattern of the 0.15 M NaCl extract from Toprina was specific enough to allow an easy differentiation of Toprina from the other microorganisms tested.

To detect and assay Toprina in blends with animal feeds, an immune serum containing antibodies reacting specifically with Toprina has been prepared by immunising rabbits with an antigen preparation extracted from Toprina with 0.15 M NaCl and precipitated by salting out the extract at 4.0 M $(NH_4)_2SO_4$. Three main antibodies reacting with Toprina antigens have been found in the anti-Toprina immune serum, but only one was specific of Toprina. The resistance of the Toprina antigens to peptic digestion and their behaviour on extraction, electrophoresis and gel filtration suggest that they might be acidic polysaccharide in nature.

Immunodiffusion analyses with the anti-Toprina immune serum of extracts obtained with 0.15 M NaCl from very heterogeneous animal feeds added with different amounts of Toprina, allowed the detection of a Toprina amount as low as 2.5%. An accurate assay of Toprina was achieved by submitting the feed extract to radial immunodiffusion with the anti-Toprina immune serum.

1. Introduction

A number of industrial processes for the production from different substrates of protein-rich biomasses are being developed in several countries.[1–10] With the main exception of a method[11] to recognise the carbon origin by measuring the radioactivity of its ^{14}C isotope that might be applied only to biomasses grown on petroleum-derived products, very little work has been devoted to the study of suitable methods to detect and assay single cell products in blends with traditional feeds or other products. In fact, such a control might be very desirable both for safety and commercial reasons.

To develop a general strategy for the solution of this problem we chose as working material the BP-yeast product consisting of dried cells of a *Candida lipolytica* strain grown on a medium containing n-alkanes. The Italian common name of this product is Toprina. Since Toprina was under trial for safety evaluation at the Istituto Superiore di Sanità (ISS), it was available to us in large amounts.

This paper deals with an electrophoretic and immunochemical characterisation of some Toprina components designed to find out at least one protein or antigen fraction specific of Toprina to be used as a molecular marker of Toprina in blends with other materials. We have also studied analytical procedures for the detection and measurement of the specific marker in very heterogeneous mixtures such as crude extracts from Toprina-containing animal feeds.

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As far as we know, although a number of serological studies on yeasts have been carried out,[12–14] no extensive characterisation study of Toprina proteins and antigens has been reported up to now.

2. Experimental

2.1. Strains

Toprina and the *Candida lipolytica* strain used for Toprina production were obtained from Italproteine, Milan, Italy. Liquipron (the Kanegafuchi yeast product consisting of *Candida* strain grown on hydrocarbons) was obtained from Liquichimica, Milan, Italy. Baker's yeast was an unclassified commercial product. All the other microorganisms were cultured at the ISS under the following conditions. The *Escherichia coli* (ISS 8001, isolated from feed) and *Bacillus subtilis* (NCTC 8236) strains were grown in nutrient broth (Difco) for 12–18 h in agitated flasks at 30°C. The *Streptomyces* sp. (ISS 1418, isolated from walnuts) was incubated for 24 h at 30°C in agitated flasks containing a 0.5% autolised yeast and 1% glucose solution. The *Penicillium brevicompactum* (ISS 1439) strain was cultured for 36–48 h at 30°C in agitated flasks containing Czapek–Dox's liquid medium with 3% glucose and 0.5% corn-steep liquor. Two strains of *Candida lipolytica* (5657, isolated from limburg cheese, and 5654, CBS Delft) were obtained from the Istituto di Microbiologia Agraria, Perugia, Italy. They were grown for 18–24 h at 30°C in agitated flasks containing a medium with the following composition: 1% corn-steep liquor, 1% Jamaican molasses, 3% sucrose, 0.05% NH_4NO_3, 0.01% KCl, 0.03% $Na_2HPO_4 \cdot 12H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$. The BP *C. lipolytica* strain was grown at 28°C in a 50-litre stainless steel fermenter for 22 h in a medium containing 0.57% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.001% $CaCl_2$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4$, 0.1% yeast extract, 0.3% urea, 3% glucose, 0.0001% silicone. The pH of this medium was 5.7. The fermentation medium (30 litres) was kept under constant mechanical agitation (400 rev/min) and air was admitted at 1 atm. overpressure with a flow rate of 50 litres/min.

Unless otherwise stated, at the end of the fermentation the cells were collected by centrifuging at 12 000 × *g*, washed 4 times with water and lyophilised.

2.2. Preparation of extracts from Toprina and other microorganisms

Unless otherwise stated, Toprina and the other microorganisms were extracted by homogenising 1 g of material in a Potter homogeniser with 10 ml of 0.15 M NaCl at 0°C. Then, the suspension was left for 3 h at 4°C. At the end of the extraction, the suspension was centrifuged at 45 000 × *g* for 30 min and the clear supernatant was freeze-dried. About 50 μl of water solution containing 20 mg/ml of lyophilate were submitted to gel electrophoresis and 15 μl were used for double immunodiffusion analysis as described below.

A larger quantity of Toprina extract was required for injection into rabbits to obtain the antigen preparation and was made by suspending 10 g of material in 100 ml of the salt solution for 3 h with mechanical mixing. The suspension was centrifuged for 30 min at 45 000 × *g*. The clear supernatant was made 4.0 M with $(NH_4)_2SO_4$ and the resulting precipitate collected by centrifuging at 45 000 × *g* for 30 min, redissolved in water, dialysed against water for 5 days at 4°C, and lyophilised.

2.3. Preparation of extracts from feeds

Two types of animal feeds (pellet and flour) containing known amounts of Toprina were analysed. The pellet type contained corn flour (60%), wheat bran (17%), vitamins (3%) and different amounts of peanut flour and Toprina to make the remaining 20% (see Figure 4D). The flour type contained soybean flour (23%), fish meal (6%), milk whey (5%), alfalfa (3%), $CaHPO_4$ (1%), $CaCO_3$ (1%), NaCl (0.5%), vitamins (0.5%) and different amounts of corn flour and Toprina to make the remaining 60% (see Figure 4D).

For extraction, 10 g of finely ground feed were suspended in 100 ml of 0.15 M NaCl and stirred for 3 h under mechanical mixing. At the end of the extraction, the suspension was centrifuged at 45 000 × *g* for 30 min and the clear supernatant was freeze-dried. Fifteen μl of aqueous solution

of the lyophilate (10 mg/ml) were used for double immunodiffusion and 12 μl for radial immunodiffusion analyses.

2.4. Preparation of anti-Toprina immune serum

Ten New Zealand rabbits, each about 3.5 kg body weight, were individually injected with 0.5 ml of antigen solution (10 mg total lyophilate) emulsified with the same volume of Freund's complete adjuvant. About 0.3 ml of this suspension was injected into the foot pad of the posterior limb in each rabbit, and the residual 0.7 ml was given by intramuscular injection. After 21 days a second injection of 2 ml of antigen solution (40 mg total lyophilate) was given intravenously. Following that, the rabbits were injected twice (after each 10 days) with 1 ml of a saline antigen solution containing 1 mg of antigen/ml. After 8 days the rabbits were bled from the marginal vein of the ear to check the antibody levels, then submitted to total bleeding. The sera were heated at 56°C for 30 min to inactivate the complement and stored at −30°C.

2.5. Immunodiffusion analyses

Agarose gel immunodiffusion analyses were carried out according to a slight modification of Wieme's micromethod as described by Piazzi *et al.*[15] Additional immunodiffusion tests were carried out, at different levels of reactions, according to Piazzi.[16] Immunodiffusion analysis performed to localise the position of Toprina antigens on polyacrylamine gel was carried out according to the procedure previously described by Minetti *et al.*[17] Radial immunodiffusion was carried out according to Lietze,[18] using 0.2 ml of antiserum per slide.

2.6. Gel electrophoresis

Disc electrophoresis was carried out in a 0.05 M Tris–0.383 M glycine buffer (pH 8.5) as previously described for wheat albumins.[17] Protein patterns were recorded by a Gilford Spectrophotometer Mod. 2400 (Gilford Instrument Laboratories Inc., Oberlin, Ohio) at 620 nm.

2.7. Gel filtration

About 1 g of the antigen preparation from Toprina was submitted to gel filtration on a Sephadex G-100 column (120 × 6 cm) equilibrated in 0.1 M ammonium acetate buffer (pH 7.8). The absorbance of the eluate at 280 nm was measured continuously (Photochrom flow-analyser, Rastelli, Italy) and recorded (Kompensograph, Siemens, Germany). Twenty × 1 ml fractions were collected and submitted to immunodiffusion analysis as described above. Fractions containing antigenic activity were pooled, lyophilised and submitted to gel electrophoresis and immunodiffusion analysis according to the procedure described by Minetti *et al.*[17]

2.8. Digestion with pepsin

Three hundred μg of pepsin were added to 1 ml of HCl solution (pH 2.2) containing 10 mg of antigen from Toprina. The mixture was incubated for 24 h at 37°C and then the pH was adjusted to 7.0 with 0.1 N NaOH.

2.9. Nitrogen determination

The nitrogen content of the extracts from Toprina and other materials were determined according to Kjeldahl.

3. Results

3.1. Nitrogen extraction

When 100 g of Toprina was dispersed in 0.15 M NaCl (1:10 w/v) with a Potter homogeniser and rapidly centrifuged to clarify the extract, about 0.95 g of nitrogen was extracted. When the homogenate was allowed to stand for 3 h at 4°C or 37°C before centrifuging, 1.1 g of nitrogen was extracted from 100 g of Toprina. No improvement in nitrogen extraction was obtained by allowing

the homogenate to stand at 4°C for periods of time up to 24 h or by using 0.01 M acetic acid, 5 M urea, or 0.01 M NaOH as extraction solvents instead of the salt solution. Light microscopy observation showed that in no case had a significant cell disruption occurred. Therefore, we tried to improve nitrogen extraction by treating the biomass in a press (X-press, AB Biox, Nacka, Sweden) at pressures up to 6 atm. Such treatment, however, was ineffective either in causing cell fragmentation or in improving nitrogen extraction.

About 1.7 g of nitrogen was extracted with 0.15 M NaCl under standard extraction conditions from 100 g of the biomass obtained by growing the BP *Candida lipolytica* strain in the laboratory under the conditions described in the experimental section. After treatment with the X-press about 4 g of nitrogen could be extracted with 0.15 M NaCl from 100 g of this material. In this case light microscopy observation showed extensive cell disruption after the X-press treatment. However, after the biomass had been heated for 15 min at 80°C and then dried overnight at 110°C, only 1.1 g of nitrogen could be extracted from 100 g of the biomass even after treatment with the X-press, which is very similar to the result obtained with Toprina.

We succeeded in obtaining a more effective nitrogen extraction from Toprina (about 7 g/100 g of material) only by suspending the biomass in 1.0 M NaOH at room temperature for 24 h.

3.2. Gel electrophoresis

When submitted to polyacrylamide gel electrophoresis in a Tris–glycine buffer system (pH 8.5), the extracts obtained from the same batch of Toprina under the different experimental conditions described in section 3.1, with the exception of the 0.1 M NaOH extract, gave electrophoretic patterns all closely related to that shown in Figure 1(a). No distinct electrophoretic pattern could be obtained from the 1.0 M NaOH extract from Toprina even after neutralisation and exhaustive dialysis against distilled water, indicating extensive denaturation of the protein in this case. A few minor qualitative differences and some significant quantitative differences were evident among the electrophoretic patterns of extracts obtained with 0.15 M NaCl from different lots of Toprina [Figure 1(a), (b), (e)]. As shown in Figure 1, large qualitative differences were evident among the electrophoretic patterns of Toprina and those of all the other microorganisms tested [Figure 1(d), (f), (g), (h), (i), (l)], including the two strains of *Candida lipolytica* 5657 and 5654 which gave identical patterns [Figure 1(c)]. The electrophoretic pattern of the BP *C. lipolytica* strain grown on the synthetic laboratory medium was more closely related to the electrophoretic pattern of the two *C. lipolytica* strains than to those of the three lots of Toprina tested.

3.3. Immunochemical studies

An anti-Toprina immune serum was prepared by injecting a salt-soluble extract from Toprina into rabbits. By submitting the anti-Toprina immune serum to immunodiffusion analysis at different levels of reaction with the 0.15 M NaCl extract from Toprina (Figure 2), we showed the presence in the immunodiffusion pattern of three main precipitation lines. This result was further confirmed by comparing (Figure 3) the electrophoretic pattern of the protein extract from Toprina [already reported in Figure 1(b)] with the immunodiffusion pattern of the same extract submitted to gel electrophoresis and then reacted with anti-Toprina immune serum (see experimental section). With this more sensitive technique, in addition to the three main precipitation lines, we showed the presence of four very faint precipitation lines not detectable in the double immunodiffusion system. No direct correspondence was found between the protein bands and the precipitation lines (Figure 3), showing that Toprina antigens cannot be identified with protein components. This interpretation was supported by the fact that, after peptic digestion of the antigen preparation, protein bands were no longer detectable in the electrophoretic pattern, whereas the number and the position of the precipitation lines in both the immunodiffusion and immunoelectrophoretic patterns were unaffected. To evaluate apparent molecular weights of Toprina antigens, the 0.15 M NaCl extract from Toprina was submitted to gel filtration on a Sephadex G-100 column and antigenic activities of the eluted fractions were determined. The absorbance at 280 nm of the same fractions was measured as well. With this procedure we showed that the three main antigens indicated in Figures 2

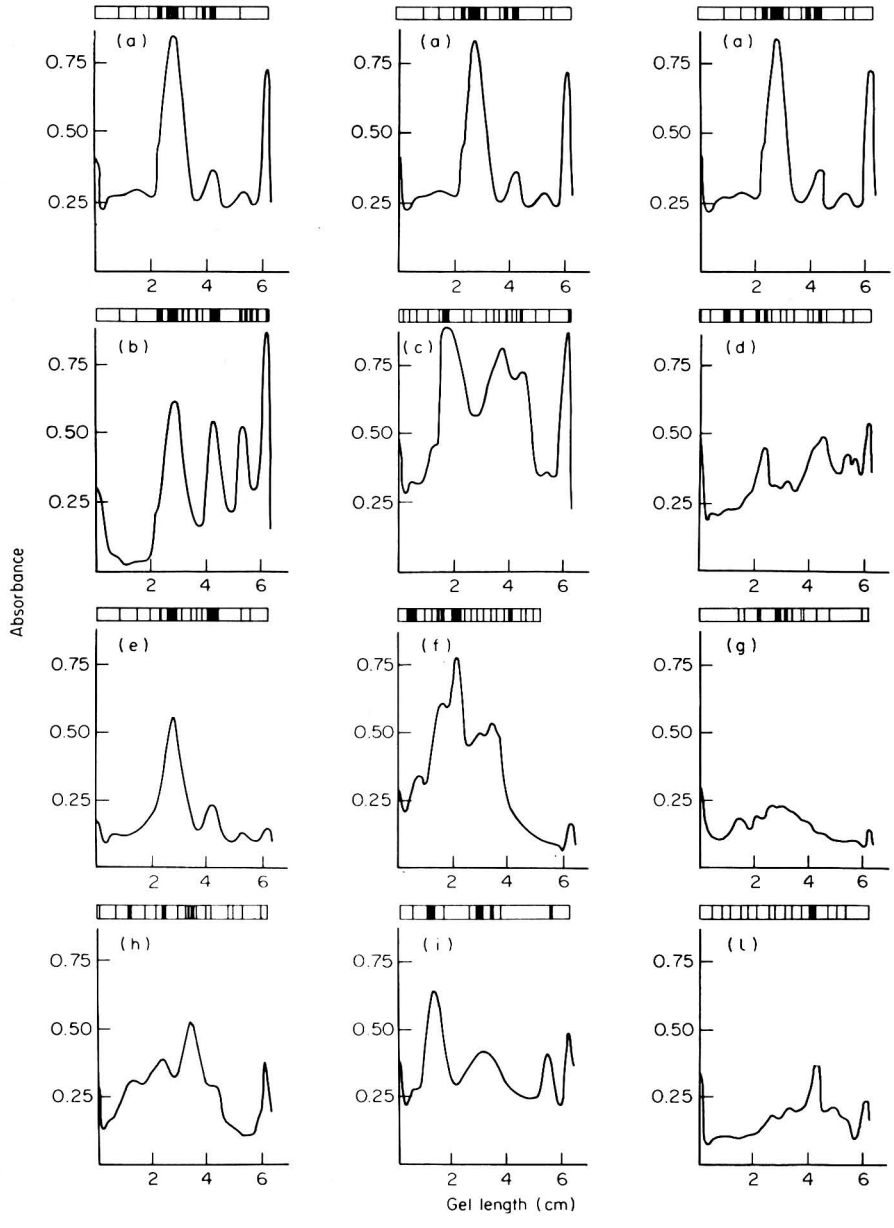

Figure 1. Gel electrophoretic patterns of 0.15 M NaCl extracts from Toprina, Liquipron, and other biomasses. (a) Toprina batch A; (b) Toprina batch B; (c) *Candida lipolytica*; (d) *Bacillus subtilis*; (e) Toprina batch C; (f) commercial baker's yeast; (g) *Bacterium coli*; (h) Liquipron; (i) *Penicillium brevicompactum*; (l) *Streptomyces* sp.

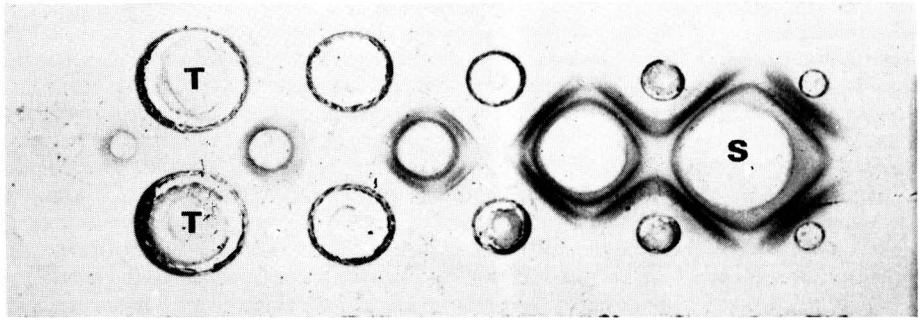

Figure 2. Double immunodiffusion analyses at different levels of reaction of 0.15 M NaCl extract from Toprina (T) with anti-Toprina immune serum (S).

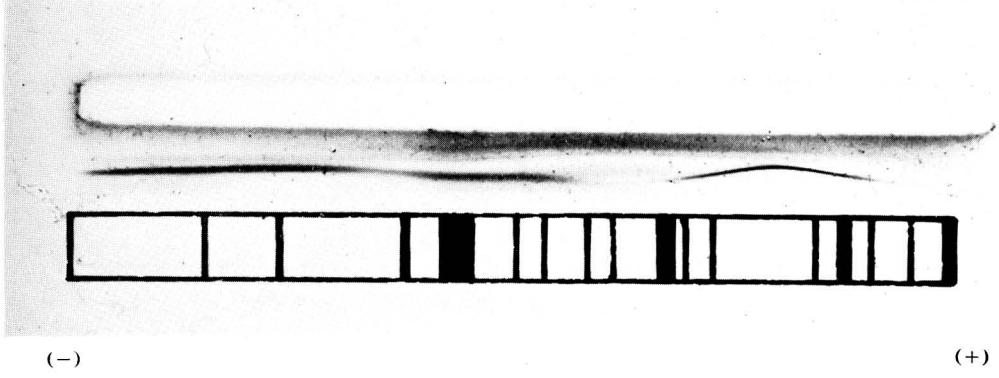

Figure 3. Comparison of gel electrophoretic protein pattern (bottom part) and immunoelectrophoretic pattern (upper part) of 0.15 M NaCl extract from Toprina.

and 3 have different apparent molecular weights. The two antigens moving slower during the gel electrophoretic fractionation (Figure 3) were eluted in the void volume of the Sephadex column, whereas the retention volume of the fast moving one corresponded to that of cytochrome *c*. Moreover, no correspondence was observed between the elution pattern of the antigens and that of the material absorbing at 280 nm.

In order to evaluate the specificity of anti-Toprina immune serum, we submitted the salt extracts from all the microorganisms of Figure 1 to immunodiffusion analysis with the immune serum. As shown in Figure 4, identical immunodiffusion patterns were given by the extracts from two lots of Toprina (Figure 4A, 2 and 4), whereas the extracts from the two strains of *C. lipolytica* only gave two precipitation lines, lacking the one closer to the antigen hole (Figure 4A, 6 and 7). The extracts from all the other microorganisms, including *Candida tropicalis* (not shown in the Figure), only gave the precipitation line closest to the antiserum hole (Figure 4B and C). Immunodiffusion analyses with anti-Toprina immune serum of salt extracts from animal feeds containing different amounts of Toprina are shown in Figure 4D. It appears that intensities of precipitation lines increased with the amount of Toprina added. Identical immunodiffusion patterns were obtained with both pellet and flour animal feeds. Only one precipitation ring was obtained when the extracts from animal feeds with added Toprina were tested with anti-Toprina immune serum by radial immunodiffusion (Figure 5A). As shown in Figure 5B, there was a linear relationship between the areas of the precipitation rings of Figure 5A and the amounts of Toprina added to the feed samples.

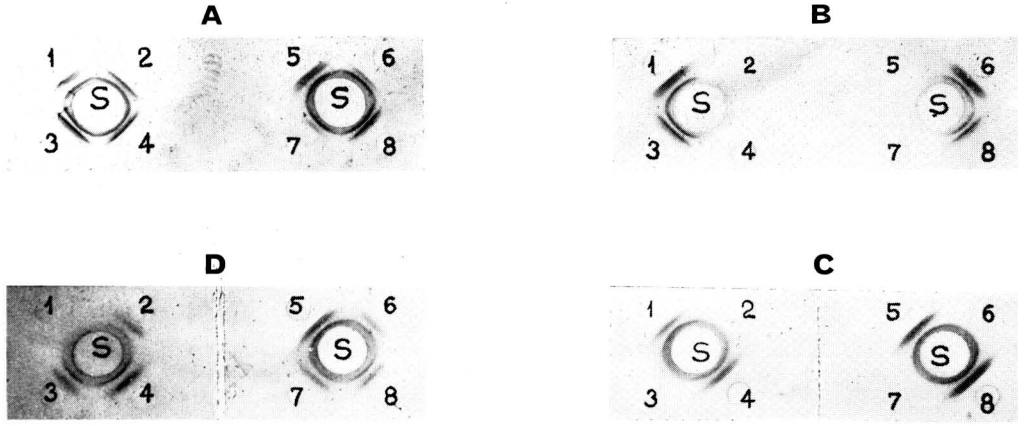

Figure 4. Double immunodiffusion analyses of 0.15 M NaCl extracts from different microbial species, Toprina, and Toprina-containing feeds with anti-Toprina immune serum.

A: 1, 3, 5, 8, controls (Toprina antigen); 2, Toprina batch A; 4, Toprina batch B; 6, *Candida lipolytica* (strain 5654); 7, *Candida lipolytica* (strain 5657).

B: 1, 3, 6, 8, controls (Toprina antigen); 2, *Penicillium brevicompactum*; 4, *Bacterium coli*; 5, *Bacillus subtilis*; 7, *Streptomyces* sp.

C: 1, 4, 5, 8, controls (Toprina antigen); 2, Liquipron; 3, commercial baker's yeast; 6, 7, mixtures of equal parts of *Penicillium brevicompactum*, *Bacterium coli*, *Bacillus subtilis*, *Streptomyces* sp. and commercial baker's yeast.

D: 1, feed without Toprina; 2, feed containing 2.5% Toprina; 3, 6, feed containing 5% Toprina; 4, 8, feed containing 10% Toprina; 5, control (Toprina antigen); 7, feed containing 15% Toprina.

4. Discussion

The data reported show that only a small amount (about 10%) of the total nitrogen content of Toprina could be extracted with a number of different procedures. The poor nitrogen extraction from Toprina appeared related to the high resistance of Toprina cells to mechanical disruption. The cells obtained by growing the BP *C. lipolytica* strain on a synthetic medium containing glucose did not show such high mechanical resistance and much higher nitrogen yields could be obtained when they were extracted under the same conditions as used for Toprina. However, the nitrogen extraction pattern of this biomass after short heating in water suspension at 80°C and drying overnight at 110°C became very similar to that of Toprina. This result suggests that the industrial processing of Toprina, which also includes a relatively high temperature drying, causes an increase of cell resistance to mechanical damage. Disrupting the cell walls might help to increase nitrogen availability when feeding Toprina to animal species not able to digest microbial cell walls.

The qualitative composition of the proteins extracted from Toprina was found to be very specific and allowed an easy differentiation of Toprina from the other microorganisms tested. Some quantitative differences found between the protein extracts from the different lots of Toprina tested might be the result of some lack of standardisation of the conditions used for biomass culture or processing. Although useful for checking the constancy of Toprina production, the gel electrophoretic patterns were too complex to be of practical help for the detection of Toprina in mixture with other feeds. In this respect, our serological investigations gave more conclusive results. We have shown that Toprina has at least one specific antigen that allows its differentiation from the other microorganisms tested, including the two strains of *C. lipolytica* that appeared most closely related to Toprina. Immunodiffusion analysis with anti-Toprina immune serum obtained by injecting a salt-soluble fraction from Toprina into rabbits allowed the detection of Toprina added to very heterogeneous animal feeds in amounts as low as 2.5%. Moreover, as long as the content of the specific antigen is constant in different lots of Toprina, a quantitative determination of Toprina can be

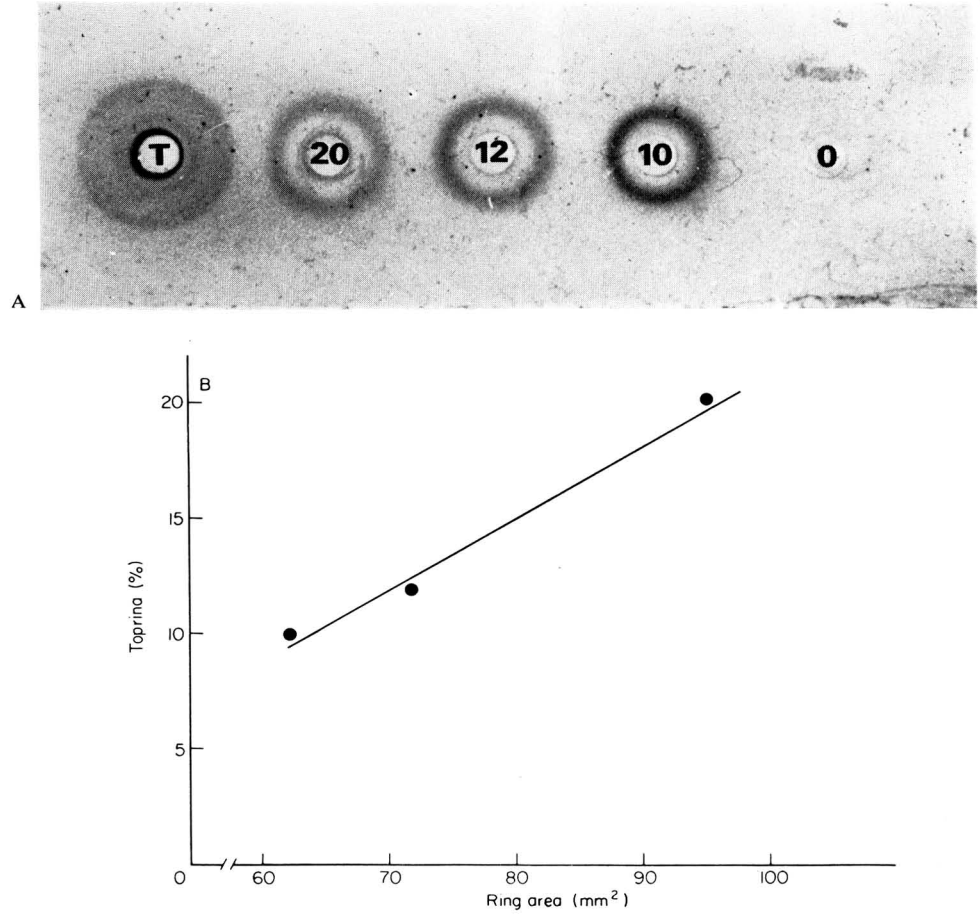

Figure 5. Radial immunodiffusion analyses of 0.15 M NaCl extracts from Toprina-containing feeds with anti-Toprina immune serum. A (from left to right), Toprina and animal feeds containing 20, 12, 10% and no Toprina, respectively. B, areas of the precipitation rings of Figure 5A are plotted against Toprina percentage of the corresponding samples.

achieved by submitting feed extracts to radial immunodiffusion with anti-Toprina immune serum.

Even though there are a number of aspects that militate against a protein nature of Toprina antigens, at this moment we have not yet established their chemical nature. The extraction, electrophoretic, and gel filtration behaviours of the Toprina antigens suggest that they might be acidic polysaccharides. This possibility is also supported by the results of Fukazawa *et al.*[13] who showed that acidic soluble polysaccharides of *Candida albicans* have a high degree of specificity and immunogenicity and that IgG produced by rabbits after immunisation with soluble polysaccharides contained a specific antibody factor capable of differentiating *C. albicans* from *C. tropicalis*.

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Effects of Volatile Bacterial Metabolites on the Growth, Sporulation and Mycotoxin Production of Fungi

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Five toxigenic fungi were grown in the presence of pure cultures of a number of different bacteria. Alterations in the gaseous environment resulting from bacterial growth produced changes in the growth, sporulation and toxin production of the fungi. Different fungi varied in their response to any one bacterium, and different bacteria produced a variety of responses from each individual fungus. The growth, sporulation and toxin production of most fungi were normally inhibited by the presence of bacteria; in a few cases toxin production was stimulated. Toxin production was able to proceed in the absence of sporulation.

The implications of bacterium–fungus interactions in certain environments are considered.

1. Introduction

Volatile metabolites from bacteria may influence growth, sporulation and morphology of fungi.[1–3] Different genera of fungi differ in their response to bacteria in dual cultures, the nature of the response being influenced by the concentrations of volatile bacterial metabolites.[2]

The volatile compounds released by bacteria include hydrocarbons, fatty acids, alcohols, aldehydes and esters,[1,4] and many unidentified compounds derived from specific bacteria.[4,5] Many of these compounds have also been derived from microorganisms other than bacteria and been shown to affect growth, spore germination and morphogenesis of fungi.[6]

Fries[6] suggested that volatile organic compounds may exert an effect on a fungus by influencing specific metabolic processes rather than by gross inhibition of metabolism, growth and development. He proposed that volatile compounds could act, in small amounts, as metabolic regulators, by a direction of metabolism along specific pathways. This suggestion could be of special importance if the accumulation of fungal secondary metabolites such as mycotoxins, on a particular substrate, could be influenced by volatile metabolites from an associated bacterial flora.

The work described in this paper was undertaken to evaluate the effect of volatile bacterial metabolites on radial growth, sporulation and toxin production of a number of toxigenic fungi.

2. Experimental

2.1. Organisms

The fungi used and the secondary metabolites they produce (bracketed) were, *Aspergillus ochraceus* ATCC 18642 (Ochratoxin A); *Penicillium citrinum* ATCC 8506 (Citrinin); *Penicillium cyclopium* ATCC 8731 (Penicillic acid); *Penicillium patulum* ATCC 18172 (Patulin) and *Penicillium islandicum* IMI 40042 (Anthraquinones).

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The bacteria used were *Klebsiella aerogenes* NCIB 10102; *Bacillus subtilis* NCIB 8872; *Lactobacillus brevis* NCIB 947; *Lactobacillus buchneri* NCIB 8007; *Pediococcus acidilacti* NCIB 6990 and *Streptococcus faecium* NCIB 943.

2.2. Media

Fungi were maintained on Bacto-Malt agar slopes and Malt Yeastrel Glucose Peptone (MYGP) agar was used as a substrate for fungal growth and toxin production.[7]

B. subtilis and *K. aerogenes* were maintained on nutrient agar in screw cap universal bottles of 27 ml capacity. The medium contained (g/litre): peptone, 10.0; Lab Lemco L29 (Oxoid), 8.0; Difco agar No. 3, 20.0; NaCl, 5.0. The pH value after sterilisation was 6.5. Other bacteria were maintained on chalk litmus milk broth containing (g/litre): yeast extract (Oxoid) 3.0; glucose, 10.0; $CaCO_3$, 10.0; litmus, 5.0; Antibiotic-free Skim milk powder L54 (Oxoid), 100 g. The pH value after sterilisation was 7.6.

Modified Rogosa (MRS) broth was used for the preparation of bacterial inocula. The medium contained (g/litre): Tryptone (Oxoid), 10.0; yeast extract (Oxoid), 5.0; dextrose, 20.0; "Tween" 80, 1.0; KH_2PO_4, 6.0; ammonium citrate, 2.0; sodium acetate, 25.0; acetic acid, 1.32; $MgSO_4 \cdot 7H_2O$, 0.575; $MnSO_4 \cdot 2H_2O$, 0.12 and $FeSO_4 \cdot 7H_2O$, 0.034.

For the evaluation of the effect of volatile bacterial metabolites on fungi, bacteria were grown on MRS agar, containing 20.0 g Difco-agar No. 3 per litre. The pH value after sterilisation was 5.8. Under the experimental conditions described, this basal medium favoured the growth of the lactic acid bacteria used, and adequately supported a comparable growth of the non-lactic acid bacteria.

Quarter-strength Ringer's solution was used throughout as a diluent.

Sterilisation of all media was carried out by autoclaving for 15 min at 121°C.

2.3. Materials

Plastic dishes (10 × 10 cm) which contained a 5 × 5 grid of $(18\ mm)^3$ compartments (Sterilin) were used in conjunction with a Ridgway-Watt automatic multipoint inoculator (Denley Instruments) and bioassay plates, 235 × 235 mm (Nunc, Denmark). Inoculum suspensions of fungi were prepared on a wrist-action flask shaker (Griffin). Inoculum suspensions of bacteria were prepared on an orbital incubator shaker (New Brunswick, G.24).

Suspensions of fungi, for microscopic examination, were prepared in an overhead vortex homogeniser (MSE). Fungi were also examined in fresh mounts prepared in water.

2.4. Measurement of growth and sporulation of fungi

Growth of a fungus was determined by the measurement of one random colony diameter. The concentricity of colonial development, under the experimental conditions employed, has been previously established.[7]

The extent of sporulation was estimated visually and scored on an arbitrary scale, from 0 (no sporulation) to 4+ (maximum sporulation), based on the intensity of colony colour imparted by spore pigmentation. Confirmation of the absence of sporulation (score, 0) was obtained by microscopic examination after homogenisation of a harvested colony in $\frac{1}{4}$ strength Ringer's solution.

2.5. Assay of fungal metabolites

The extraction of toxins from individual fungal colonies and the determination of toxin concentrations were carried out by the methods described by Barr and Downey.[7]

2.6. Preparation of inocula of bacteria and fungi

Fungi were grown on MYGP agar and harvested with a sterile glass microscope slide, after 96 h incubation at 30°C. The fungal growth, containing mycelium and spores, was suspended in $\frac{1}{4}$ strength Ringer's solution and shaken 10 min at full speed. Dilutions were made in $\frac{1}{4}$ strength Ringer's solution to obtain an inoculum size of approximately 50 000 propagules/ml. Fungi were

inoculated, as previously described,[7] into Sterilin dishes containing 2 ml MYGP agar in each compartment.

Bacteria were grown in 25 ml MRS broth, contained in a 100 ml Erlenmeyer flask, and harvested after 48 h incubation at 30°C on a reciprocal shaker working at 300 rev/min. One ml broth was used to inoculate each 100 ml MRS agar, held molten at 45°C. Each 100 ml agar was poured into the base of a NUNC bioassay plate and allowed to set on a level surface to give a seeded agar layer of uniform thickness. MRS agar seeded with a bacterial culture was incubated 24 h at 30°C to establish bacterial growth prior to association with fungi.

2.7. Experimental design

Inoculation of fungi was restricted to alternate peripheral compartments of Sterilin dishes to minimise possible interaction between fungi. This allowed the inoculation of eight fungi on each Sterilin dish. The results derived from five of these fungi, representative producers of the five different toxins investigated, are described here.

For each bacterium under investigation, three bioassay plates containing seeded agar were prepared. Three Sterilin dishes were inoculated with fungi, each dish inoculated in a different randomised pattern, and one Sterilin dish placed in each of the three bioassay plates.

To obtain an association between bacteria and fungi, Sterilin dishes with lids removed were placed in a position facing the seeded MRS agar. This position was achieved by placing a sterile aluminium test tube cap in the central compartment of the Sterilin dish, and impressing the cap into the seeded MRS agar layer to form a pillar support. This supported the plane of fungal growth 20 mm above the layer of bacterial growth. Under control conditions, Sterilin dishes were placed in association with sterile unseeded MRS agar.

In parallel experiments, which are to be reported in detail elsewhere, fungi were grown in the presence of different concentrations of acetic acid. This was achieved by substituting the MRS agar layer with Whatman 3 MM paper, saturated with different dilutions of acetic acid prepared in sterile distilled water. Sterilin dishes, inoculated with fungi in the manner described above, were placed in a position facing the saturated paper, and supporting the plane of fungal growth 15 mm from the paper.

Bioassay plates, containing Sterilin dishes, were incubated 72 h at 30°C before evaluation.

3. Results

Statistical analysis of variance of fungal colony diameter readings demonstrated that no significant variation occurred between colonies derived from replicate inoculations and exposed to any one bacterial culture. Very significant differences ($P < 0.001$), however, were recorded in the response of individual fungi to volatile metabolites produced by different bacteria.

The results obtained reflect the response of fungi to specific bacterial metabolites and do not represent a response to volatile acetic acid derived from the growth medium. In the presence of Whatman 3 MM paper saturated with different concentrations of acetic acid, the growth of all five test fungi was inhibited only by acetic acid concentrations in excess of 1% (v/v), a level unlikely to be approached under the conditions described here. In the presence of different concentrations of acetic acid, no differential effect on growth, sporulation or toxin production by the fungi was observed.

3.1. Effect of bacteria on radial growth of fungi

Radial growth of fungi was significantly influenced by the metabolism of bacteria on MRS agar (Tables 1–5). Growth of each toxigenic fungus showed a specific pattern of sensitivity to the six test bacteria. No one fungus was characterised by a significantly greater sensitivity or significantly greater resistance to the metabolites of the different test bacteria.

Colony morphology of toxigenic fungi with the exception of *P. cyclopium*, was not influenced greatly by the presence of bacteria; the spread of peripheral substrate mycelium of all fungi was

slightly increased in the presence of all bacteria. In the presence of bacteria only *P. cyclopium* produced colonies with a raised spiky appearance resulting from the formation of strands of interwoven, infrequently branched, non-sporing, aerial hyphae.

The greatest inhibition of growth of all *Penicillium* spp. tested was produced by *K. aerogenes*, which was not active against *A. ochraceus*. *B. subtilis* almost completely inhibited growth of *P. cyclopium*, and significantly inhibited *P. islandicum*, but did not significantly inhibit any of the other fungi.

The heterofermentative *Lactobacillus buchneri* significantly inhibited growth of all fungi tested, while another heterofermentative species, *L. brevis*, was significantly active only against *A. ochraceus*. The homofermentative Lactobacillaceae, exerted the least effect on growth of the test fungi, *S. faecium* inhibiting growth of two *Penicillium* spp., while *Pediococcus acidilacti* did not inhibit any of the test fungi.

Growth stimulation of the toxigenic fungi in the presence of the test bacteria did not occur.

3.2. Effect of bacteria on sporulation of fungi

The sporulation of *P. islandicum* and *A. ochraceus* was least affected by the presence of bacteria, while sporulation of *P. cyclopium* and *P. citrium* was significantly affected by all six test bacteria (Tables 1–5).

The sporulation response of each fungus may be influenced to a different degree by the various test bacteria (Tables 1 and 2). Generally *K. aerogenes*, *B. subtilis*, *L. buchneri* and *L. brevis* exerted the greatest inhibition, and *S. faecium* and *Pediococcus acidilacti* the least inhibition of sporulation.

Table 1. Effect of bacteria on growth, sporulation and patulin production by *Penicillium patulum*

Bacterium	Colony diameter (mm)[a]		Patulin (μg/colony)		
	Mean	% of control	Mean	% of control	Extent of sporulation[b]
Klebsiella aerogenes	5.5	61.1	50.0	9.8	0
Bacillus subtilis	8.2	91.1	55.0	10.8	0
Lactobacillus brevis	7.9	87.7	200.0	39.3	1+
Pediococcus acidilacti	8.6	95.5	120.0	23.6	2+
Lactobacillus buchneri	7.0	77.7	241.7	47.5	1+
Streptococcus faecium	7.4	82.2	1035.0	203.5	2+
Control (no bacterium)	9.0		508.3		4+

[a] Standard error of means, 0.6 mm.

[b] Extent of sporulation was estimated visually by spore colour *en masse* and confirmed by microscopic examination.

The sporulation of some fungi was inhibited under conditions which did not inhibit their radial growth. This is in accord with observations that sporulation is more sensitive than growth to changes in the gaseous environment.[8]

3.3. Effect of bacteria on the production of secondary metabolites by fungi

Very significant differences in secondary metabolite production were recorded when fungi were cultured in the presence of test bacteria.

Generally inhibition of toxin production accompanied significant inhibition of radial colony growth, the exceptions being the production of patulin, penicillic acid and citrinin in the presence of *S. faecium*. The production of toxic metabolites by fungi appeared to be more sensitive than radial growth to changes in the gaseous environment, and very significant decreases in toxin production

Table 2. Effect of bacteria on growth, sporulation and ochratoxin A production by *Aspergillus ochraceus*

Bacterium	Colony diameter (mm)[a]		Ochratoxin A (μg/colony)		Extent of sporulation[b]
	Mean	% of control	Mean	% of control	
Klebsiella aerogenes	14.6	94.1	25.0	33.3	1+
Bacillus subtilis	15.6	100.6	42.0	56.0	3+
Lactobacillus brevis	13.5	87.1	6.7	8.8	0
Pediococcus acidilacti	14.8	95.5	8.3	11.1	3+
Lactobacillus buchneri	12.3	79.3	41.6	55.4	1+
Streptococcus faecium	15.0	96.7	74.0	98.6	3+
Control (no bacterium)	15.5		75.0		4+

[a] Standard error of means, 0.6 mm.

[b] Extent of sporulation was estimated visually by spore colour *en masse* and confirmed by microscopic examination.

Table 3. Effect of bacteria on growth, sporulation and penicillic acid production by *Penicillium cyclopium*

Bacterium	Colony diameter (mm)[a]		Penicillic acid (μg/colony)		Extent of sporulation[b]
	Mean	% of control	Mean	% of control	
Klebsiella aerogenes	0.0	0.0	10.0	3.3	0
Bacillus subtilis	2.2	14.3	17.3	5.8	0
Lactobacillus brevis	14.4	94.1	116.6	38.7	1+
Pediococcus acidilacti	15.3	100.0	250.0	16.6	1+
Lactobacillus buchneri	12.8	83.6	83.3	27.7	0
Streptococcus faecium	14.6	95.4	425.0	141.6	0
Control (no bacterium)	15.3		300.0		4+

[a] Standard error of means, 0.6 mm.

[b] Extent of sporulation was estimated visually by spore colour *en masse* and confirmed by microscopic examination.

occurred in the absence of significant radial growth limitation. Thus, a 90% reduction in patulin production in the presence of *B. subtilis* (Table 1), an 89% reduction in ochratoxin A production in the presence of *Pediococcus acidilacti* (Table 2), and an 83% decrease in penicillic acid production in the presence of *Pediococcus acidilacti* (Table 3), occurred under conditions which did not significantly inhibit radial growth of the toxin producing fungi. Indeed, except in the case of anthraquinone production by *P. islandicum*, the presence of *Pediococcus acidilacti* greatly reduced toxin production without any accompanying inhibition of radial growth.

In the presence of *S. faecium* the production of penicillic acid, by *P. cyclopium*, patulin by *P. patulum* and citrinin by *P. citrinum* were stimulated. Citrinin production was also stimulated in the presence of *L. brevis*. The stimulation of citrinin and patulin production in the presence of *S. faecium* was accompanied by a significant inhibition of growth of the toxin producing fungi. The results (Tables 1–5) show that growth and toxin production by five toxigenic fungi may be influenced in different ways by the presence of bacteria. Toxin production may be stimulated or inhibited under conditions which may or may not influence the growth of the producing organism.

The interaction between sporulation and toxin production was also evaluated. The results (Tables 1–5) indicate that considerable toxin production (between 25% and 50% of control production) could occur when sporulation was minimal or completely inhibited. The complete inhibition of sporulation by *P. citrinum* and *P. cyclopium* was in some cases accompanied by a stimulation of toxin production (Tables 3 and 4).

Table 4. Effect of bacteria on growth, sporulation and citrinin production by *Penicillium citrinum*

Bacterium	Colony diameter (mm)[a]		Citrinin (μg/colony)		Extent of sporulation[b]
	Mean	% of control	Mean	% of control	
Klebsiella aerogenes	7.3	55.3	0.0	0.0	1+
Bacillus subtilis	12.2	92.4	83.3	24.9	0
Lactobacillus brevis	12.5	94.7	450.0	137.1	0
Pediococcus acidilacti	13.4	101.5	175.0	52.4	1+
Lactobacillus buchneri	11.0	83.3	183.3	54.8	0
Streptococcus faecium	10.8	81.8	411.5	123.2	0
Control (no bacterium)	13.2		334.0		4+

[a] Standard error of means, 0.6 mm.
[b] Extent of sporulation was estimated visually by spore colour *en masse* and confirmed by microscopic examination.

Tabje 5. Effect of bacteria on growth, sporulation and anthraquinone production by *Penicillium islandicum*

Bacterium	Colony diameter (mm)[a]		Anthraquinones (per colony)[c]		Extent of sporulation[b]
	Mean	% of control	Mean	% of control	
Klebsiella aerogenes	6.9	57.8	5.7	29.7	2+
Bacillus subtilis	9.2	77.4	15.3	78.8	3+
Lactobacillus brevis	12.0	100.8	18.1	83.1	3+
Pediococcus acidilacti	11.5	96.6	21.3	109.3	3+
Lactobacillus buchneri	9.7	81.5	11.7	60.9	2+
Streptococcus faecium	10.9	91.6	12.9	66.6	2+
Control (no bacterium)	11.9		19.4		4+

[a] Standard error of means, 0.6 mm.
[b] Extent of sporulation was estimated visually by spore colour *en masse* and confirmed by microscopic examination.
[c] Amount of anthraquinone produced per colony expressed as absorbancy at 450 nm in chloroform.

Sporulation was always inhibited to some degree under conditions inhibitory to toxin production. However, sporulation by one toxigenic fungus could react in different ways to the presence of bacteria which exerted similar effects on toxin production. Table 2 shows that ochratoxin A production by *A. ochraceus* is similarly affected by the presence of *L. brevis*, which completely inhibits sporulation, and by *Pediococcus acidilacti*, which does not significantly affect sporulation.

4. Discussion

The growth, sporulation and toxin production of five fungi were influenced by volatile compounds released by bacteria actively growing in MRS agar.

Sporulation of fungi is considered to be particularly sensitive to changes in the gaseous environment.[8] Here significant variation in the sporulation response of *A. ochraceus* and *P. patulum* in the presence of different bacteria, and significant differences in the activity of any one bacterium against the range of fungi tested occurred. This suggests that variation in the response of fungi to the presence of different bacteria resulted from specific differences in the nature of the volatile bacterial metabolites produced and not simply from environmental changes such as alterations in the levels of oxygen or carbon dioxide.

Indeed, the bacteria used in this investigation are known to produce a wide variety of volatile

metabolites, normally derived from the oxidation of carbon substrates.[4-5,9-10] Many of these volatile metabolites, such as acetic acid, propionic acid and butyric acid have been characterised[5] and are widely known to possess antifungal activity, while other compounds, which may include basic volatile metabolites,[1] remain unidentified.

However, the results obtained show that the modification of the gaseous environment may exert a differential effect on growth, sporulation and the production of specific metabolites by fungi, as suggested by Fries.[6] It has been demonstrated that volatile bacterial metabolites may selectively inhibit sporulation in the absence of growth limitation, or stimulate toxin production under conditions of growth limitation.

The differential response of growth, sporulation and toxin production by toxigenic fungi has been noted in this work. Schaeffer[11] proposed the possibility that toxin production by fungi might be linked to sporulation in a manner similar to that suggested for specific metabolites associated with the sporulation of *Bacillus* spp.[12] and *Streptomyces venezuelae*.[13]

However rubratoxin production by *Penicillium rubrum*[14] has been shown to occur during the growth phase prior to spore formation and the results given here demonstrate that the production of penicillic acid and citrinin (by *P. cyclopium* and *P. citrinum* respectively) could occur in the absence of spore formation. Nevertheless, the independence of sporulation and toxin production cannot be deduced from these results since sporulation was not demonstrated in the total absence of toxin synthesis.

The interactions described between bacteria and fungi are of importance since the bacteria used in the study are widely distributed in nature and particular bacteria may be associated with specific substrates. In this respect coli-aerogenes bacteria[15] and Lactobacillaceae[16-17] may exert an effect on the metabolism of the fungal flora associated with storage of moist grain and silage respectively. Under certain conditions, the production of volatile metabolites by these bacteria could be expected to occur and to influence fungi of a particular indigenous fungal population, by affecting the synthesis of mycotoxins.

The effects of volatile bacterial metabolites are likely to be beneficial by inhibiting fungal growth, by limiting sporulation and thus reducing dissemination of a fungus, and by limiting toxin production. However, under certain environmental conditions the metabolism of certain bacteria may contribute to a stimulation of mycotoxin production and accumulation.

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Evaluation of the ESCA Technique as a Screening Method for the Estimation of Protein Content and Quality in Seed Meals

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The ESCA (Electron Spectroscopy for Chemical Analysis) technique has been evaluated as an analytical screening method for the determination of protein content and quality of grain meals of some legumes and cereals. To this end the grain meals of a number of different species were analysed for total nitrogen, meals of varieties of cowpea (*Vigna unguiculata*) were examined for sulpho-amino acid content, and the levels of basic amino acid in meals of some maize and barley varieties were measured by this technique; some amino acids, polyamino acids and pure proteins were also examined as standards.

The results obtained for 15 grain meals by the ESCA technique, expressed as the ratio of the intensity of the N_{1s} photoelectron signal to that of the C_{1s} signal, were compared with %N as determined by a semi-automated micro-Kjeldahl method, and the correlation coefficient between the results from these two methods was $r = 0.98$. The binding energies of S_{2p} electrons were determined for some amino acids and proteins, and the amino acid sulphur was differentiated from oxidised sulphur. The N_{1s}/S_{2p} intensity ratios for four pure proteins correlated very well with the calculated stoichiometric N/S ratios ($r = 0.999$). The N_{1s}/S_{2p} intensity ratios were also determined for seven cowpea varieties and the correlation coefficient between these values and the ratios N/S_{aa}, measured by chemical methods, was $r = 0.77$.

The N_{1s} photoelectron peaks of some varieties of barley and maize meals showed a pronounced shoulder on the high binding energy side. On deconvolution into two components the main peak was assigned to nitrogen in the peptide bond and the smaller peak was assigned to the protonated nitrogen on the side chains of the basic amino acid residues.

1. Introduction

In screening protein in grains it is necessary to determine not only the quantity of protein but als the amount of any essential amino acid that might be present at a low enough level in the protei to limit its nutritional value. Analytical techniques should be chosen that require as little materi and analysis time as possible, particularly if it is necessary to perform one analysis to determin protein quantity and another to measure its quality, and the method should be able to measur protein in seeds or meals rather than require its extraction.

The potential of ESCA (Electron Spectroscopy for Chemical Analysis)[1] as a tool for such a analysis of the protein in grains was examined some years ago by Klein and Kramer.[2,3] Their wor showed that ESCA can be used to determine the nitrogen content of grains, and that two measure of the protein "quality" might be possible. These were the sulpho-amino acid content, given in term of total sulphur, and the lysine content as reflected in the measurement of basic amino acids, whic has been shown to give a measure of the lysine level in some cereal varieties and lines.[4] The poss bility that ESCA might give all this information in a single experiment on a very small sampl suggests its potentiality as an extremely powerful screening tool in plant breeding experiments.

Accompanying the increasing use of the ESCA technique in numerous areas of scientific research there has been a rapid improvement in instrumentation, resulting in much higher sensitivity and significantly improved resolution. In the light of these advances, a further investigation of the use of ESCA in this field is warranted. Accordingly, the grain meals of a number of cereal and legume species were analysed for their total nitrogen content, meals of varieties of cowpea (*Vigna unguiculata*) were examined for their sulpho-amino acid levels, and the basic amino acid levels of meals of some maize and barley varieties were also measured by this and other chemical methods. A number of amino acids, polyamino acids and pure proteins were also examined as standards.

2. Experimental

2.1. Materials and chemical methods

Amino acids, polyamino acids and pure proteins were obtained from Sigma London Chemical Company, Ltd, England. The seeds of varieties of cowpea were obtained from IITA, Ibadan, Nigeria and different varieties are designated according to their IVu numbers. The samples of barley seeds were from the Swedish Seed Association, Svalöf, by courtesy of L. Munck, and the samples of maize from O. E. Nelson Jr, Laboratory of Genetics, University of Wisconsin, Madison, USA. Other seeds were obtained locally. Meals were prepared from seeds by milling with either a Janke and Kunkel K. G. mill, type A10, or a ball mill, Pitchford Industries, Inc.

Total nitrogen, total sulphur and amino acid composition of the meals were determined on a dry weight basis according to the procedures previously described.[5] All determinations were performed in duplicate and single amino acid analyses were performed on two separate hydrolysates. *S*-methyl-l-cysteine was eluted with proline and therefore its content in some cowpea meals was calculated on the basis of the ratio of absorption at 570 nm to that at 440 nm.[6] Lysozyme was oxidised with performic acid.[5,7]

2.2. ESCA measurements

ESCA spectra were obtained on an AEI-ES200 AA electron spectrometer using Mg $K\alpha_{1,2}$ radiation, the photon energy of which is 1253.6 eV. The meals were mounted on the sample probe tip using double sided Scotch tape. The amino acids, polyamino acids, and proteins were examined as finely ground powders pressed on to the tape. All the samples were studied at ambient temperature. The pressure in the spectrometer source at the sample position was typically about 10^{-8} to 5×10^{-8} Torr. The X-ray flux absorbed in the outermost 100 Å of material, which is the volume examined in the ESCA experiment, was somewhat less than 100 rad/hour. No sign of decomposition due to the ultra-high vacuum or the X-ray irradiation was noted for any of the samples, even after a time which was significantly longer than that required to obtain a spectrum.

The binding energies of the core electrons for the amino acids, polyamino acids, and proteins were measured relative to the C_{1s} photoelectron line arising from hydrocarbon contamination (285.0 eV). (Such contamination is a common feature in ESCA spectra, and is due to background hydrocarbon in the spectrometer, for instance from the vacuum pumps or outgassing from the walls and seals, being gradually deposited on to the sample. The peak is readily identified as its intensity slowly increases with the time the sample spends in the spectrometer.) For the meals, the main C_{1s} photoelectron peak was taken as the reference at 285.0 eV. Unresolved peaks in the spectra were deconvoluted on a du Pont 310 Curve Resolver assuming Gaussian line shapes. The analogue integrator in the curve resolver was used to measure the relative intensities of the photoelectron lines arising from the core levels of different elements in the samples. The reproducibility in these measurements was better than $\pm 5\%$. For a number of samples possible effects of the hydrocarbon contamination on the intensity ratios were checked by monitoring the photoelectron line intensities over a time much longer than normally required to obtain satisfactory spectra. The rate of build-up of the contamination was found to be too slow to produce any noticeable effects under the experimental operating conditions.

3. Results and discussion

3.1. Crude protein content of meals

Quantitative nitrogen analysis of a number of meals was performed using the C_{1s} photoelectron signal as an internal standard, the ratio of the intensity of the N_{1s} line to that of the C_{1s} band giving a measure of the nitrogen content for each meal. The results are tabulated in Table 1 along with the

Table 1. The nitrogen content of various milled grains

Meal	N_{1s}/C_{1s} intensity ratio $\times 100$ (ESCA)	%N (micro-Kjeldahl)
Cowpea:		
I Vu 530	7.2±0.1	3.67
I Vu 1354	7.6±0.2	3.92
Prima	8.0±0.2	4.17
I Vu 2093	8.4±0.2	4.46
I Vu 3284	8.5±0.2	4.47
Westbred	8.6±0.3	4.56
I Vu 37	8.75±0.2	4.99
I Vu 1456	8.9±0.1	5.31
Lima Bean IPI 197	8.3±0.2	4.13
Broad Bean (*Vicia faba major*, var. "Triple White")	9.0±0.2	5.08
Wheat	4.9±0.4	2.04
Oats	5.0±0.2	2.40
Barley Bomi	4.9±0.2	2.03
Risø 1508	6.0±0.2	2.41
5V 71/669	5.5±0.2	2.84

The errors given for the ESCA results are standard deviations in repeated measurements on several samples for each variety.

corresponding values of micro-Kjeldahl analyses of the same meals. There is a very good correlation between the two methods of analysis ($r=0.98$), and for the 15 grain meals listed in Table 1 the best-fit regression line is $N_{1s}/C_{1s}=2.1645+1.3648$ %N. For the eight varieties of cowpea which were examined the correlation coefficient is 0.95. It is apparent that this ESCA method of analysis can reliably be used not only to show differences in the grain nitrogen content among different species, but also to monitor the much smaller variations occurring among different varieties of the same species.

The escape depths of electrons photoejected from atomic core levels are such that the ESCA experiment analyses only the outermost ~ 50 Å of material,[8] and for meals prepared as described above the particle size is such that only a thin layer on the surface of each particle is being examined. The excellent correlation between the ESCA results and those of the bulk nitrogen analyses shows that the milling procedure used is satisfactory in producing particle sizes such that the surface protein content is representative of that of the bulk for the grains listed in Table 1.

However, data obtained with the oil-seeds peanut, soybean and maize have not been included, since the N_{1s}/C_{1s} intensity ratios for these meals were lower than expected on the basis of the data in Table 1 and the bulk nitrogen analyses. It seems likely that during the milling process the oil in the grains was pressed out and coated the surfaces of the resulting meal particles. A very thin surface film, ~ 10 Å thick, would be sufficient to attenuate drastically the photoelectron signals originating from under the film. For the above mentioned grains, the amount by which the ESCA results deviated from plots of the data contained in Table 1 was roughly proportional to the oil

content of the grains:[9] no N_{1s} signal was observed for the peanut meal, the soybean result was out by a factor of about 3, and the maize by a factor of ~1.25. Obviously an analysis by ESCA of the protein content of these and other grains with high oil levels must be preceded by extraction of the fat.

Another consequence of the small electron escape depths is that the volume of material actually being examined is small, $\sim 10^{-6}$ to 10^{-7} cm^3; hence only very small samples are required, provided of course that the sample can be spread out uniformly. This small sample requirement may be important when only limited amounts of material are available. For instance, it is possible in principle to split a grain seed and examine the inner surface, or indeed to excise, or drill out and examine, a small portion of the seed and still be able to germinate the seed. The results of such a study might or might not be representative of the entire seed, but on the other hand, the technique affords the possibility to study protein distribution within a seed.

3.2. Determination of the quality of the protein in meals by ESCA

3.2.1. Sulpho-amino acid levels

The binding energies of the S_{2p} electrons in some amino acids and proteins are listed in Table 2. It is apparent that with the presently available instrumentation the S_{2p} photoelectron signals from

Table 2. S_{2p} binding energies observed for some amino acids and proteins

Sample	S_{2p}	Binding energy (eV)
Cysteine	163.4	
Cystine	163.5	
Methionine	163.2	
Polymethionine	163.2	
α-Chymotrypsinogen	163.2	167.6
Insulin	163.6	
Myoglobin	163.2	167.8
Lysozyme	163.4	
Oxidised lysozyme[a]		168.1

[a] Performic acid oxidation.

For the proteins, the S_{2p} peak at higher binding energy is assigned to some form of inorganic sulphur, or oxidised sulphur (e.g. SO_4^{2-}, SO_3^{2-}, etc.)

methionine, cystine, and cysteine cannot be distinguished. It was felt that by oxidising all the sulphur in a protein the oxidised methionine (present as methionine sulphone) could perhaps be resolved from the oxidised cystine and cysteine, which would be present as cysteic acid. For this reason lysozyme was oxidised with performic acid[7] and, as seen from the data in Table 2, the S_{2p} peak was shifted by 4.7 eV to higher binding energy. No residual peak remained at 163.4 eV, indicating that the oxidation was essentially complete. Unfortunately the linewidth of the peak was unchanged and there was no indication that the different sulpho-amino acids could be resolved by this technique. However, it is possible to differentiate the amino acid sulphur from oxidised sulphur (e.g. SO_4^{2-} or oxidised organic sulphur) which gives a peak shifted to higher binding energy by ~4 eV in the proteins α-chymotrypsinogen and myoglobin, as shown in Figure 1. Although it was outside the scope of this study, it would be possible to determine oxidised sulphur quantitatively.

Lysozyme, insulin, α-chymotrypsinogen, and myoglobin were used to test the applicability of ESCA to the quantitative measurement of the sulpho-amino acid content of proteins. Table 3 contains the experimental intensity ratio of the N_{1s} to the S_{2p} photoelectron peak, along with the

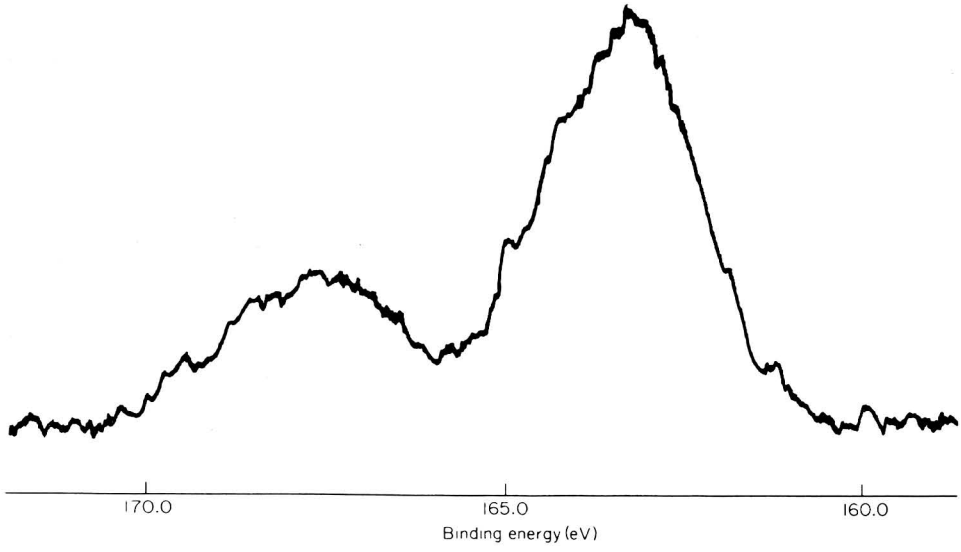


Figure 1. The S_{2p} region of the ESCA spectrum of α-chymotrypsinogen, showing the photoelectron peak from oxidised sulphur at higher binding energy than the peak due to amino acid sulphur.

Table 3. N_{1s}/S_{2p} intensity ratios for some proteins, and the corresponding stoichiometric N/S ratios

Sample	N_{1s}/S_{2p} intensity ratio (ESCA)	N/S (stoichiometry)
α-Chymotrypsinogen	20.3	25.7
Insulin	6.7	10.8
Myoglobin	80.8	102
Lysozyme	12.2	19.2
Oxidised lysozyme	11.8	19.2

corresponding stoichiometric N/S ratio, for each protein. The two sets of data correlate well ($r = 0.999$), showing that the ESCA technique can provide a very accurate measure of the sulpho-amino acid content of a protein.

It is, in principle, possible to perform a similar analysis of the protein in a meal; such a study is of interest because the limiting amino acids in grain legumes are methionine and cystine, and appropriate screening methods are required for breeding programmes which aim to improve the levels of these amino acids in seeds. Seven varieties of cowpea, in which the sulpho-amino acids are the first limiting amino acids,[10] were examined by the use of ESCA for their sulphur content. Only one sulphur peak was observed, indicating that any inorganic or oxidised organic sulphur in the samples was present at a level below the detection limit. The extremely low sulpho-amino acid levels necessitated the use of fairly long sampling times in recording the S_{2p} spectra, and the unfavourable signal to noise levels limited the accuracy of the sulphur determinations. Table 4 presents the ESCA results, expressed as the ratio of the intensity of the N_{1s} peak to that of the S_{2p} peak for each meal. The N/S_{aa} ratio was also determined for the meals, where S_{aa} represents sulphur in cysteine, methionine and S-methyl-cysteine.[6] Also included is the ratio N/S were S represents the total sulphur in a meal, the N, S and S_{aa} values being determined by micro-Kjeldahl analysis, turbidimetric analysis and ion-exchange chromatography respectively.[5,6] The correlation coefficient

Table 4. The analysis of the sulpho-amino acid content of some cowpea meals. The ESCA results, given by the intensity ratio of the N_{1s} to the S_{2p} photoelectron peak, are compared with the ratio of the N to the S_{aa} and also with the ratio of the N to the S as determined by the analytical procedures described in the text

Cowpea variety	N_{1s}/S_{2p} intensity ratio (ESCA)	N/S_{aa}[a]	N/S[b]
I Vu 530	31.7±4.0	20.5	18.0
I Vu 3284	28.9±4.1	20.1	18.6
I Vu 1354	28.8±3.7	18.6	20.0
Prima	36.8±4.4	20.0	20.3
Westbred	37.7±3.5	23.5	23.1
I Vu 37	35.3±2.0	23.9	24.3
I Vu 2093	41.4±2.7	23.9	27.2

[a] S_{aa} = % sulphur Σ(methionine + cysteine + *S*-methylcysteine).

[b] S = % total sulphur.

The errors given for the ESCA results are standard deviations in repeated measurements on a number of samples for each meal.

between the ratios N_{1s}/S_{2p} from ESCA measurements and the ratios N/S_{aa} is $r=0.77$, and that between the N_{1s}/S_{2p} and N/S values is $r=0.82$; the corresponding best-fit regression lines are $N_{1s}/S_{2p} = -1.4865 + 1.6678\ N/S_{aa}$ and $N_{1s}/S_{2p} = 9.0025 + 1.1722\ N/S$.

The chance of a random occurrence of the first correlation is between 3 and 3.5%, indicating that the ESCA technique can be used as a screening method for measuring the sulpho-amino acid levels in these meals. Furthermore, this comparative method indicates that total sulphur is probably a correct measure of the sulpho-amino acid content of cowpea meals.

The uncertainties in the ESCA data in Table 4 originate almost entirely in the measurement of the intensities of the S_{2p} photoelectron peaks in the meals; they could probably be reduced by longer sampling times.

3.2.2. Basic amino acid levels

The nutritional value of many cereal grains is limited by the low level of lysine.[4] It is therefore important in breeding experiments to screen for the amount of this amino acid, and this is usually done by measuring the total basic amino acids content. The results of an ESCA study of the basic amino acids and their polymers are given in Table 5. From these it is evident that the nitrogen in the side-chain of the basic amino acids, provided it is protonated, can be distinguished from the nitrogen of the peptide bond and from the side-chain nitrogen of tryptophan, glutamine, and asparagine. At the pH values which obtain in biological systems the side chain nitrogen of the basic amino acids is protonated and as a result the N_{1s} photoelectron peaks of the meals showed a pronounced shoulder on the high binding energy side, as shown in Figure 2 for some barley meals. It proved possible to deconvolute these bands into two components, a main peak with binding energy ~400.5 eV, due to the nitrogen in the peptide bond, and a smaller peak assigned to the protonated nitrogen on the side-chains of the basic amino acid residues in the grain proteins (binding energy ~402.5 to 403.0 eV). Spectra were obtained for normal and high lysine varieties of barley, shown in Figure 2, and maize meals. Quantitative determination of the relative areas of the two deconvoluted peaks gave a measure of the basic amino acid content for each meal as shown in the data in Table 6. There is a correlation between the ESCA measurements and the results of the amino acid analysis for each species, but the ratio of the two N_{1s} photoelectron peaks does not

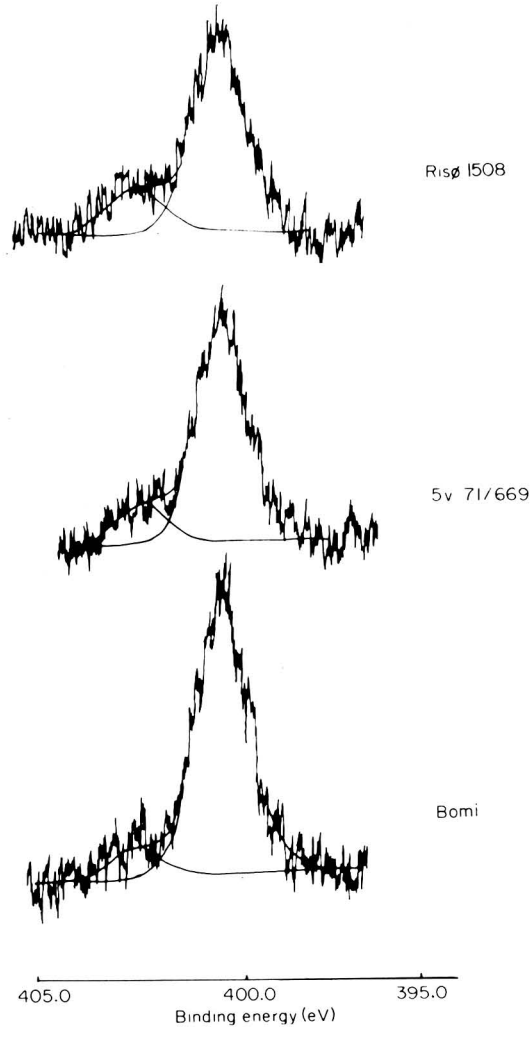
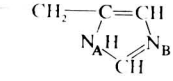
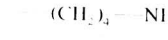
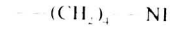
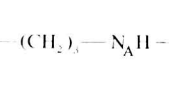


Figure 2. The N_{1s} photoelectron regions of the ESCA spectra of three varieties of barley meals. The bands have been deconvoluted into two peaks, as discussed in the text.

give an absolute value for the basic amino acid content. It is, therefore, worthwhile considering other factors that could contribute to the observed N_{1s} line-shapes.

In the first place, a certain proportion of the photo-electrons originating from within the sample bulk undergo inelastic scattering as they pass through the material. This results in a "tail" on the low kinetic energy (high binding energy) side of the photoelectron peaks, just where the side-chain nitrogen of interest would be found. Using model compounds such as the polyamino acids and proteins the shape of the "tail" was determined, and, since the tailing is expected to be the same in the N_{1s} bands of the models and meals, its contribution to the N_{1s} photoelectron signals of the meals was determined. In fact the contribution to the observed shoulder from the inelastic tail is small. The precise magnitude of its effect is difficult to measure, but it certainly contributes less than 20% of the intensity measured by the deconvolution procedure. This amount is sufficient to explain

Table 5. The experimental N_{1s} binding energies, in electron volts, of the basic amino acids and their polymers, and of other amino acids having side chain nitrogen

Sample	α–N	Peptide bond N	Side chain N	N_A	N_B
Histidine:					
Free base	402.0	—		401.0	399.5
Hydrochloride	401.9	—		401.9[a]	
Polyhistidine	—	400.4		401.0	399.2
Lysine:					
Free base	402.0	—		400.1	
Hydrochloride	401.9	—		401.9	
Polylysine hydrobromide	—	400.4		402.0	
Arginine:				400.3	400.8
Free base	400.3[b]	—		400.1	400.7
Hydrochloride	402.0	—		400.4	401.4
Polyarginine hydrochloride	—	400.4		400.3	
				400.3	
Asparagine	402.0	—		400.5	
Glutamine	401.9	—		400.6	
Tryptophan	402.1				
Polytryptophan	—	400.6			

[a] In histidine hydrochloride, N_B is protonated and the side chain carries a net positive charge.

[b] In arginine, the proton from the carboxyl group is transferred to the guanidinium group rather than the α-amino group. Hence it exists as $-NH_2$. For all the other peptides listed here, it is in the form $-NH_3^+$.

Table 6. The analysis of the basic amino acid content of some barley and maize meals. The ESCA results are expressed as the ratio of the intensity of the smaller component of the N_{1s} band to the intensity of the complete N_{1s} band

Meal	Basic amino acid His.	Lys.	Arg.	(g/16 g N)[b] Σ (His. + Lys. + Arg.)	(ESCA)[a] × 100
Barley:					
Bomi	2.5	3.9	5.7	12.1	11 ± 1
5v 71/669	2.2	4.6	5.6	12.4	14 ± 1.5
Risø 1508	2.5	5.3	7.1	14.9	16 ± 1.5
Maize:					
11B 2372	2.8	2.5	4.6	9.9	10 ± 0.5
459 W64A+	2.5	2.5	3.8	8.8	11 ± 1
3473 W64 A02	2.8	4.5	6.5	13.8	17 ± 2.5
43134 fl 2	2.7	3.5	5.2	11.4	17 ± 2.0

[a] The errors are standard deviations in repeated measurements on several samples for each meal.

[b] Single analyses were performed on separate 22 h acid hydrolysates of each meal. The ranking in levels of basic amino acids was similar to that found in the literature for maize varieties[12] and barley Bomi and Risø 1508.[13]

why the ESCA measurements fail to give absolute values for the basic amino acid content in agreement with the values determined by the amino acid analysis. However, since the "tail" contributes about the same proportion to the N_{1s} band of each meal, it is not surprising that trends in the basic amino acid content in a given grain variety are observable by the ESCA technique.

The meals themselves contain other nitrogen compounds which could conceivably give rise to the observed shoulder. The ESCA spectrum of RNA (from *Escherichia coli*: BDH Ltd) showed N_{1s} peaks centred at 401.0 and 399.6 eV and it is unlikely that there is a significant contribution to the shoulder from this source. Inorganic nitrogen present as nitrate or nitrite would have a significantly

higher binding energy and would not contribute to the signal of interest. A contribution from NH_4^+ ions in the meals cannot be ruled out, however. Similarly the $-NH_3^+$ groups in small peptides, amino acids, and on the protein end groups would have a N_{1s} binding energy identical to that of the nitrogen of the basic amino acid side-chains. Such contributions to the shoulder are expected to be of minor importance, and the trends observed in Table 6 suggest that this is so.

Factors such as these which may contribute to the observed shoulder naturally affect the precision with which the basic amino acid content can be found, and the tail from the inelastically scattered electrons lowers the precision of the deconvolution process. Furthermore, it is difficult to deconvolute in a reproducible manner a small peak close to a much more intense one. In spite of these difficulties, it is apparent from the spectra illustrated in Figure 2 and the data tabulated in Table 6 that the size of the shoulder relative to the total N_{1s} peak does give some measure of the basic amino acid levels in the grain proteins, and it can be used as a ranking procedure (high, medium or low).

The N_{1s} binding energy of $\sim$400.5 eV obtained with meals agrees well with that of the peptide bond nitrogen at 400.5 eV in the polypeptides, but the binding energy of the smaller peak, $\sim$402.5 to 403.0 eV, is somewhat higher than that of the nitrogen on the protonated side-chains of the basic amino acids (see Table 5). Now, it is well known that the binding energy of an electron in an ion depends strongly upon the lattice in which the ion exists and also upon the counterion.[11] Indeed, such a dependence can be seen in the data for arginine in Table 5. For the free base and the hydrochloride salt of the amino acid the binding energies of the side-chain nitrogens are essentially identical, since in both cases the counterion is the $-COO^-$ function (in arginine the proton from the carboxyl is transferred to the guanidinium group rather than to the amino group on the α carbon). In polyarginine HCl, however, the counterion is the chloride ion. In addition to the change in counterion, the potential of the "zwitterionic" lattice that exists for the amino acids is substantially different for polyarginine hydrochloride. Similarly, changes in potential are expected to occur on removing a protein from a reasonably well-defined lattice such as exists for a pure protein and placing it into the much more amorphous environment of a biological system. The lattice potential most certainly changes substantially, and the nature of the counterion and/or its distance from the protonated side-chain could also be different. A change in the binding energy of 0.5–1.0 eV could certainly result from such effects.

4. Conclusions

In evaluating an analytical technique it is necessary to balance the advantages against its disadvantages. For the analysis of meals as described in this work, the principal advantage of the ESCA technique is the large body of information available from a single experiment. From the intensities of the various photoelectron peaks it has been possible to determine with some precision the protein content of the meals and in addition, the content of sulpho-amino acids and of total basic amino acids. The sulphur of the sulpho-amino acids could also be distinguished from oxidised sulphur.

Although different correlation coefficients were obtained between the N_{1s}/S_{2p} intensity ratios and sulpho-amino acids and total sulphur respectively, these cannot be considered significantly different since they are based on a sample of only seven varieties. To test the hypothesis that the correlation between the N_{1s}/S_{2p} and N/S_{aa} ratios should be better than between the N_{1s}/S_{2p} and N/S, it is necessary to increase greatly the sample size and preferably, if possible, to cover a broader range of variability in the sulpho-amino acid content of the meals.

A significant advantage of ESCA is the very small amount of material required for the analysis and the minimal sample preparation necessary; a few milligrams of material suffice and a seed is presented in a suitable form merely by milling. In the analysis of the protein in grains with high oil levels it is necessary also to extract the fat prior to obtaining the ESCA spectra, since an oil film 10 Å thick would attenuate signals. The fact that the technique is non-destructive is important when the amount of material available is limited. No internal standard needs to be added since all quantitative results can be expressed in terms of ratios of the intensities of ESCA lines.

The time required to obtain the spectra depends upon the information desired. Spectra of quality

sufficient to extract the N_{1s}/C_{1s} intensity ratios with the accuracy given in Table 1 were obtained in less than 10 min. To improve the statistics in the N_{1s} signal sufficiently to allow the deconvolution required for assessing the basic amino acid content, a further eight minutes was used. Another hour was necessary to obtain S_{2p} photoelectron signals suitable for quantitative measurement. These times could, of course, be lowered, but this would undoubtedly be accompanied by an increased uncertainty in the results. The accuracy of the sulphur determination could be improved by more extensive signal accumulation. However, this is not so for the other data, for which the uncertainties arise mainly from the reproducibility of the deconvolution and the line-shape fitting necessary for the intensity determinations.

The main disadvantage of the ESCA method lies in the expensive and complicated equipment required. Skilled operators are required and maintenance can be difficult and costly. However, the process of accumulation of spectra and data processing can be fully automated. Whether the advantages of the ESCA method are sufficient to outweigh the cost factor remains to be seen; the technique is certainly capable of giving valuable information on the protein content of meals.

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Comparative Evaluation of the Enzyme Multiplicity in a Diploid, a Triploid and a Tetraploid Sugar Beet Variety[a]

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Specific activities and isoenzyme multiplicities of glutamic dehydrogenase, esterase, peroxidase, acid phosphatase, acid and neutral invertases were determined in a diploid, a triploid and a tetraploid sugar beet variety. The different ploidy status was accompanied by variations in the level of activity of glutamic dehydrogenase, peroxidase and invertase. Polyploidy induced a number of isoenzymes higher for peroxidase and lower for esterase, acid phosphatase and glutamic dehydrogenase. Specific isoenzyme bands characterised the invertase of the triploid variety.

1. Introduction

Spontaneous formation of hybrids and polyploids has been a main instrument of adaptation of plant species to environmental changes along the evolutionary pattern. The artificial production of both hybrids and polyploids has been widely used by plant breeders for the difficult task of introducing in cultivated species new favourable characters without loss of stability towards the environmental conditions. An example of the success of these breeding techniques is the stabilisation of Triticale hybrids.[1] In the case of sugar beet, controversy has existed about the effects of induced polyploidy on the qualitative and quantitative characters connected with the industrial utilisation of the roots. In Europe the optimal ploidy level appeared to be the triploid, characterised by maximum root size and sugar yield; on the other hand in the USA important differences connected with ploidy level have not been demonstrated. The present work springs from the belief that a clearer appreciation of breeding practices can arise from a better knowledge of their impact at the molecular level. At this level the main phenotypic consequence of a genetic manipulation is the change in the relative quantity of the various enzymes and in the level of multiplicity of each enzyme.

While the effect of heterosis on the enzyme make-up in crosses has been sufficiently elucidated,[2–6] little is known about the corresponding effect of the ploidy level. Qualitative and quantitative changes in the isoenzyme profiles of esterases in *Triticum* genus have been observed by Bhathia[7] and similar results have been obtained by Reddy and Garber[8] for esterases and peroxidases in *Nicotiana*. Isoenzymes of a polyploid wheat[9] and gene localisation in tetra and hexaploid wheat[10] have also been studied.

Malate dehydrogenase isoenzymes have been compared in haploid and diploid *Datura* by Ganapathy and Scandalios.[11] Murray and Williams[12] have shown that in leaves of *Briza media* L., the esterase level in triploid and tetraploid is four times higher than in diploid varieties, whereas in seeds two bands of esterases are present in diploids and only one in tetraploids. In sugar beet, studies on peroxidases in di-, tri- and tetraploid Russian varieties[13] have shown that a higher activity is constantly present in tetraploids. Platon *et al.*[14] have evaluated peroxidase, ascorbic-oxidase, polyphenoloxidase and catalase in Romanian beets. Four varieties of sugar beets, differing in ploidy level and sugar content, have been studied by Dubucq *et al.*[15] which demonstrated significant differences both in the activity and in the number of isoenzymes. The growth rate and

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the sugar yield have been shown to be correlated to the peroxidase level by Gaspar and Bouchet.[16] Owing to the important role in the metabolism and in the post-harvest physiology of sugar beets, the following enzymes have been considered in the present work: glutamic dehydrogenase (EC 1.4.1.2), esterase (EC 3.1.1.2), acid phosphatase (EC 3.1.3.2), neutral and acid invertase (EC 3.2.1.26) and peroxidase (EC 1.11.1.7).

The level of activity and the number of isoenzymes have been compared in a di-, tri-, and tetraploid variety.

2. Experimental

2.1. Materials and methods

Seeds of diploid, triploid and tetraploid sugar beet (*Beta vulgaris* L.) were obtained by the courtesy of the Van der Have N.V. Plant Breeding Department, Rilland, The Netherlands.

Cultivation was carried out in plots of a single soil type (Terra Rossa) of the College of Agriculture farm, Padua, having the following characteristics: pH 7.9; free $CaCO_3$ 2.6%; sand, silt and clay 24; 26 and 47% respectively. After harvesting beet roots were stored in perforated plastic bags at $-20°C$ and only those completely free of visible mould were used.

A 50 g sample of root was homogenised in an ice-refrigerated Ultraturrax apparatus with 100 ml of 0.01 M phosphate buffer pH 7.2 containing 2 mM EDTA, 2 mM cysteine–HCl and 10 mM Na_2SO_3. After centrifugation at $35\,000 \times g$ for 20 min, soluble proteins were precipitated from the supernatant with $(NH_4)_2SO_4$ up to saturation, resuspended in the same buffer without additives and dialysed against distilled water at 3°C for 48 h. Protein samples were stored with liquid nitrogen.

Enzyme activities were evaluated according to: Bernt and Bergmeyer[17] for glutamic dehydrogenase with 0.2 M Tris–HCl buffer pH 9.6, 0.86 M sodium glutamate and 7 mM NAD^+; Rudolph and Stahmann[18] for esterase (α-naphthylacetate as substrate); Linhardt and Walter[19] for acid phosphatase; Lodha *et al.*[20] for peroxidase; Ricardo and Rees[21] for acid and neutral invertase with sucrose concentration of 10% and 1 mM EDTA. The sucrose content was estimated from the difference in the amounts of reducing sugar found in the presence and in the absence of commercial invertase (Serva, Feinbiochemica 100 EU/mg). Total nitrogen was determined with a micro-Kjeldahl method and protein by Lowry's method.[22]

Protein samples were submitted to electrofocusing by the procedure of Wrigley[23] under the following conditions: 90 volts, 10 mA per tube, 15 h run. The staining of the electrophoretic bands was obtained with the following methods: glutamic dehydrogenase in 0.2 M Tris–HCl as suggested by Hartmann *et al.*[24]; esterase in α-naphthylacetate[18]; acid phosphatase by Allen *et al.*[25]; peroxidase with 0.5% *O*-dianisidine in 1 M HCl;[20] invertase by Lück[26] with sucrose concentrations of 10% in 0.187 M Na_2HPO_4–0.007 M citric acid pH 7.5 + 1 mM EDTA for the neutral invertase, in 0.091 M Na_2HPO_4–0.055 M citric acid pH 4.5 + 1 mM EDTA for the acid invertase.

Gels used as control were treated with boiling distilled water for 10 min.

3. Results

The three varieties of beets examined show (Table 1) a sugar content decreasing in the following order; tetraploid > diploid > triploid, while no significant differences appear in the total nitrogen content. The true protein decreases in the order: diploid > tetraploid > triploid. High sugar content is accompanied by high activity of peroxidase and to a small extent acid invertase, in tetraploid, whereas in di- and triploid (Table 2) this correlation does not remain valid.

The highest non-protein nitrogen content of the diploid is accompanied by the highest value of the glutamic dehydrogenase activity.

Our results, which are in accordance with the data found by Lobotskaya *et al.*[13] and Dubucq *et al.*[15] as regards the correlation between tetraploidy and a high level of peroxidase, demonstrate also that triploidy is related to the highest level of non-protein nitrogen and glutamic dehydro-

Table 1. Characteristics of the di-, tri- and tetraploid sugar beets used in the experiments

Beet variety	Water content (%)	Total nitrogen (% dry matter)	Sucrose (% fresh weight)	Protein (Lowry) (% dry matter)
Diploid	83.8	1.19	10.3	0.29
Triploid	85.6	1.18	9.4	0.10
Tetraploid	82.4	1.19	12.1	0.12

Mean of 5 determinations.

Table 2. Enzyme activity in roots of a di-, tri- and tetraploid sugar beet variety

	Diploid	Triploid	Tetraploid
Glutamic dehydrogenase (μmoles L-glutamate $\times$ min^{-1} $\times$ mg^{-1} protein at 25°C)	2.68×10^{-3}	4.15×10^{-3}	3.88×10^{-3}
Acid phosphatase (μmoles phenolphthalein $\times$ min^{-1} $\times$ mg^{-1} protein at 37°C)	0.68	0.55	0.59
Esterase (change in absorbance $\times$ min^{-1} $\times$ mg^{-1} protein at 37°C)	0.48	0.47	0.47
Peroxidase (change in absorbance $\times$ min^{-1} $\times$ mg^{-1} protein at 25°C)	0.20	0.21	1.15
Neutral invertase (μmoles sucrose $\times$ min^{-1} $\times$ mg^{-1} protein at 30°C)	1.67×10^{-2}	1.92×10^{-2}	1.85×10^{-2}
Acid invertase (μmoles sucrose $\times$ min^{-1} $\times$ mg^{-1} protein at 30°C)	3.35×10^{-3}	3.21×10^{-3}	4.63×10^{-3}

Mean of 5 readings.

genase. The enzyme multiplicity is quite different for the three levels of ploidy. The number of isoenzyme bands (Figure 1) decreases from the diploid to the tetraploid status for esterase, acid phosphatase and glutamic dehydrogenase.

In contrast peroxidase shows a greater number of bands in the triploid, while the diploid differs from tetraploid in one band only.

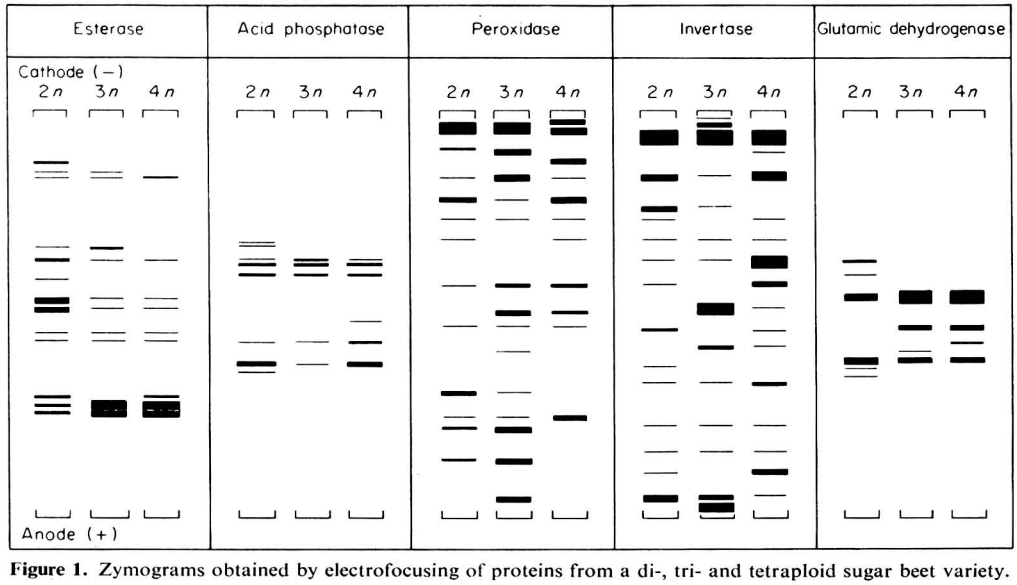

Figure 1. Zymograms obtained by electrofocusing of proteins from a di-, tri- and tetraploid sugar beet variety.

Invertase is present with the same number of bands in diploid and triploid; in tetraploid one more band is present: however, the triploid is characterised by specific bands, two in the cathodic and one in the anodic zone.

4. Conclusions

The different ploidy status in three sugar beet varieties is accompanied by differences in the level of activity of several enzymes (glutamic dehydrogenase, peroxidase, acid invertase). Even the multiplicity of the single enzymes varies with the ploidy level, but in different ways for the various enzymes.

While the formation of hybrids following crossing leads constantly to a higher multiplicity of the enzymes, the polyploidy induces in the sugar beet in some cases an increase, in others a decrease of the isoenzyme number.

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Enzymatic Determination of Butane-2,3-diol in Wines

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Butanediol is first oxidised to acetoin by butane-2,3-diol dehydrogenase from a strain of *Sarcina*, in the presence of NAD^+ and an excess amount of 2,6-dichlorophenol-indophenol at an alkaline pH. Then the acetoin formed is reduced back to butane-2,3-diol by the same enzyme in an acidic condition, and NADH thereby oxidised is measured spectrophotometrically. Direct measurement of NADH formation in the first reaction, oxidation of butane-2,3-diol, has not yet been successful. Colorimetric measurement of the reduction of 2,6-dichlorophenol-indophenol was also unsuccessful. Ethanol in sample solutions should have been removed by evaporation before the enzymatic oxidation. Presence of acetoin caused no difficulty in the oxidation of butane-2,3-diol, but made it necessary to correct the measured values for the original acetoin contents. The isomers of butane-2,3-diol present in wines have been reported to be D(−) and meso, on both of which the butane-2,3-diol dehydrogenase from *Sarcina* appears to be able to act. The butane-2,3-diol contents of some Japanese commercial wines were determined by this enzymatic method. Concentrations ranged from 324 to 768 mg/litre.

1. Introduction

The authors previously reported[1] an enzymatic method for the determination of acetoin (3-butanol-2-one) using butane-2,3-diol dehydrogenase (BDHG, EC 1.1.1.4) from a strain of *Sarcina hansenii*, and determined acetoin contents of a number of grape musts and wines by this method. Acetoin formed in wine, however, is readily converted to butane-2,3-diol (BD) by yeast. Therefore the assay method for BD, and not only for acetoin, is essential to the study of acetoin formation in wine. In this report a new method of BD determination is described.

The reaction of BDHG is reversible. In the presence of reduced form nicotinamide-adenine dinucleotide (NADH) in an acidic condition, the equilibrium is much in favour of BD formation, but in the presence of oxidised form nicotinamide-adenine dinucleotide (NAD^+) and an excess amount of 2,6-dichlorophenol-indophenol (2,6-DCPI) as a hydrogen acceptor in an alkaline condition, the reaction proceeds to the right and BD can be converted to acetoin.

$$\begin{array}{rcl} \text{Butane-2,3-diol} & \rightleftharpoons & \text{Acetoin} \\ NAD^+ & \rightleftharpoons & NADH \\ \text{Reduced 2,6-DCPI} & \leftarrow & \text{2,6-DCPI} \end{array}$$

The acetoin thus produced can be determined as described in the former paper.[1] The specificity of this enzymatic oxidation makes it possible to determine BD separately from the other wine components, e.g. sugars or glycerol.

2. Experimental

2.1. Materials

The enzyme (BDHG) used was extracted and partially purified from a strain of *Sarcina hansenii* as previously reported.[1] Acetoin, BD, NAD^+ and NADH were also as described in the former paper.[1]

2.2. Determination procedure

The procedure consists of the following two steps.

Step 1. Enzymatic oxidation of BD to acetoin: pipette 1.0 ml of a sample solution containing BD less than 60 μg, 0.5 ml of 0.2 M-phosphate buffer (pH 8.0) containing 2 mM 2,6-DCPI sodium salt (freshly dissolved), 0.1 ml of 0.1% NAD^+, and 0.1 ml of the BDHG solution (protein 800 μg) into a little tube (12 × 120 mm), and make the total volume 2.7 ml with distilled water. After stoppering and mixing, let stand at 30°C for 30 min with occasional shaking. During the incubation period the solution must keep its deep blue colour; the fading of the colour means that the amount of BD in the sample solution is too large. Add 0.1 ml of 8 N-H_2SO_4 and heat for 2 min in boiling water to remove protein and excess colour. After cooling, add 0.2 ml of 3.5 N-NaOH. The resulted pH value of the solution should be 5.0–6.0; addition of excess NaOH colourises the solution. Remove the sediment by centrifuging (7.5×10^3 *g*, 5 min). Take 0.5–1.0 ml aliquot of the supernatant for the next step.

The partially purified BDHG preparation from *Sarcina* usually has an activity of NADH dehydrogenase and the hydrogen atoms derived from BD can be easily delivered to 2,6-DCPI. If completely purified enzyme is used, addition of NADH dehydrogenase may be necessary.

Step 2. Enzymatic determination of the acetoin produced: the acetoin produced in step 1 is reduced back to BD in the presence of BDHG and NADH at pH 6.5, and the decrease in absorbance at 340 nm in 5 min from the start of the reaction (ΔA) is measured as previously described.[1] The value ΔA has linear relation to the amount of BD in the sample solution. Standard curves are essential with every analysis because of variation in temperature, enzyme activity and other conditions. The following equation was obtained from typical one of our standard curve:

$$(\text{BD } \mu\text{g}) = 62.11 \times (\Delta A)$$

Standard deviation was within 2% of experimental average for each concentration of BD.

3. Results

3.1. Effect of pH on the enzymatic oxidation of butane-2,3-diol

The rate of the enzymatic oxidation of BD was estimated at various pH values by spectrophotometric measurement of NADH formation. The rate of reaction varied with the buffer used. With borate–NaOH or Tris–glycine buffer, the reaction was strongly inhibited. Carbonate–bicarbonate buffer gave a superior activity, but was unstable to heating or addition of strong acids. The highest activity was given by phosphate buffer. The maximal velocity was obtained at pH 9.50–9.75 as shown in Figure 1, but the normal pH range of phosphate buffer is about 6–8, and at pH values above 8.0 the buffer action may be unsatisfactory. In this paper phosphate buffer, pH 8.0 was used.

3.2. Stoichiometry of the oxidation of butane-2,3-diol

At pH 8.0 (phosphate buffer), the amount of acetoin formed in the step-1 reaction reached about 75% of the theoretical value. This ratio of conversion was practically constant for each amount of BD under 70 μg as shown in Table 1. Consequently the value of ΔA measured in step-2 reaction was quite proportional to the amount of BD. Increased yields of acetoin could be obtained with other lots of enzyme or at higher pH values.

3.3. Effect of 2,6-dichlorophenol-indophenol concentration on the oxidation of butane-2,3-diol

The equilibrium of the reaction of BDHG is much in favour of BD formation in usual conditions. To push the reaction towards BD oxidation, rapid and smooth transport of hydrogen out of the system is needed. In this paper, 2,6-DCPI was used as a hydrogen acceptor for this purpose. The addition of a large excess amount of 2,6-DCPI was important to facilitate the oxidation of BD; over 1.4 μmol 2,6-DCPI were needed for the oxidation of 0.44 μmol BD as shown in Table 2.

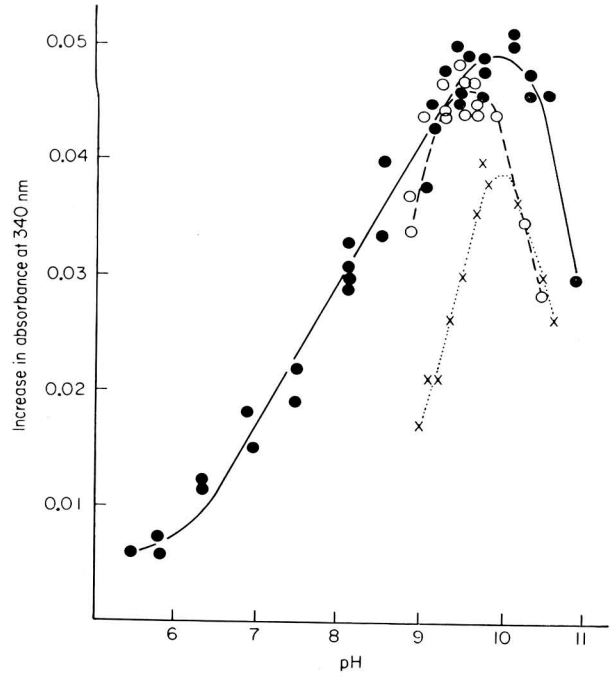

Figure 1. Effect of pH on the reaction rate of butane-2,3-diol oxidation. The reaction mixtures contained 400 μmol buffer, 0.3 μmol of NAD^+, 40 μmol BD, and 0.05 ml enzyme solution (protein 16 μg) in total volume 3.05 ml. Increase in absorbance at 340 nm; from 15 to 30 s after the start of the reaction at 30°C. ●——●, phosphate buffer; ○- - -○, carbonate–bicarbonate buffer; × ··· ×, glycine–NaOH buffer.

Table 1. Yield of acetoin obtained by the enzymatic oxidation of butane-2,3-diol

Butanediol added (μg)	Acetoin formed[a] (μg)	Yield of acetoin (% of the theoretical amount)
0	0	—
10	7.0	71.8
20	14.6	75.1
30	22.0	75.2
40	29.9	76.7
50	36.5	75.2
60	44.3	75.7
70	51.0	74.9
80	56.9	73.1
90	61.6	70.3
100	64.3	66.0

[a] During 30 min reaction at 30°C. Composition of the reaction mixtures are as described in the text.

Table 2. Effect of the amount of 2,6-dichlorophenol-indophenol (hydrogen acceptor) on the enzymatic determination of butane-2,3-diol

Substrate[a]		Oxidation of butanediol[c]			Results of butanediol determination	
Butanediol (μmol)	2,6-DCPI (μmol)	Colour of the solution after oxidation	Amount of acetoin formed (μmol)	Yield of acetoin (%)	Amount of butanediol measured (μmol)	Recovery (%)
0.442[b]	0.2	colourless	0.086	19.4	0.115	26.0
0.442	0.4	colourless	0.123	27.7	0.165	37.3
0.442	0.6	colourless	0.166	37.6	0.223	50.5
0.442	0.8	colourless	0.192	43.5	0.257	58.1
0.442	1.0	colourless	0.236	53.5	0.317	71.7
0.442	1.2	colourless	0.282	63.9	0.379	85.7
0.442	1.4	light blue	0.321	72.6	0.431	97.5
0.442	1.6	blue	0.322	72.9	0.433	98.0
0.442	1.8	blue	0.339	76.7	0.456	103.2
0.442	2.0	blue	0.327	73.9	0.438	99.1

[a] Dissolved in 0.2 M-phosphate buffer (pH 8.0) with 0.15 μmol NAD^+.
[b] 40 μg.
[c] Step-1 reaction in the text.

3.4. Removal of ethanol

The BDHG preparation contained some activity of alcohol dehydrogenase, and the presence of ethanol had an interference with the oxidation of BD, consuming 2,6-DCPI wastefully. Moreover, it results in formation of acetaldehyde, which is one of the interfering substances with the enzymatic determination of acetoin.[1] Therefore ethanol in the sample solution should have been distilled off before measurement by careful rectification (by usual simple distillation, a portion of BD will be distilled and lost with ethanol). A more simple and easy method to eliminate ethanol is to evaporate the sample in a little dish on a water bath under controlled conditions. The bath temperature should not exceed 90°C; higher temperatures caused some loss of BD. The optimum evaporation conditions found in our experiments were as follows: in a little dish (50 mm diam.), for 30–35 min, and on a water bath at 70°C. The volume of the sample solution decreased from 1 ml to 0.3–0.5 ml in these conditions. The removal of ethanol may be unnecessary if completely purified enzyme is available.

3.5. Influence of the presence of acetoin

The above mentioned evaporation treatment to eliminate ethanol could decrease the amount of acetoin in the sample solution only to about half for each acetoin concentration from 50 to 500 mg/litre. So the influence of the presence of acetoin was investigated. As shown in Table 3, acetoin is never oxidised by BDHG and remains unchanged in the sample solution, nor does it disturb the oxidation of BD. Therefore, the amount of BD can be exactly determined, even in the presence of acetoin, from the results of acetoin analyses carried out before and after the oxidation of BD.

3.6. Isomers of butane-2,3-diol

Three stereoisomers of BD exist: D(−), L(+) and meso isomers. Stereospecificity of the BDHG from *Sarcina* for these isomers has not yet been established. The BD isomers present in wines have been separated and identified as D(−) and meso by gas chromatography.[2] As shown in Figure 2, two peaks, which correspond to D(−)- and meso-BD, could be found on a gas chromatogram of a wine. Both of these peaks completely disappeared by treating the wine with the *Sarcina* enzyme. This appears to indicate that the *Sarcina* enzyme can act on both D(−) and meso isomers.

Table 3. Effect of the presence of acetoin on the enzymatic determination of butane-2,3-diol

Sample solution		Amount measured		Recovery	
Acetoin (μg/ml)	Butanediol (μg/ml)	Acetoin[a] (μg/ml)	Butanediol[b] (μg/ml)	Acetoin (%)	Butanediol (%)
3.80	—	3.85	—	101.3	—
7.60	—	8.05	—	105.9	—
11.40	—	11.80	—	103.5	—
15.20	—	15.85	—	104.3	—
19.00	—	18.80	—	98.9	—
3.80	9.05	—	9.00	—	99.4
7.60	9.05	—	8.55	—	94.5
11.40	9.05	—	7.70	—	85.1
15.20	9.05	—	8.95	—	98.9
19.00	9.05	—	9.20	—	101.7

[a] Measurement was made after the enzymatic oxidation by BDHG.

[b] Determined as follows: using the sample solution before oxidation, decrease in absorbance at 340 nm was measured (ΔA-1) in the presence of BDHG and NADH. After the enzymatic oxidation of the sample, decrease in absorbance was again measured (ΔA-2) by the same method. The difference between these two values was attributable to the acetoin formed from BD by the oxidation. The value (ΔA-2 minus ΔA-1) was converted to amount of BD with a standard curve.

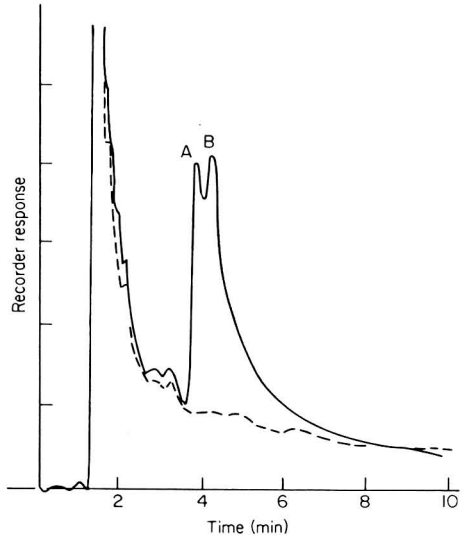

Figure 2. Consumption of butane-2,3-diol isomers in wine by the *Sarcina* enzyme shown gas chromatographically The D(−)-isomer peak A and meso-isomer peak B (——) disappeared (– – –) during the enzyme reaction. The reaction conditions were as described in the text. The gas chromatography operating conditions were as follows: column: 2 m × 3 mm i.d. stainless steel packed with Ucon-75-H-90 000 (10% w/w) coated Chromosorb W-AW (60–80 mesh), carrier gas: nitrogen 32 ml/min; temperatures: column 125°C, injector 200°C; sample: 3 μl of the ethanol-removed wine, directly injected on to the column.

3.7. Butane-2,3-diol contents in some Japanese wines

Amounts of BD in some Japanese commercial table wines were determined by the enzymatic method presented here. Table 4 shows the results. Concentrations of BD ranged from 324 to 768 mg/litre, while acetoin ranged from 0 to 43 parts/10^6. Red wines contained more BD than white wines on an average. Wines containing much BD generally contained much acetoin.

Table 4. Acetoin and butane-2,3-diol in some Japanese commercial table wines measured by the enzymatic method

Type	Number of samples	Acetoin (mg/litre)		Butanediol (mg/litre)	
		Range	Mean	Range	Mean
Dry white	17	2–14	7	324–747	508
Dry red	13	0–43	13	426–768	581
Total	30	0–43	10	324–768	538

4. Discussion

Most of the methods employed for the determination of BD consist of removal of interfering substances, followed by oxidation of BD to acetaldehyde by periodate or to acetoin by bromine, and colorimetric determination of the amount of acetaldehyde or acetoin formed. These methods are, in general, hard, tedious, and lacking in accuracy owing to the difficulty of complete separation of interfering substances. Besides, the optimum conditions for the oxidation of BD are difficult to establish. Gas chromatographic determination is more easy, but direct injection of samples on to the g.c. column should be avoided when the samples contain high concentration of sugars. By the enzymatic method presented here, specific oxidation of BD can be easily performed without removal of sugars or glycerol, and many samples can be analysed in a short time.

The activity of BDHG can be found in various microorganisms other than the strain of *Sarcina hansenii* used by the authors. The enzymes of these microbes, however, are not always the same in nature. Results of comparative studies on these enzymes and studies on the possibility of utilising the microbes other than *Sarcina hansenii* as enzyme sources for this determination procedure will be reported later.

Direct spectrophotometric measurement of NADH formed by the enzymatic oxidation of BD is not successful as yet, owing to the low reaction rate (absorbance continues to increase slowly). The adaptation of this direct method to fluorophotometry is now under investigation. A colorimetric method by measuring the fading of the blue colour of 2,6-DCPI, which is linked with the oxidation of BD, was also unsuccessful because of its susceptibility to influences of pH or other substances.

The levels of BD found in the Japanese wines were relatively low and within a narrow range compared with other results.[2] This is presumably due to the various vinification conditions in Japan; grape varieties, sugar levels of musts, fermentation temperatures and others.

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Release of Carbon Dioxide from Dough During Baking

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Dough was baked in a closed container from which the volatiles were swept with a stream of nitrogen. Carbon dioxide was determined colorimetrically by absorption in an alkaline indicator solution contained in a special cell, in which the flow of gases caused rapid circulation of the absorbent. Graphs of carbon dioxide release against time in oven were sigmoid in form, showing induction periods averaging 4.5 min for mechanically developed (CBP) doughs mixed with fat, and 2.3 min without fat. Analysis of the variance of induction periods and of loaf volumes showed that, in each case, the factor "fat *versus* no fat" was highly significant. Induction periods were highly correlated with loaf volumes. These results show that loaf volumes are considerably influenced by changes in the permeability of doughs to carbon dioxide at the start of baking. The possibility was considered that carbon dioxide release may occur at a definite transition temperature which is affected by the addition of fat.

1. Introduction

The addition of a small proportion of bakery fat (shortening), containing high-melting components, is essential for the production of commercially acceptable bread by processes involving mechanical development of the dough,[1–3] such as the Chorleywood bread process (CBP). The composition of the shortening for CBP can vary widely provided that sufficient is used (0.09–2.14% of the flour weight) to provide about 0.02% of unmelted fat at 100°F (37.8°C).[3] The beneficial effects of fat in improving loaf volume and crumb texture manifest themselves in the oven; this had been established earlier with another no-time bread making system.[4] The course of dough expansion in the oven was investigated in our laboratories by means of time-lapse cinematography,[5] which made it clear that doughs containing fat started to rise more rapidly immediately after entering the oven, and continued to rise for longer than doughs made without added fat. Three suggestions were made to account for these observations: the presence of fat might cause (a) lowered permeability of crumb cells to steam or carbon dioxide; (b) delay in the increase in viscosity associated with starch gelatinisation; (c) delayed protein denaturation.

The present work extends these studies by measuring the release of carbon dioxide from doughs during baking in a closed vessel through which nitrogen was passed to sweep out the volatile products. Carbon dioxide was determined by an adaptation of Emmert's colorimetric method.[6] This work was briefly described in a lecture delivered to the American Oil Chemists' Society at their Fall Meeting, 1974.[7]

2. Experimental

2.1. Materials

2.1.1. Flours

All flours were obtained from commercial mills. Flours A and B were treated with oxidising improver at the mill, the others were untreated. C, D and E were experimental flours, milled from English, Manitoban, and mixed English and Manitoban grists respectively. Flours A and B were fresh,

C–E had been stored in N_2-filled cans at −20°C for 1.5 years; F had been stored for 2, and G for 6 years, both at ambient temperatures, in moisture-proof sacks. Protein contents: A, 11.6; B, 10.9; C, 10.3; D, 13.6; E, 12.3; F, 13.3; G, 12.6%.

2.1.2. Bakery fat

"Covo" (Van den Berghs and Jurgens Ltd, Craigmillar Division) was used throughout.

2.1.3. Absorbent for carbon dioxide

Aqueous sodium hydroxide (0.5 N, 10 ml) mixed with 2 ml Titan Yellow indicator solution (BDH Ltd, 1% w/v in ethanol–water, 1:9 v/v). This indicator changes colour, from red to yellow, in the pH range 13.0–12.0.

2.2. Closed baking container

The special baking container is shown in Figure 1, *A*. Its base was similar in shape to a conventional baking tin (dimensions: bottom, 144 × 56 mm; top, 184 × 120 mm; height, 150 mm; capacity, 2400 ml;

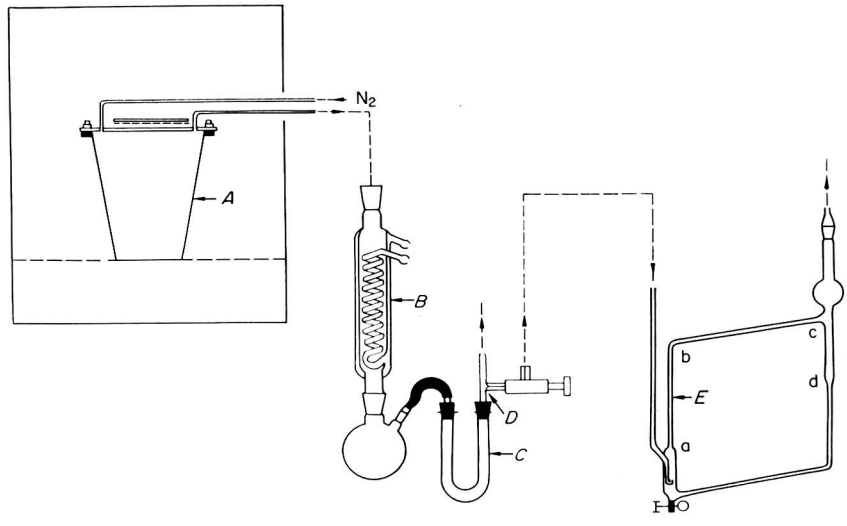

Figure 1. Schematic diagram of apparatus for determining CO_2 released from doughs during baking. *A*, baking container with heated lid (electrical connections not shown); *B*, double-surface condenser; *C*, drying tube containing blue silica gel; *D*, gas-stream splitter; *E*, absorption cell containing alkaline Titan Yellow solution, caused to circulate by N_2 bubbles rising in limbs a–b and b–c; limb c–d (o.d., 8.0 mm) is in light-path of the EEL colorimeter.

constructed in 16 gauge aluminium). A wide flange, reinforced with steel, carried ten bolts for securing the flat lid (aluminium, thickness 3.5 mm), sealed with a silicone rubber gasket (SR70, Esco (Rubber) Ltd, London). Additional heating for the lid was necessary for the production of loaves of normal appearance using this container. Two flat heating elements, as used in domestic irons, were clamped to the lid with a second aluminium plate, and were connected in series (output at 240 V, 225 W). The lid was also fitted with inlet and outlet tubes for N_2, extended horizontally about 150 mm so as to pass through a hole in the oven door.

2.3. Continuous determination of CO_2 released on baking

N_2 (3 litres/min, measured by a flowmeter at the inlet) was used to sweep steam and other volatiles from the baking tin. These were partially condensed by a glass double-surface condenser, *B*, and

the N_2 stream was further dried with coarse silica gel granules in the U-tube, *C*. A glass T-piece, *D*, with the open outlet constricted to *ca* 1 mm diameter, and the other attached to a needle valve, acted as a gas-stream splitter. The slower stream (100 ml/min) was passed through a special absorption cell, *E*, which utilised the "air-lift" principle to circulate the alkaline absorbent solution. A second gas flowmeter was attached to the outlet of the "circulating cell" *E*.

The right-hand limb (c–d) of the cell, *E*, was inserted into a modified EEL colorimeter[8] fitted with a blue filter No. 623, λ_{max} 490 nm. The output from the colorimeter was connected via an adjustable potentiometer to a recorder. With distilled water in the cell, the internal meter was set at 100% transmission (100% T) by adjusting the shutter of the colorimeter, and the recorder was set to full-scale deflection (20 mV range) by means of the potentiometer. The apparatus was calibrated with known mixtures of 0.5 N-NaOH and 0.5 N-Na_2CO_3 (10 ml) with indicator (2 ml), a constant flow of N_2 (100 ml/min) being maintained in cell *E* while measurements were being made. The efficiency of the absorption of CO_2 was determined by injecting known quantities from a gas burette into the rapid gas stream. In these experiments, the baking tin was replaced by a flask containing boiling water, and the CO_2 was introduced between this flask and condenser, *C*. The standard absorbent mixture (section 2.1.3.) was used.

2.4. Baking procedure

With each sample of flour, two doughs were prepared by the CBP,[5] one with fat (2 lb/sack: 0.71% of flour weight) and one without fat, the basic recipe being: flour 840 g; yeast 18 g; salt 15 g; water as required by the water absorption of the flour, *ca* 480 ml; potassium bromate 0.021 g (flours C–G); ascorbic acid 0.126 g; fat (when included) 6 g. One-third of the mix was taken, moulded by machine, rested for 10 min, remoulded, and sealed in the container, *A*. The proof period was 50 min at 36°C; during the last few minutes the container was transferred to a water-bath at 36°C and water-saturated N_2 (3 litres/min) passed through for 1.5 min to expel CO_2 released during proof. It was then placed in the preheated oven, timing commenced, electrical and N_2 connections rapidly made, and the flow of N_2 restarted. Colorimetric measurements began within 1 min, and baking continued for 32 min. The removal of CO_2 from the vicinity of the dough was not thought to influence the course of CO_2 production. Indeed, rapid diffusion from the surface is also believed to occur in normal baking conditions.

The oven (Simon laboratory baking oven, static) was set at 215°C. Full power (225 W) was applied to the lid heating element, for 12 min, and reduced power (90 W) for the next 20 min. Preliminary tests with a thermocouple below the lid had shown that this heating programme reproduced the temperature changes which occur above a conventional open tin when it is inserted into this oven.

3. Results

3.1. Baking in a closed vessel

Bread baked in the special container was of reasonable quality, though generally slightly underbaked, and with larger volume than that made from the same flours under the usual laboratory conditions. Average volumes of loaves obtained by each of the baking methods are shown in Table 1. Flours C–F, in which the response to fat was positive (i.e. loaf volume increased) under ordinary conditions of baking, behaved in the same way when baked in the closed container. The fresh flours A and B were known to behave normally, but their actual fat responses under ordinary conditions were not determined. The badly deteriorated flour G showed a small negative fat response under both conditions.

3.2. Performance and calibration of CO_2-measuring system

Alkaline phenolphthalein[6] was found to be unsuitable for use in the circulating absorption cell, as the initial rapid reaction when CO_2 was injected was followed by a slow return of colour on

Table 1. Loaf volumes (ml) of bread baked in the closed vessel or in open tins (means of 2 bakings)

	Flour						
	A	B	C	D	E	F	G
Baking in closed vessel:							
Without fat	1290	1340	1100[a]	1395	1290[a]	1230	1315
With fat	1605	1630	1550[a]	1715	1630[a]	1480	1210
Baking in open tins:							
Without fat	—	—	1135	1310	1180	1110	1060[a]
With fat	—	—	1450	1630	1555	1400	1040[a]

[a] Single values.

continued passage of N_2. This disadvantage was avoided by using an absorbent in which a colour change occurred at a higher pH. In tests with Titan Yellow, blank readings showed only a slight upward drift (0.3% T in 30 min period, max. rate of drift 0.05% T/min) provided that a steady flow of N_2 was maintained. If the circulation of absorbent was interrupted, an immediate downward drift in readings occurred (initial rate 1% T/min, later decreasing), but the original reading was restored if circulation was resumed within a short time. This behaviour was possibly due to a photolytic change in the small volume of reagent remaining in the light path.

In calibrating the apparatus with 0.5 N-NaOH–Na_2CO_3 mixtures, 1.0 ml 0.5 N-Na_2CO_3 corresponded to the absorption of 0.25 mmol CO_2 by 10 ml 0.5 N-NaOH. The graph of light absorbance ($=\log_{10} 100/\%T$) against mmol CO_2 was curved, but it was found that there was a linear relation between $100/\%T$ and CO_2 concentration (Figure 2). The regression coefficient, $b = 1.40 \pm 0.03$,

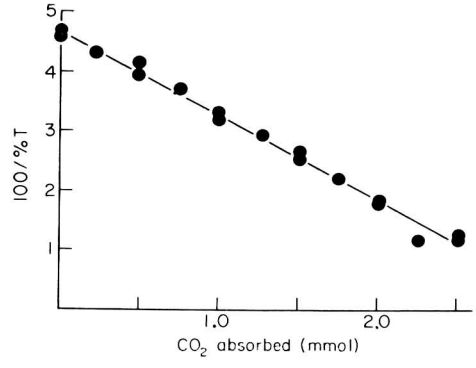

Figure 2. The relationship between CO_2 absorbed by 0.5 N-alkaline Titan Yellow solution (12 ml) and $100/\%T$, measured at λ_{max} 490 nm in circulating cell *E*, with N_2 (100 ml/min) flowing. Regression equation: $Y = 4.64 - 1.40\ X$ ($r = 0.997$) where Y is $100/\%T$, X is CO_2 absorbed (mmoles).

was used to calculate the CO_2 absorption from %T values obtained in subsequent experiments. In those using a gas burette, the amounts of CO_2 injected and absorbed were highly correlated ($r = 0.995$, 18 d.f.), the recovery being $84 \pm 2\%$.

In operating a gas-stream splitter in conjunction with the circulating absorption cell, the splitting ratio was found to be very sensitive to fluctuations in input flow-rate. This effect was due to the inherent pressure-stabilising property of the "air-lift" in the circulating cell; in such a device, flow-rate is not proportional to pressure. This source of inaccuracy was minimised by increasing the flow resistance of the splitter, so that an inlet pressure of 40–45 cm of water above atmospheric ($4.0–4.5 \times 10^3$ Nm^{-2}) was needed to maintain the flow-rate of 3 litres/min. This slight increase in atmospheric pressure over the dough did not affect its baking behaviour.

3.3. Release of carbon dioxide during baking

The course of CO_2 release during baking followed sigmoid curves typified by those in Figure 3. These curves were drawn after converting %T values from the recorder traces into mmoles of

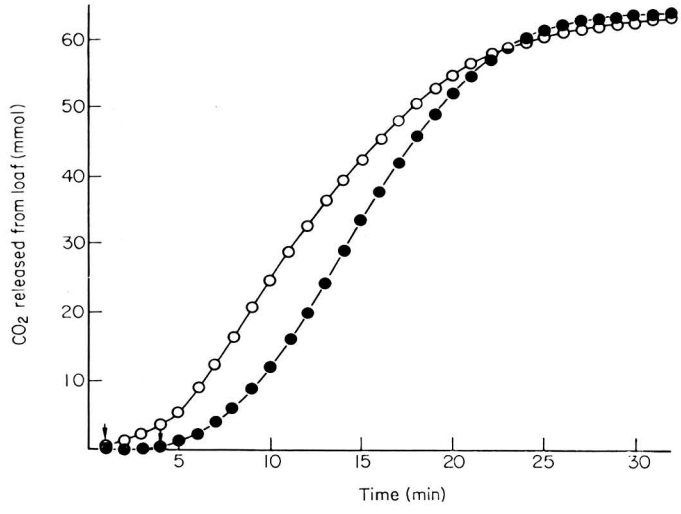

Figure 3. The CO_2 released from doughs during baking in the closed vessel. Samples: CBP doughs prepared from 280 g flour E. ○–○, Dough containing no fat; ●–●, dough containing 2 g fat; ↓ signifies end of induction period.

evolved CO_2, including corrections for gas-stream splitting (1:30) and percentage absorption (84%). They indicated the occurrence of an induction period during which there was little release of CO_2: the end of the induction period was arbitrarily defined as the time at which the rate of change in %T reached 1.5 × the maximum rate of drift, i.e. *ca* 0.08% T/min.

Table 2 shows the induction periods obtained, with doughs mixed from the seven flours, with and without fat. The average values were, for doughs with fat, 4.5 min; for those without fat,

Table 2. Induction periods (minutes) preceding release of CO_2 from doughs baked in the closed vessel (means of 2 bakings)

	Flour						
	A	B	C	D	E	F	G
Without fat	2.0	3.0	2.0	3.5	1.5	1.5	2.5
With fat	5.0	4.5	6.0	6.0	4.5	3.5	2.0

2.3 min. Separate two-way analyses of variance for the loaf volumes (24 bakings) and for the induction periods (28 bakings) were carried out. They indicated that, of the two main effects "between flours" and "fat *versus* no fat", the former was not significant either for loaf volumes or for induction periods, when compared with the appropriate error variance. However, the fat effect was significant at the 1% level of probability for loaf volumes, and at the 0.1% level for induction periods. Examination of the variance and covariance for the same two properties, where appropriate pairings were available, indicated a significant correlation between them ($r = 0.91^{***}$, 8 d.f.).

4. Discussion

The phenomena that cause the rapid expansion of dough when it is placed in the oven, including the thermal expansion of the air and CO_2 already in the cells, production of extra CO_2 by the yeast, vaporisation of ethanol and other organic volatiles, and the progressive evolution of steam, have often been described.[4,9] Expansion may be limited (a) by a temperature-dependent increase in the permeability of intact gas-cell walls, until the rate of leakage equals the rate of gas production, and (b) by a decrease in wall thickness due to the expansion of the dough, leading perhaps to the actual formation of holes. The present work has shown the importance in determining the final loaf volume, of changes in the permeability of dough to CO_2 during the first few minutes of baking. Conditions during the initial expansion of dough, while there is minimal loss of CO_2, are not necessarily related to those in later stages. For this reason, the definition of the induction period (section 3.3) was adopted, based on an arbitrary initial rate of CO_2-release. It was considered that the conventional definition, the intercept with the time-axis of a tangent to the linear part of the graph of CO_2-release *versus* time (Figure 3), might give misleading results.

The precise nature of the barrier to CO_2 release is unknown. The presence of shortening fat may both decrease its initial permeability to CO_2 and also increase its resistance to thermal change. Microscopic studies have shown that gas cell walls (after proof) consist of gluten layers in which the large starch granules are embedded tangentially.[9,10] It is generally held that the gluten network is the component most involved in gas retention,[9,11] particularly in preserving the mechanical integrity of the cell walls under stress. Arising from studies of the extensive binding of lipids by gluten during mixing, a mechanism for the improving action of fat has been proposed by Daniels *et al.*,[12] based on competition between hard fat components and natural flour lipids for "binding sites". A part of this hypothesis is the view that lipids in the free (petrol-soluble) state are beneficial in breadmaking. The importance of the free lipid fraction is supported by recent histological[13,14] and chemical[7,12] evidence that much of the shortening fat added to dough remains in the free state, as globules of varying size. Owing to the difference in composition between flour lipids and shortening fat, the free lipids of dough prepared with fat include a higher proportion of saturated fatty acids than do those without fat.[7] In another field of study, increasing the proportion of saturated components in a lipid film between two phases, increased the resistance of this barrier to the passage of solutes across it.[15] Despite the obvious difference between the dispersion of free flour lipids and that of lipids in a film, there appears to be a parallel between their responses to increasing saturation. However, in doughs made with fat from flours deteriorated by long storage (e.g. flour G), the free lipids are even more saturated, though smaller in quantity, than those in similar fresh flour doughs.[7] With these doughs gas retention during baking is poor, and the "fat response" is negative. It is clear that factors other than the saturated lipid content of the free lipid are involved in this case.

Consideration of the form of the CO_2-release curves has led to the hypothesis that the end of the observed induction period corresponds to a change in dough properties at a definite temperature. If a small region, or volume element, of the dough is considered, it is suggested that an abrupt change in permeability to CO_2 occurs at this "transition temperature", but that in a real system being heated from the outside, the inward progression of the transition zone at a finite rate would lead to less sharp overall changes, and consequently to the sigmoid form of CO_2-release curves actually observed. According to this hypothesis, changes in induction period correspond to changes in the transition temperature; in particular, the presence of a suitable shortening would raise it.

Some support for this hypothesis is provided by the work of Baker and Mize[4] on the time-course of the rise of internal pressure and of loaf height in doughs heated by electrical resistance heating. Discontinuities were observed in the curves, occurring later in the doughs with fat than in those without fat, and these may correspond to the transition temperatures postulated above. These authors interpreted their results in terms of melting of the shortening.

An alternative explanation may invoke an interaction between shortening and starch, which is perhaps not strong enough to be classed as lipid binding in terms of solvent extraction. There is evidence that the fat globules tend to concentrate at the surface of the starch granules.[9] Saturated

monoglycerides and triglycerides have markedly beneficial effects in the baking of gluten-free starch loaves,[16,17] the former mainly with respect to crumb structure and the latter with respect to loaf volume. Addition of the corresponding unsaturated lipids did not benefit starch loaves. There is thus a close similarity between the responses to fat observed in baking ordinary flour doughs and gluten-free starch batters. Saturated monoglycerides were preferentially bound to starch, and unsaturated monoglycerides were preferentially bound to protein, in continuous-mix bread.[18] Saturated monoglycerides are adsorbed to starch in dilute aqueous suspension.[19] However, although commercial shortenings usually contain monoglycerides, work with "model fats" consisting of pure triglycerides, shows that additions of monoglycerides are not obligatory for the production of volume-improving effects in the CBP.[7] In connection with the deterioration of flour during storage, the observation that unesterified fatty acids are preferentially bound to starch[18] is of interest, in view of the extensive lipolysis which occurs in such flours. Strong binding of fatty acids to starch may modify its lipophilic properties, and by changing the degree of interaction with the added fat, may indirectly influence the baking properties of the flour.

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Organic Sulphur Fractions in Scottish Soils

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Over 90% of the sulphur in some acid surface soils derived from different parent materials in north-east Scotland was in organic combination. N:S ratios in the majority of the soils fell within the narrow limits of 6.0–7.5, and showed less variation than C:S ratios. Organic sulphur was poorly correlated with organic phosphorus, and high correlations with categories of soluble aluminium and iron seemed to reflect similar relationships between these elements and the whole organic matter. A group of calcareous soils derived from shelly sand deposits contained a lower proportion of organic sulphur. An average 64% of organic sulphur in the acid soils occurred as organic sulphate, compared with only 23% in the calcareous soils. Organic sulphate was less well correlated with carbon and nitrogen than was total organic sulphur. Total carbon-bonded sulphur was best determined as the difference between total organic sulphur and organic sulphate, because direct measurement by Raney-nickel reduction underestimated the amount present in most of the soils, due to the presence of chemically unreactive compounds. Good correlations between the reactive carbon-bonded sulphur and carbon, nitrogen and organic sulphur, suggested that a well-defined group of compounds was being measured. There were no consistent effects of drainage conditions on organic sulphur contents, or on the amounts and proportions of organic sulphur present as sulphate or in carbon-bonded form. The freely drained soils derived from basic igneous drift, however, contained more organic sulphur than poorly drained samples, but this is attributed to differences in the organic matter content.

Several features of the results indicated that organic sulphur, unlike organic phosphorus, was predominantly an integral part of the soil organic matter.

1. Introduction

Most of the sulphur in non-calcareous soils of the humid regions usually occurs in organic combination and there is a growing interest in the nature and properties of this fraction.

A large number of organic sulphur compounds have been isolated from plants, animals and microorganisms,[1] but few have so far been found in soils. Some soil sulphur, occurs in amino acids, polypeptides or proteins, and in two Australian soils, 26% of the total organic sulphur was found in amino acids after hydrolysis.[2]

Treatment of soils with Raney-nickel and sodium hydroxide has shown that a large proportion of organic sulphur is in carbon-bonded form. Many organic compounds containing a C—S bond, including the amino acids methionine and cystine, are reduced to inorganic sulphide by this reagent whereas organic sulphates do not react. However, some compounds with a C—S bond, for example alkyl sulphones and sulphoxides, are not reduced.[3]

The remainder of the organic sulphur has not been fully characterised, but can be reduced to inorganic sulphide by a reagent containing hydriodic, formic and hypophosphorous acids, which does not liberate sulphur directly bonded to carbon, and the reactive fraction is thought to consist of organic sulphates.[4] These exist in nature in several forms, including sulphate derivatives of polysaccharides, lipids and phenols.

Little is known about the organic sulphur fraction in Scottish soils, except that it accounts for

most of the total sulphur. In view of the importance of sulphur as a plant nutrient, and as a constituent of soil organic matter, the distribution and properties of the organic sulphur fraction have been examined in a selection of contrasting soils from north Scotland.

2. Materials and methods

2.1. Soils

Sets of freely drained surface soils, derived from four different parent materials, were obtained from field experiment sites in north-east Scotland. They represented the Insch, Foudland, Countesswells and Stonehaven series, belonging to the Associations of the same name (Table 1) described

Table 1. Ranges and mean values of soil properties (ten soils from each Association)

Soil Association	Soil series	Parent material	pH	Organic carbon (%)	Nitrogen (%)	Sulphur (mg/kg)
Insch	Insch	Basic igneous drift	5.3–6.5 5.8	3.7–6.6 4.9	0.31–0.48 0.39	530–800 654
Foudland	Foudland	Slate drift	5.0–6.0 5.6	3.2–7.2 4.6	0.28–0.43 0.34	420–750 562
Countesswells	Countesswells	Granite drift	5.1–6.1 5.6	2.6–7.0 4.8	0.27–0.41 0.31	360–690 510
Stonehaven	Stonehaven	Old Red Sandstone drift	5.3–6.1 5.5	1.7–6.5 3.6	0.17–0.46 0.28	300–780 483
Fraserburgh	Fraserburgh	Calcareous shelly sands	7.0–7.5 7.4	1.6–6.5 2.8	0.18–0.46 0.24	460–1790 985

by Glentworth and Muir.[5] Because of differences in lithology of the Old Red Sandstone drift, the Stonehaven group has wider ranges of properties than the others, and three of the soils have subsequently been allocated to other Associations. Ten calcareous soils of the Fraserburgh Association and series, derived from shelly sand deposits, were also examined.

To study influences of drainage, comparisons have also been made between each of the above freely drained series and a corresponding poorly drained series from the same Association. Altogether ten pairs of soils were examined, and to eliminate effects of differential agricultural treatments, each pair was sampled within the same field.

The soils were sampled to 20 cm depth, air-dried and the fraction less than 2 mm retained for analysis.

2.2. Methods

Inorganic sulphate was extracted from the acid soils with 0.016 M-potassium dihydrogen phosphate[6] and from calcareous soils with 0.1 M-hydrochloric acid,[7] and was estimated turbidimetrically[8] using a seed suspension to increase the sensitivity.[9]

Total soil sulphur was measured after ignition of the soil at 400°C with magnesium nitrate,[8] and total organic sulphur calculated as the difference between total sulphur and inorganic sulphate, assuming that no other inorganic forms of sulphur were present. Total sulphate, in soils and extracts, was measured by reduction to hydrogen sulphide with a mixture of hydriodic, formic and hypophosphorous acids, the hydrogen sulphide being measured by the methylene blue method.[10] Total organic sulphate was obtained by subtraction of inorganic sulphate from total sulphate. Carbon-bonded sulphur was estimated directly using Raney-nickel in the presence of sodium hydroxide,[3] and indirectly as the difference between total organic sulphur and total organic sulphate.

Organic sulphur compounds soluble in acetylacetone were extracted from 2 g samples of soil which had been pretreated by shaking twice for 30 min with 40 cm^3 0.1 M-hydrochloric acid and

then for 5 min with 30 cm^3 water. The acid extracts and washings were discarded. The residue was treated with 90 cm^3 0.2 M-aqueous acetylacetone at pH 8.0 and after standing overnight the pH was restored with a few drops 3 M-sodium hydroxide. The soils were dispersed for 10 min with a KG100 ultrasonic generator (Kerry's Ultrasonic, Hitchin, Herts.) fitted with a titanium probe, 20 mm in diameter, in which no sulphur could be detected (<0.5 μg S in 25 mg). The mixture was then shaken gently for 20 h and centrifuged at 25 000 g for 10 min. The supernatant solution was decanted, the residues treated for a second time with acetylacetone, and the two extracts combined and diluted to 200 cm^3 with water. Suitable aliquots were evaporated to dryness for sulphur analyses.[11]

Soluble iron and aluminium were extracted with acid-oxalate solution, pH 3.2,[12] and with 2.5% acetic acid for 2 h at the solvent: soil ratio of 40:1. The oxalate extracts were ignited to destroy organic matter and the residues dissolved in dilute hydrochloric acid. Iron and aluminium were measured colorimetrically, the former with 2-2′-dipyridyl and the latter with aluminon.[13] Organic-carbon was measured by the Walkley–Black method[14] and nitrogen by the Kjeldhal method.

3. Results and discussion

3.1. Total organic sulphur

3.1.1. Acid soils

Although the total organic S for the 40 soils (Table 2) ranged from 286 to 768 mg S/kg, it formed a uniformly high proportion of between 90 and 98% of the total soil S, with virtually no difference

Table 2. Ranges, mean values and standard errors of total organic sulphur in acid and calcareous soils

Soil series	mg S/kg soil		% of total S	
	Range	Mean	Range	Mean
Insch	502–768	612 ± 29.3	90.5–96.2	93.5
Foudland	378–714	527 ± 36.6	90.0–96.4	93.5
Countesswells	335–673	484 ± 36.9	90.2–97.7	94.5
Stonehaven	286–744	455 ± 44.3	90.6–95.9	93.6
Fraserburgh	170–730	330 ± 32.1	11.3–79.3	41.1

between the four groups. The absolute organic S contents, however, showed effects of parent material. The group means fell clearly in the sequence Insch > Foudland > Countesswells and Stonehaven and, despite twofold internal variations, the differences between the Insch soils and the Countesswells and Stonehaven series were significant at the 5% level. The same trend was evident in the total N content (Table 1), but not in the organic C values, although the Stonehaven soils were again the lowest, in keeping with the more intensive cultivation and arable cropping practised in the area.

The mean N:S ratios for the four groups (Table 3) fell within the fairly narrow range of 6.0, for the Stonehaven soils, to 6.6, for the Countesswells series, in agreement with the observation of Russell[15] that this ratio shows relatively little variation in well managed agricultural surface soils. Of the 40 soils, 32 gave values between 6.0 and 7.5. The C:S ratio showed greater variation, the means ranging between 78.8 and 97.4.

As expected, organic S was highly and significantly correlated with both organic C and total N, (Table 4) but the level of correlation of about 0.8 ($P<0.001$) for carbon was lower than the corresponding value of about 0.9 ($P<0.001$) for nitrogen. In marked contrast to this, the relationship between organic S and organic P was very poor. This is compatible with the fact that none of the

Table 3. Ranges and mean values of carbon, nitrogen and sulphur ratio

Soil series	C:N	C:S	N:S
Insch	11.6–15.1	72.5–97.4	5.8–7.6
	12.5	79.6	6.4
Foudland	11.4–17.3	74.5–122.6	5.9–7.1
	13.6	86.6	6.4
Countesswells	8.8–20.0	61.9–131.5	5.4–9.6
	14.7	97.4	6.6
Stonehaven	8.2–18.7	35.2–97.5	4.3–7.1
	13.4	78.8	6.0
Fraserburgh	9.7–15.7	62.4–130.5	5.7–13.5
	11.4	94.0	7.8

Table 4. Overall correlation coefficients for 40 acid soils

Total organic S *vs*:		Carbon *vs*:	
carbon	0.80***	acetic soluble Al	0.74***
nitrogen	0.90***	oxalate soluble Al	0.57***
organic phosphorus	0.43*	acetic soluble Fe	0.44**
acetic soluble Al	0.60***	oxalate soluble Al	0.31*
oxalate soluble Al	0.70***	Nitrogen *vs*:	
acetic soluble Fe	0.40*	acetic soluble Al	0.82***
oxalate soluble Fe	0.32 NS	oxalate soluble Al	0.81***
acetic soluble Al + Fe	0.60***	acetic soluble Fe	0.33*
oxalate soluble Al + Fe	0.66***	oxalate soluble Al	0.44*

NS = not significant. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

phosphate esters so far identified in soils contain sulphur, and also with the very variable proportion of organic P to organic matter in these soils.[16] The overall mean ratios of organic C:N:P:S were 136:10:1.6:2.5, similar to those reported in agricultural soils in other temperate areas.[17,18]

The group means (Table 3) showed that the C:N, C:S and N:S ratios all tended to be higher in the Countesswells series than in the others. This trend was not attributable to the current pH (Table 1), but in keeping with the lower base status of the parent material, it indicated a lower degree of decomposition of the organic matter in the Countesswells soils, which can be classified as cultivated podzols.

Soil organic matter consists of a heterogeneous mixture of components at various stages of decomposition, but in these soils much is in the form of very stable humus, whose resistance to breakdown is attributable largely to association with clay minerals and hydrous oxides of iron and aluminium. In keeping with this the organic carbon and nitrogen in the 40 soils were highly correlated with the amounts of iron and aluminium extracted with acetic acid or acid oxalate (Table 4), in agreement with earlier findings.[19] For example, the overall correlations of acetic and oxalate soluble aluminium with organic C were 0.74 ($P < 0.001$) and 0.57 ($P < 0.001$), respectively, compared with 0.82 ($P < 0.001$) and 0.81 ($P < 0.001$) for N. The corresponding iron correlations were 0.44 ($P < 0.01$) and 0.31 ($P < 0.05$) with organic C, compared with 0.33 ($P < 0.05$) and 0.44 ($P < 0.01$) with total N. These relationships supported the earlier conclusion that major proportions of the soluble aluminium in these soils were intimately associated with organic matter but that there was much less evidence of this for iron.

Organic S also showed significant overall correlations, at the 0.6–0.7 level ($P < 0.001$), with acetic and oxalate soluble aluminium and aluminium plus iron (Table 4), and again the corresponding correlations with iron were much poorer. There was little doubt, however, that the S relationships

were largely reflections of similar correlations between the organic matter and aluminium and iron, rather than an indication of the specific binding of S-containing organic moieties by the metals.

3.1.2. Calcareous soils

The total organic sulphur content of these samples was examined in an earlier study.[16] They contained considerable amounts of inorganic sulphate in insoluble or occluded form, associated with calcium carbonate. Consequently the proportion of organic sulphur was much less than in the acid soils, ranging from 11.3 to 79.3% of the total sulphur, with a mean of 41.1%. The mean absolute amount of total organic sulphur, 330 mg/kg, was also lower than in any of the acid soils.

3.2. Total organic sulphate

Total organic sulphate, calculated as the difference between hydriodic acid reducible sulphur and inorganic sulphate, accounted for 44–84% of the total organic sulphur in the acid soils. There was little difference between the soil series, the mean values ranged from 59.6% in the Countesswells soils to 67.4% in the Stonehaven series (Table 5). On average, organic sulphate comprised 64%

Table 5. Organic sulphate and carbon-bonded sulphur in the soils

			Carbon-bonded sulphur			
	Organic sulphate		Measured directly		Measured by difference	
Soil series	Range and mean mg S/kg soil	Mean % of total organic S	Range and mean mg S/kg soil	Mean % of total organic S	Range and mean mg S/kg soil	Mean % of total organic S
Insch	330–545 397 ± 19.8	65.5	66–159 109 ± 9.2	18.0	80–349 215 ± 25.5	34.5
Foudland	258–360 322 ± 12.0	62.9	60–136 96 ± 7.6	18.2	84–396 206 ± 33.7	37.1
Countesswells	184–330 279 ± 13.9	59.6	79–132 102 ± 6.9	21.1	80–342 204 ± 30.1	40.4
Stonehaven	191–424 302 ± 22.6	67.4	62–140 91 ± 8.3	20.0	95–320 154 ± 25.1	32.6
Fraserburgh	0–180 80 ± 10.9	23.1	40–170 76 ± 6.8	25.5	120–550 242 ± 18.9	76.9

of the total soil sulphur in the acid soils, somewhat higher than corresponding values for ranges of soils in Australia (52%)[1] and the USA (50%).[18] When the acid soils were considered as a whole there were significant relationships between organic sulphate and carbon and nitrogen (Table 6) but they were much poorer than the corresponding relationships for total organic sulphur (Table 4). The correlation between organic sulphate and organic phosphorus on the other hand, though poor, was higher than between total organic sulphur and organic phosphorus, indicating that an association existed between these ester forms, or that they were predominantly derived from the same sources.

In the calcareous soils organic sulphate varied from virtually zero to 45.5% with a mean value of 23.1%, much lower than in the acid soils. It was possible, however, that the values for the calcareous soils could have been low due to mineralisation of organic sulphates by the 0.1 M-hydrochloric acid used to extract inorganic sulphate from these soils. Mean ratios of carbon and nitrogen to organic sulphate were 194:1 and 15:1, respectively, compared to 140:1 and 10:1 for the acid soils. In the calcareous soils organic sulphate had low non-significant correlations with organic C, N, S and P.

Table 6. Correlation coefficients for relationships between organic sulphur fractions and organic C, N, S, P and soluble Al

Relationships	40 acid soils	10 calcareous soils
Organic sulphate *vs*:		
carbon	0.50**	0.23 NS
nitrogen	0.58***	0.23 NS
organic sulphur	0.69***	0.21 NS
organic phosphorus	0.47**	0.22 NS
acetic acid sol. Al	0.37*	
oxalate sol. Al	0.47**	

	Measured directly	Measured by difference	Measured directly
Carbon bonded S measured directly and measured by difference *vs*:			
carbon	0.84***	0.75***	0.64*
nitrogen	0.85***	0.77***	0.77**
organic sulphur	0.86***	0.85***	0.85**
organic sulphate	0.69***	0.61***	
organic phosphorus	0.18 NS	0.21 NS	0.16 NS
acetic acid sol. Al	0.66***	0.56***	
oxalate sol. Al	0.71***	0.54***	

NS = not significant. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

3.3. Carbon-bonded sulphur

In 1962 De Long and Lowe[3] described a method for measuring carbon-bonded S in soils involving reduction with Raney-nickel in alkaline solution. They noted that the reagent released most forms of organically combined sulphur, with the exception of alkyl sulphones and ester sulphates, and reacted also with elemental sulphur and some mineral sulphides. Carbon-bonded S determined by this method (Table 5) ranged from 60 to 159 mg S/kg soil in the acid soils, compared with 40 to 170 in the calcareous group. The mean carbon-bonded S accounted for 19% of the total organic sulphur in the acid soils, and for 25% in the calcareous soils, compared with 12, 27 and 35% for three Canadian mineral soils,[20] 17 and 26% for Canadian Chernozemic and grey-wooded surface soils,[21] and 11% for Iowa soils.[18] In the 40 acid soils, organic sulphate plus directly determined carbon-bonded sulphur ranged from 64.3 to 102.2% of the total organic sulphur with a mean of 83.3%. Within each group the range was fairly wide, but the means were similar, ranging from 81% in the Countesswells and Foudland series to 88% in the Stonehaven soils. In the calcareous soils the sum ranged from 20.0 to 72.0% with a mean of 48.5%, much less than in the acid soils, indicating either a difference in the nature of the organic matter, or in its accessibility to the reducing agent.

As illustrated in Table 5, there were large discrepancies between the directly determined values for carbon-bonded sulphur and those calculated by difference, especially in the calcareous soils. These discrepancies may have been due to the presence of unreactive compounds which contained a C—S bond not reduced by Raney-nickel. Methionine sulphone and cysteic acid, for example, are not reduced in this way,[22,23] but although these compounds have been detected in soil extracts they are unlikely to occur in sufficient amount to account for such large differences. The presence of large amounts of iron and manganese can cause low recovery, by oxidising the reducing agent[23] but the amounts of these elements present, in relation to the reducing conditions employed, makes this an unlikely source of error.

It was suggested that lack of reactivity of carbon-bonded sulphur can also arise from insolubility rather than inherent chemical stability.[21] Measurements were therefore made on extracts of some of the acid soils to find what proportion of the extracted organic sulphur could be accounted for

as organic sulphate plus carbon-bonded sulphur. To minimise possible chemicals change, aqueous acetylacetone at pH 8 was used.[11] One soil showing a large difference between the calculated and directly measured values was selected for analysis from each of the acid groups.

Total sulphur in the extracts accounted for 73–103% of the total soil organic sulphur (Table 7), with an average of 87%, and included virtually all the organic sulphate and also all the directly

Table 7. Sulphur values in soil acetylacetone extracts (mg S/kg soil)

Association and series	Total organic sulphur			Organic sulphate		Carbon-bonded sulphur measured directly	
	Soil A	Extract B	B as % of A	Soil	Extract	Soil	Extract
Insch	600	500	83.3	440	400	80	80
Foudland	710	630	88.7	340	330	120	110
Countesswells	600	620	103.0	290	290	120	130
Stonehaven	740	540	72.9	330	310	140	140

measured carbon-bonded sulphur. In no instance, however, even in the one soil from which the total soil organic sulphur was extracted, did the sum of organic sulphate and directly measured carbon-bonded sulphur account for the total sulphur in the extract. This showed that chemically unreactive carbon-bonded sulphur compounds were present and that the discrepancies between the measured and calculated values were not simply due to inaccessibility to the reducing agent.

It is clear, therefore, that there are uncertainties in the direct estimation of carbon-bonded sulphur and that a more realistic approach is to use the value obtained by subtracting total organic sulphate from total organic sulphur. On this basis total carbon-bonded sulphur in the acid soils accounted on average for 36.2% of the total organic sulphur, compared with 77% for the calcareous group and 41% reported for Australian soils.[1]

Table 6 shows that in the acid soils both values for carbon-bonded sulphur were highly correlated with carbon, nitrogen, total organic sulphur, and total organic sulphate. They were also significantly correlated with oxalate and acetic acid soluble aluminium. In all cases, however, the correlations were closer for the directly measured carbon-bonded sulphur suggesting that the latter constituted a well defined category of soil sulphur, possibly amino acid sulphur. Irrespective of the method of measurement, correlations between carbon-bonded sulphur and other soil constituents (Table 6) were higher than for organic sulphate, indicating that carbon-bonded sulphur was a more stable and integral component of the soil organic matter.

In the calcareous soils carbon-bonded sulphur was significantly correlated with carbon, nitrogen and total organic sulphur but the low and non-significant correlation with organic phosphorus was even poorer than in the acid soils.

3.4. Heat-soluble sulphur

Williams and Steinbergs[24] noticed that when a soil was moistened and dried, first on a water bath and then in an oven at 102°C, the amount of sulphur extracted with sodium chloride increased considerably. They suggested that most of the increase was due to the release of organic sulphur, probably in the form of sulphate.

Treatment of eight of the acid soils in this way gave increases in total extractable sulphur ranging from 0 up to 33 mg S/kg soil (Table 8). The maximum increase was 157%, but in five of the eight soils the increases were between 10 and 23%. The absolute changes in inorganic sulphate extracted were mostly small and inconsistent, but the organic sulphate increased in every case. The absolute increases, however, were very variable, and sometimes exceeded the increases in total sulphur, indicating that heat treatment reduced the extraction of carbon-bonded sulphur in some of the

Table 8. Sulphur extracted by sodium chloride from heated and unheated acid soils (mg S/kg soil)

Soil Association and series	Untreated soil			Heat treated soil		
	Total S	Organic sulphate	Inorganic sulphate	Total S	Organic sulphate	Inorganic sulphate
Insch	47	21.8	8.0	47	30.3	6.0
Insch	43	19.6	5.5	51	27.9	8.4
Foudland	40	14.3	5.5	44	17.5	5.5
Foudland	21	3.8	6.1	54	32.8	6.1
Countesswells	42	4.5	8.1	50	20.6	8.7
Countesswells	38	10.4	8.7	42	17.1	6.6
Stonehaven	57	12.6	21.0	76	32.1	17.0
Stonehaven	70	20.1	8.0	86	25.6	12.4

soils. Organic sulphate constituted a mean 29.4% of the total sulphur extracted from the unheated soils, rising to 46.7% after heat treatment.

3.5. Influence of drainage

Comparisons of freely drained and poorly drained soil series within the soil Associations have shown that several properties of the acid soils were affected by the drainage status.[25] For example, the freely drained members were invariably richer than their poorly drained counterparts in both organic phosphorus and soluble aluminium (Table 9), and differences were normally greatest in

Table 9. Carbon, aluminium, organic phosphorus and organic sulphur values for four pairs of freely drained and poorly drained soils

Series	Drainage	C (%)	Acetic soluble Al (%)	Total organic P (mg/kg)	Total organic S (mg/kg)	Organic sulphate S (mg/kg)	Carbon-bonded S (mg/kg) (measured by difference)
Countesswells	Free	3.49	0.064	620	335	231	104
Terryvale	Poor	3.90	0.041	530	338	231	107
Insch	Free	6.24	0.126	1170	783	411	373
Myreton	Poor	3.10	0.026	770	470	331	139
Foudland	Free	4.21	0.027	730	468	289	179
Fisherford	Poor	3.49	0.023	420	427	231	196
Stonehaven	Free	2.07	0.033	470	215	184	31
Leadmore	Poor	1.86	0.010	300	255	184	71

the Insch Association. In contrast to this, there was no consistent effect on the organic sulphur content, nor on organic sulphate and carbon-bonded S, and only in the Insch Association were the sulphur values lower in the poorly drained soil. The values are not included, but there was also no consistent effect of drainage status on the C:S ratio, nor on the proportion of organic sulphate. In these cultivated surface soils, therefore, there was no evidence of greater aeration and oxidation in the freely drained samples.

Analyses, not included, of other pairs of samples confirmed the above pattern. Unlike organic phosphorus, the apparent influences of drainage on organic sulphur could be largely attributed to corresponding variations in the content of organic matter, rather than to any specific effects on

organic sulphur components. Like the relationships with carbon, nitrogen, aluminium and iron, the results therefore emphasised that the organic sulphur fraction was predominantly an integral part of the soil organic matter.

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Sulphate Contents and Sorption in Scottish Soils

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Phosphate-extractable inorganic sulphate contents of freely drained acid surface soils representing four contrasting soil series in north-east Scotland ranged from 13 to 60 mg S/kg soil. Of this an average of 59% was adsorbed under the conditions of extraction used. The soils were therefore well supplied with available sulphate and there are no known cases of deficiency in the area. Average group contents of adsorbed sulphate were closely related to the abilities of the soils to adsorb added sulphate, and unlike phosphate retention, sulphate sorption seemed to depend more on active iron than aluminium.

1. Introduction

Inorganic sulphate is the form of soil sulphur that is most readily available to plants.[1] In most soils, however, organic forms of sulphur predominate and readily soluble and adsorbed sulphate constitute only a small proportion of the total soil sulphur. Sulphur deficiency is not a problem in Scotland, but information on sulphur relationships is desirable to characterise major soil Associations and series, and to complement studies on soil phosphate. An examination has therefore been made of the inorganic sulphate contents of ranges of agricultural soils from contrasting soil series in north-east Scotland. Relationships were sought between their abilities to adsorb added sulphate and parent material, pH and amounts of soluble iron and aluminium.

2. Materials and methods

2.1. Soils

Ten freely drained, cultivated soils from each of four soil series were sampled to 20 cm depth at field experiment sites. The samples were air-dried and sieved to less than 2 mm before analysis. Further details and analytical results are given in Table 1. The soil Associations and series have

Table 1. Mean properties, with standard errors, of groups of ten freely drained acid surface soils

Soil Association		Insch	Foudland	Countesswells	Stonehaven
Soil series		Insch	Foudland	Countesswells	Stonehaven
Parent material		Basic igneous till	Till derived from slate and agillaceous schists	Granitic till	Old Red Sandstone till
pH (1:2 H_2O)		5.8	5.6	5.6	5.5
Sand (2–0.05 mm) %		58.5±0.9	47.8±3.4	64.8±1.9	54.0±1.8
Silt (0.05–0.002 mm) %		12.8±0.6	20.7±2.0	11.9±0.6	16.7±1.0
Clay (<0.002 mm) %		13.7±0.8	13.8±1.2	11.5±0.9	16.8±1.1
Organic carbon %		4.94±0.28	4.64±0.44	4.75±0.49	3.64±0.43
Acetic acid soluble (mg/kg)	Fe	10.7±1.4	12.5±1.3	11.5±2.3	8.33±2.2
	Al	920±100	726±50	694±50	493±95
Acid oxalate soluble	Fe %	0.96±0.06	0.99±0.08	0.65±0.05	0.52±0.05
	Al %	0.83±0.06	0.47±0.04	0.45±0.03	0.42±0.08
Dithionite soluble	Fe %	1.77±0.15	1.69±0.49	1.02±0.44	1.07±0.38
	Al %	0.47±0.02	0.37±0.02	0.33±0.01	0.29±0.03
P retention capacity %		0.92±0.05	0.72±0.05	0.55±0.03	0.55±0.08

been described by Glentworth and Muir.[2] Because of differences in lithology of the parent material, three of the soils included in the Stonehaven group have since been assigned to another Association, but for present purposes it suffices that they are all derived from Old Red Sandstone till.

2.2. Iron and aluminium extractions

Extractable iron and aluminium were determined by three different methods: (a) 2.5 g soil was shaken with 100 cm^3 2.5%, by volume, acetic acid for 2 h and filtered; (b) 3.0 g soil was shaken for 1 h with 100 cm^3 solution, pH 3.2, containing 24.9 g ammonium oxalate and 12.6 g oxalic acid per litre, and after centrifuging the supernatant was decanted off, the residue washed with 50 cm^3 of the extractant, and the treatment repeated;[3] (c) 2.0 g soil was shaken for 1 h first with 50 cm^3 solution containing 2.0 g sodium dithionite and then with 0.05 M-hydrochloric acid, and the treatments repeated.[4] Aliquots of the extracts were dried and ignited at 500°C for 10 min to destroy organic matter and the extracting reagent, and the residues taken up in dilute hydrochloric acid for analysis.

2.3. Inorganic sulphate extractions

Readily soluble sulphate was extracted by shaking 10.0 g soil for 30 min with 30 cm^3 water, and also by shaking 5.0 g soil with 25 cm^3 0.01 M-calcium chloride solution for the same time. Inorganic sulphate was determined in the filtrates.

To determine adsorbed sulphate, 20.0 g soil was shaken for 30 min with 100 cm^3 0.016 M-potassium dihydrogen phosphate solution (500 mg P/litre) and the sulphate determined in the filtrate; water soluble sulphate was subtracted to give the adsorbed sulphate fraction.[5]

2.4. Effect of pH on sulphate sorption

This was studied for two soils of the Insch series by adding 100 cm^3 of potassium sulphate solution containing 2.5 mg S to 5 g soil (equivalent to 500 mg S/kg soil) and adjusting the pH within the range 2 to 7 by adding hydrochloric acid or potassium hydroxide solution. The suspensions were shaken for 16 h at 20°C and the pH measured. Sulphate sorbed was taken as the difference between the initial and final concentration in solution. The results in Figure 1 show that sorption reached a maximum at pH 3, when 30% of the added sulphate was removed from solution, and fell rapidly

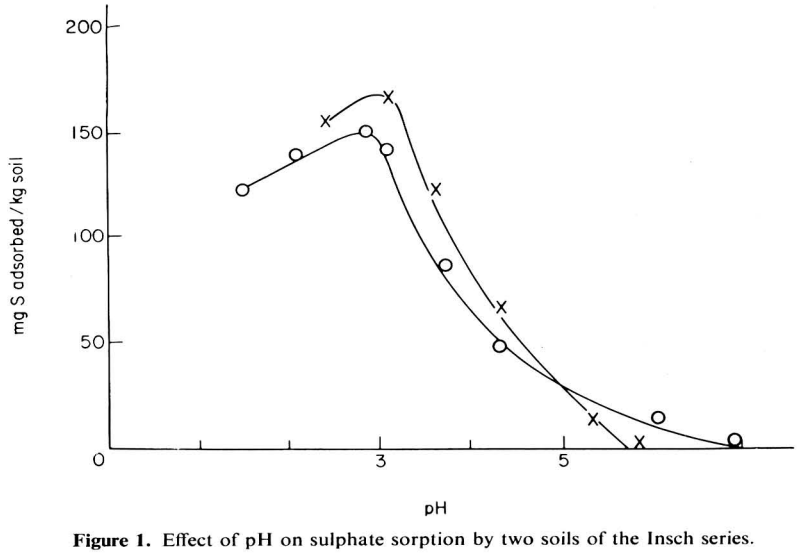

Figure 1. Effect of pH on sulphate sorption by two soils of the Insch series.

as the pH rose to the levels normally prevailing in limed soils. The pH has therefore an important effect and must be standardised when comparing different soils.

2.5. Sulphate sorption index

Full characterisation of sulphate sorption by soils requires isotherms showing the relationship between adsorbed sulphate and the concentration in solution. The preparation of such isotherms, however, is time consuming, and for examination of large numbers of soils a simple comparative index of their abilities to sorb sulphate is useful. Using the information in Figure 1, the following method was developed: 5 g soil was shaken for 16 h at 20°C with 100 cm^3 of potassium sulphate solution containing 2.5 mg S and 2%, by volume, acetic acid to ensure an optimum pH of 3.0 in the suspensions. Adsorbed sulphate was calculated from the difference in concentration between the initial solution and the filtrate. The amount of added sulphate was chosen to be sufficiently large to swamp differences in the content of native adsorbed sulphate, but for accuracy the latter was added to that removed from solution to give the total adsorbed.

2.6. Sulphate retention capacity

To obtain a standard comparative measure of the maximum capacity to adsorb sulphate at pH 3, the following method, analogous to that described by Piper[6] for phosphate retention capacity, was adopted: 5.0 g soil was treated with 25 cm^3 0.5 M-potassium sulphate solution adjusted to pH 3 for 1 h on a water bath at 50°C with occasional shaking. The suspension was then allowed to stand overnight at room temperature and filtered through a Buchner funnel. The soil was transferred completely on to the filter and leached with five successive 10 cm^3 portions of the potassium sulphate solution. It was then washed free of excess potassium sulphate solution with five 10 cm^3 portions of 60% aqueous acetone and allowed to dry. The adsorbed sulphate was extracted from the treated sample as described in section 2.3. Tests on a selection of soils rich in calcium showed that pretreatment with 0.1 M-potassium chloride did not significantly affect the results, indicating that precipitation of calcium sulphate was not a problem.

2.7. Chemical analyses

Inorganic sulphate was estimated turbidimetrically[7] using a seed suspension[8] to increase sensitivity. Iron was determined colorimetrically by the 2:2′-dipyridyl method after reduction with hydroxylamine hydrochloride. Aluminium was determined colorimetrically by the aluminon method.[9]

3. Results and discussion

3.1. Readily soluble sulphate

Water and 0.01 M-calcium chloride solution extracted similar amounts of sulphate ranging from 6 to 28 mg S/kg soil (Table 2), but calcium chloride was preferable because it flocculated the soil

Table 2. Means, standard errors and ranges of inorganic sulphate contents and sorption values (mg S/kg soil) for groups of ten freely drained acid surface soils

Sulphate value	Soil series			
	Insch	Foudland	Countesswells	Stonehaven
Water soluble	12.1±9.9 (5.9–15.7)	13.4±0.4 (10.5–14.9)	11.3±0.8 (7.5–16.1)	13.8±1.9 (7.3–27.7)
$CaCl_2$ soluble	11.3±1.1 (5.9–14.7)	11.6±0.7 (9.0–15.2)	11.5±1.0 (6.7–17.4)	12.7±2.0 (6.2–27.3)
Adsorbed	29.6±3.6 (12.8–46.2)	22.5±2.4 (15.2–37.4)	15.9±2.6 (2.2–27.2)	17.7±4.3 (3.5–48.1)
Sorption index	124±4.8 (103–146)	99±9.0 (58–140)	46±8.5 (10–91)	49±6.8 (26–92)
Retention capacity	4680±182 (3700–5450)	4120±203 (3000–5450)	2590±325 (1130–4100)	2500±363 (900–4440)

colloids and the suspensions filtered easily. This range was comparable with values found by workers in USA[10, 11] and Australia.[12] The four soil groups gave very similar mean values. The only statistically significant difference, $P < 0.05$, was the slightly higher water soluble sulphate content of the Foudland compared with the Countesswells group, but the difference was small and hinged on the narrower spread of the values concerned. This close similarity of the group means was compatible with the broadly similar textural and drainage characteristics of the soils and with the general uniformity of climatic conditions and agricultural practices in north-east Scotland.

3.2. Adsorbed sulphate

This varied from 2 to 48 mg S/kg soil. The general level was much higher than values reported for Iowa soils,[11] which contained virtually no adsorbed sulphate, and for Australian soils,[12] where the values seldom exceeded 10 mg/kg and were mostly less than 5. Since adsorbed sulphate is available for plant growth and sulphur uptake,[10, 13] the results showed that these representative agricultural soils from north-east Scotland were well supplied with available sulphur. The mean group values showed marked effects of parent material and fell clearly in the sequence Insch > Foudland > Countesswells and Stonehaven, with the differences between Insch and Countesswells, Insch and Stonehaven, and Foudland and Countesswells, attaining statistical significance.

3.3. Sulphate sorption index

Mean values in Table 2 show that the Insch soils adsorb most sulphate, followed by the Foudland soils, the average for which was twice that for the Countesswells and Stonehaven groups, which gave similar values. Despite wide internal variations, the differences between the Insch group and the others and between Foudland group and the Countesswells and Stonehaven soils were all statistically significant, $P < 0.05$. The corresponding variations above in adsorbed sulphate content can therefore be regarded as expressions of differences in the relative abilities of the soils to adsorb sulphate, and the sorption index provided a simple measure of this characteristic.

Table 3 shows correlations between the sulphate sorption index and soluble iron and aluminium determined by the oxalate and dithionite methods. Except in the Stonehaven soils, where the

Table 3. Correlation coefficients between sulphate sorption index and soluble iron and aluminium for 40 freely drained acid surface soils

Soil series	Oxalate soluble			Dithionite soluble		
	Fe	Al	Fe+Al	Fe	Al	Fe+Al
Insch	0.81**	0.01 NS	0.43 NS	0.69*	0.10 NS	0.68*
Foudland	0.95***	0.65*	0.90***	0.81**	0.46 NS	0.85**
Countesswells	0.56 NS	−0.17 NS	0.36 NS	0.93***	−0.06 NS	0.89***
Stonehaven	0.77***	0.93***	0.94***	0.35 NS	0.93***	0.67*
Overall	0.82***	0.52**	0.77***	0.76***	0.50	0.80***

NS = not significant. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

relationship was better with aluminium, the main factor determining sulphate sorption appeared to be the content of reactive iron. This was in marked contrast to the corresponding phosphate relationships where aluminium was in all cases the dominant retention agent.[14, 15] Except in the Countesswells group, oxalate-extractable iron correlated better with the sorption index than did dithionite-extractable iron. The dithionite reagent extracted considerably more iron (Table 1) and the lower correlations in the Insch, Foudland and Stonehaven groups were presumably attributable to dissolution of categories of iron which were less active in sulphate sorption. In the Countesswells soils, on the other hand, the reverse appeared to be true, possibly due to the presence of more

highly crystalline goethite, which might have been active in sulphate retention and was reduced by the dithionite but was not attacked by the oxalate.

Acetic acid was a weaker extractant for iron and aluminium than either oxalate or dithionite (Table 1) and with one exception the amounts extracted did not correlate with the sorption index (results not shown). The exception was the Stonehaven group, where aluminium gave a highly significant correlation of 0.93, $P<0.001$, again indicating that sulphate sorption in these soils depended more on aluminium than iron. This group also differed in that its sorption index correlated much better with oxalate soluble than with dithionite soluble iron. One factor underlying both these contrasts was likely to be the presence of hematite, which was probably not highly active in sulphate sorption and was excessively extracted by the dithionite reduction. Further work is necessary to decide whether these interpretations are correct.

3.4. Sulphate retention capacity

The saturation value (Table 2) was 40–50 times greater than the sorption index. Even so, the two values were correlated ($r=0.59$, $P<0.01$, for the 40 soils), and the mean group values again followed the sequence Insch < Foudland > Countesswells and Stonehaven. Differences between the Insch group and the others, and between the Foudland group and the Countesswells and Stonehaven soils were statistically significant ($P<0.001$). As illustrated by the values in Table 1, the corresponding phosphate retention capacity was also found[14] to follow this sequence. The two measurements were strongly correlated ($r=0.73$, $P<0.001$, for the 40 soils) and on a molar basis the mean sulphate values were consistently about half the phosphate values. This suggested that the same sites were involved in the adsorption of the two anions, with sulphate being sorbed as SO_4^{2-} and phosphate as $H_2PO_4^-$.

There was an instructive contrast between the two measurements of sulphate sorption. For both oxalate and dithionite soluble iron, the correlations with the saturation sulphate retention capacity (Table 4), were consistently poorer than with the sorption index (Table 3). On the other hand, the

Table 4. Correlation coefficients between sulphate retention capacity and soluble iron and aluminium for 40 freely drained acid surface soils

Soil series	Oxalate soluble			Dithionite soluble		
	Fe	Al	Fe+Al	Fe	Al	Fe+Al
Insch	0.53 NS	0.12 NS	0.38 NS	0.49 NS	0.18 NS	0.49 NS
Foudland	0.56 NS	0.79**	0.72*	0.20 NS	0.47 NS	0.30 NS
Countesswells	0.34 NS	0.55 NS	0.68*	0.18 NS	0.39 NS	0.25 NS
Stonehaven	0.71*	0.86**	0.86**	0.23 NS	0.83**	0.55 NS
Overall	0.55**	0.65**	0.70***	0.28 NS	0.52**	0.41**

NS = not significant. * $P<0.05$. ** $P<0.01$. *** $P<0.001$.

correlations with both aluminium categories were clearly better for the saturation value than the sorption index, except in the Stonehaven soils where the aluminium relationships were all very good. Because the saturation procedure favours weaker retention agents and sites, the results suggest that, as with phosphate, retention of sulphate by iron compounds is stronger than by aluminium compounds. Sulphate sorption from the relatively dilute solutions used to measure the sorption index would therefore be expected, as was the case, to be associated mainly with the more active iron sites, whereas weaker binding at less active sites, especially aluminium, should make a greater contribution to the saturation value. As well as activating weaker sites, however, the saturation procedure may involve different sorption mechanisms.

4. Conclusions

Scottish soils probably receive about 10–15 kg S/ha per annum in rainfall and unknown amounts by gaseous absorption from the atmosphere. In the past, large amounts have also been added in superphosphate and ammonium sulphate, but additions from these sources have declined because of the use of purer NPK fertilisers. These additions can be expected to be offset by extensive removal in crops and by drainage losses, since inorganic sulphate is not strongly retained by the soil and does not therefore accumulate to any great extent. Sulphate retention also decreases as soil pH is raised by liming. Even so, the results show that these soils contain sufficient reserves of adsorbed sulphate to meet the requirements of crops and explain why sulphur deficiency has not been reported in the area. Since the soil organic matter contents are stable, there is presumably equilibrium between the organic and inorganic sulphur fractions, although details of mineralisation of organic forms are not known.

The longer term supply of sulphur from these soils is governed by the reserve of adsorbed sulphate, and this depends on the differing abilities of the soils to retain added sulphate. The main retention agents are active iron and aluminium compounds, and except in the Old Red Sandstone soils, which require further study, the dominant factor is iron. This largely explains the effects of parent material. It is also compatible with the finding from other results, not included, that the drainage status, which affects mainly the aluminium content, had little effect on sulphate sorption. The emphasis in the inorganic sulphur relationships of these soils is therefore on the soil Association rather than the soil series. In this respect the situation differs from that for phosphate sorption, which is markedly dependent on both, because it hinges primarily on aluminium.[14]

As with phosphate,[15,16] rapid comparative sorption measurements are valuable for general characterisation of different soils.

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Effect of Irrigation on the Lipid Composition of Taramira Seed

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The effect of irrigation on the lipid composition in maturing taramira (*Eruca sativa*, Mill) seed was studied. Decreasing the number of irrigations had an adverse effect on oil setting. Restricted irrigations affected adversely the biosynthesis of erucic acid in the oil at initial stages, though at later stages of seed development, irrigation had no such effect.

1. Introduction

A study of the changes in fatty acid composition during ripening may enhance our understanding of the complexity of enzyme systems involved in the production of fatty acids and may be useful in oilseed breeding programmes. We have already studied these changes in a number of oilseeds.[1–3] Moisture stress is known to affect the lipid quality of maturing wheat grains.[1] The present study is extended to relate the effect of irrigation on the lipid changes in maturing taramira (*Eruca sativa*, Mill) seed.

2. Materials and methods

The experiment was performed at the farms of Punjab Agricultural University, Ludhiana. Seeds were sown in eight plots (5 × 2m) on 26 October 1970. Two of these were irrigated three times (on 26 October, 29 November and 28 December); two plots were irrigated twice (on 29 November and 28 December); two were irrigated only once (on 26 October) and the remaining two plots were not irrigated. An open irrigation system was adopted and each time the irrigation was 7.5 cm deep. The total rainfall during the entire ripening period of the crop was 4.8 cm.

The first sampling was done 10 days after maximum flowering (DAF) and the subsequent samplings were at 20, 30 and 40 DAF. Extraction of total lipids from the seeds, separation of lipid components by thin-layer chromatography and fatty acid separation by gas–liquid chromatography were done by the methods used by Sukhija and Bhatia.[2]

3. Results and discussion

Irrigation had a pronounced effect on oil setting. With zero irrigation the 40-day-old seed, whose lipid composition is assumed to be identical to that of matured seed,[2] contained only 18.5% oil as compared to 25.5, 29.3 and 33.4% with one, two and three irrigations applied; the periods between 10 and 20 DAF; and 30 and 40 DAF were the peak periods for oil synthesis (Table 1).

Partial glycerides, free fatty acids and free sterols showed poor resolution on t.l.c. and were estimated collectively. Similarly, hydrocarbons, sterol esters and pigments were also estimated together. The data are presented in Table 2. Under different irrigations the concentration of the triglyceride component rose from 22.2 to 70.2% and concomitantly, the other non-glyceride components declined during the period of 10–20 DAF (Table 2). As expected, during the same period

Table 1. Per cent lipid in ripening taramira (*Eruca sativa*, Mill) seed under different number of irrigations (on dry weight basis)

No. of irrigations applied	Days after flowering (DAF)			
	10	20	30	40
0	0.6	13.1	14.0	18.5
1	0.9	14.1	17.6	28.5
2	3.1	17.2	18.2	29.3
3	3.5	18.2	21.6	33.4

Table 2. Relative per cent of non-polar lipid classes of seed oil of ripening taramira (*Eruca sativa*, Mill) seed under different number of irrigations (densitometric determinations)

No. of irrigations applied	Days after flowering (DAF)	Lipid components		
		Partial glycerides, free fatty acids, free sterols	Triglycerides	Sterol esters, hydrocarbons, pigments
0	10	69.1	14.1	16.9
	20	20.4	63.1	16.5
	30	22.3	63.4	10.3
	40	20.6	72.4	7.0
1	10	67.5	17.5	15.0
	20	18.2	67.6	14.2
	30	17.9	71.9	10.2
	40	16.7	77.3	6.0
2	10	65.4	20.3	14.3
	20	17.8	68.6	13.6
	30	17.0	72.0	11.1
	40	15.1	78.0	6.1
3	10	64.0	22.2	13.8
	20	16.7	70.2	13.1
	30	15.0	74.0	11.0
	40	6.1	87.1	6.8

the oil content increased from 3.5 to 18.2% (Table 1). This increase in oil content is definitely due to *de novo* synthesis of the triglyceride component along with the oil, since the conversion of the non-triglycerides present at 10 DAF could not possibly explain such a phenomenal increase in the oil content during the next 10 days. Similar observations have been previously recorded by Sekhon and Bhatia[4] in *Sesamum indicum* and by Bhatia and Sukhija[3] in linseed (*Linum usitatissimum*).

With three irrigations, erucic acid content of the total oil was 8.25% at 10 DAF and, subsequently, rose to 13.9% at 20 DAF (Table 3). The average daily increase in erucic acid content of the oil was 0.6% between 10 and 20 DAF whereas a daily increase of 1.1% of this acid between 20 and 40 DAF was recorded which indicated this period as the peak period of erucic acid synthesis. At 10 DAF, the seeds under zero irrigation had a negligible amount of oil with only a trace of erucic acid which indicates a rather slow synthesis of oil with a lower amount of erucic acid during the initial stages of seed development. However, at later stages of ripening, the rate of synthesis of erucic acid in the seed was the same irrespective of the number of irrigations applied. This observation indicates that the restricted irrigations had little effect on overall synthesis of erucic acid in the developing seed.

Table 3. Percentage (wt basis) fatty acid composition of ripening taramira (*Eruca sativa*, Mill) seed under different number of irrigations

	No. of irrigations 10 DAF				No. of irrigations 20 DAF				No. of irrigations 30 DAF				No. of irrigations 40 DAF			
Fatty acids	3	2	1	0	3	2	1	0	3	2	1	0	3	2	1	0
Lower fatty acids	1.3	2.0	2.5	3.1	0.4	1.8	4.4	5.3	1.2	1.6	2.2	4.0	0.4	—	0.4	—
16:0	38.0	20.0	19.7	22.4	8.7	8.1	8.0	7.9	6.3	6.0	6.0	8.6	5.7	5.5	4.0	3.4
16:1	—	—	—	—	1.8	3.2	4.7	5.5	1.3	2.3	3.4	4.2	1.1	1.0	1.6	1.5
16:2	1.5	—	—	—	0.5	—	—	—	0.2	0.8	0.8	Tr.	0.2	0.2	0.2	0.2
16:3	—	—	—	—	—	—	—	—	—	—	0.2	—	—	—	0.1	0.1
18:0	4.0	3.6	2.9	3.1	2.1	1.8	1.7	1.5	1.4	1.4	1.2	1.2	1.3	1.2	0.9	0.7
18:1	10.7	7.4	9.4	8.8	22.5	21.3	20.8	25.0	21.0	20.0	18.5	16.3	22.1	18.3	17.4	20.2
18:2	23.8	26.0	24.0	24.8	26.7	24.8	24.8	24.2	12.1	13.2	12.6	11.0	14.5	14.0	13.9	13.0
18:3	12.5	34.7	37.0	37.9	Tr.	3.6	Tr.	Tr.	26.1	25.1	25.1	26.1	20.5	20.3	20.3	19.4
20:1	—	—	—	—	—	—	—	—	2.3	2.4	3.9	4.5	—	7.5	10.3	12.1
22:1	8.3	6.3	5.6	Tr.	13.9	12.0	1.7	8.2	28.1	27.3	26.1	24.2	34.1	32.0	31.1	29.3

Tr. = Traces.

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Changes in Some Nitrogenous Constituents of Potato Tubers During Aerobic Autolysis

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Potato tubers of four cultivars were ground and allowed to undergo aerobic autolysis. Tubers were fractionated before and after autolysis, and distributions of dry matter, N and of free and bound amino acids measured. "Chemically available lysine" and chlorogenic acid were also determined. Extent of "enzymic browning" correlated with loss of free tyrosine but not with "chemically available lysine" content. This last may have been influenced by enzymic oxidation of chlorogenic acid. Losses of S-containing amino acids were observed. Free 4-aminobutyric acid increased and free glutamic acid decreased, suggesting α-decarboxylation of the latter.

1. Introduction

Interactions of polyphenols and their oxidation products with amino acids and amino acid residues in the protein of subaereal parts of plants are well known.[1–4] Potatoes contain the polyphenol chlorogenic acid, polyphenol oxidases (also capable of oxidising tyrosine to dihydroxyphenylalanine[5,6]), amino acids and proteins. The enzymic browning of cut potatoes is a well-known reaction involving some of these constituents and has been extensively investigated by Mapson and colleagues.[7–10] The extent of browning was shown to be related to the free-tyrosine content of the potato.[9] The present investigation was undertaken to determine the effect of aerobic autolysis on the other free and bound amino acids. Four varieties of potatoes were chosen, representing a wide range of browning activity, and the previously developed method of fractionating plant material[11] was used before and after autolysis. Each fraction was analysed for dry matter, nitrogen and amino acids (after hydrolysis, for protein-containing fractions); chlorogenic acid was determined in the aqueous fractions and "chemically available lysine" in the protein fractions.

2. Experimental

2.1. Materials

The four varieties of potato (Désirée, Golden Wonder, Orion and Redskin) were grown in the same field near Wymondham, Norfolk. They were mechanically dug and hand-lifted at maturity (in late September 1973), carefully sorted and put into a store at 5°C within a week. Extractions and autolyses took place 19, 38, 30 and 38 days (respectively) after harvesting.

2.2. Sample preparation

Two 100 g samples of each variety were taken from opposite halves of approximately 300 g of ongitudinally halved tubers. Each sample was slurried in liquid nitrogen.[11]

2.3. Aerobic autolysis

One of the slurries from each of the varieties was allowed to autolyse aerobically by placing it in a crystallising dish (12 × 6 cm) at room temperature for 24 h. Microbial growth was prevented by

[a] Present address: Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen AB9 8DG, Scotland.

the addition of a few drops of chloroform. Any water lost by evaporation was replaced prior to extraction and the colour of the slurry after 24 h was noted.

2.4. Extraction

Extraction of the slurries was made according to the method of Laird *et al.*,[11] except that the autolysed slurries were extracted and stored under air and not oxygen-free nitrogen. All the fractions were stored at +1°C until required for analysis.

2.5. Hydrolysis

Suitable quantities of each fraction were hydrolysed under reflux in not less than 0.2 ml/mg dry matter of 6 M-HCl for 18–24 h.

2.6. Amino acid analysis

2.6.1. Total amino acids

Hydrolysates were analysed on a Beckman 120C Amino Acid Analyzer.[12]

2.6.2. Free amino acids

Portions of the aqueous extracts were analysed for free amino acids on a Technicon NC1 Amino Acid Analyzer according to the method of Nunn and Vega.[13]

2.6.3. Tryptophan

Tryptophan was determined on evaporated portions of the phenol–acetic acid–water (PAW) extracts subjected to alkaline hydrolysis according to Hugli and Moore.[14] The tryptophan was separated on a 18 × 0.9 cm column of Zeo-Karb 225 ion-exchange resin (particle size 10–20 μm) eluted with pH 5.4 sodium citrate buffer.

2.6.4. Chemically available lysine

As described by Allison *et al.*[15]

2.7. Nitrogen content

A semi-micro Kjeldahl method[16] was used to determine the nitrogen content of aliquots of the hydrolysates.

2.8. Chlorogenic acid content

As described by Mapson *et al.*[9]

2.9. Dry-matter determinations

As described by Laird *et al.*[11]

3. Results and discussion

3.1. General

Laird *et al.*[11] showed that the bulk of the dry matter and nitrogen of potato was distributed between three fractions: aqueous phase; phenol–acetic acid–water (PAW) extract; and residue. In view of this result, only these three fractions have been analysed in this work.

3.2. Dry-matter and nitrogen distributions

The dry-matter results are presented in Table 1 and the nitrogen results in Figure 1 (see also Table 2). The dry-matter results show variations between varieties but the overall trend on autolysis was a loss of dry matter from the PAW fraction and slight gains in the aqueous and residue fractions.

Table 1. Dry-matter distribution in fractions from potato tubers (g/100 g fresh wt)

	Orion		Désirée		Redskin		Golden Wonder	
	Fresh	*Autolysed*	*Fresh*	*Autolysed*	*Fresh*	*Autolysed*	*Fresh*	*Autolysed*
Aqueous phase	2.78	2.96	3.17	2.90	2.60	2.89	2.51	2.82
PAW extract	2.08	1.82	1.62	1.40	1.90	1.93	3.02	2.97
Residue	18.16	20.16	18.46	18.41	18.16	18.16	22.86	23.79
Total	23.02	24.94	23.25	22.71	22.66	22.98	28.39	29.58

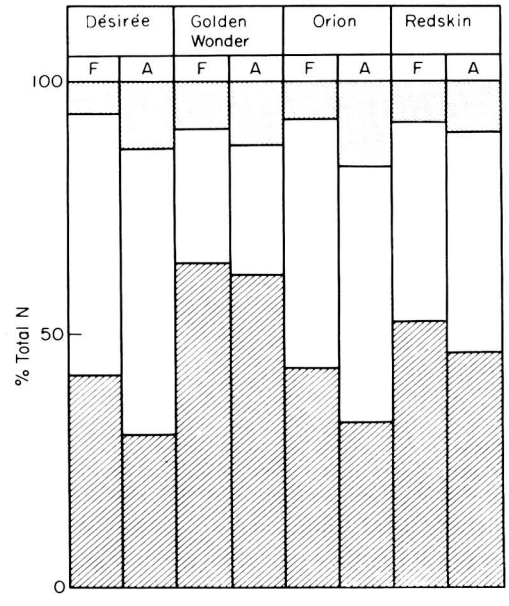

Figure 1. Distribution of nitrogen between main potato fractions before and after autolysis. Aqueous phases, unhatched; PAW extracts, hatched; residues, dotted; F, fresh; A, after autolysis.

The nitrogen-distribution changes on autolysis were more marked. All varieties showed a loss of nitrogen from the PAW fraction and a gain by the residue fraction. Apart from Golden Wonder, the other varieties showed a gain of nitrogen in the aqueous fraction on autolysis. These results show that protein is being hydrolysed but it is not clear if this material becomes bound to the insoluble fraction or if there is a more complex interchange with material which was extracted in the aqueous phase before autolysis.

3.3. Amino acid analysis

Selected results from the amino acid analyses are shown in Table 2.[a]

3.3.1. Free amino acids

The most obvious results of autolysis in all varieties were a loss of glutamic acid and tyrosine and a gain of 4-aminobutyric acid. The loss of glutamic acid and, in three varieties, glutamine was

[a] The complete set of amino acid-analysis results has been deposited as a Supplementary Publication (SUP 90019: 5 pp.) at the National Lending Library, Boston Spa, Yorkshire LS23 7BQ, England, from whom copies can be obtained.

Table 2. Selected analytical results for potato tubers

	Orion		Désirée		Redskin		Golden Wonder	
	Fresh	Autolysed	Fresh	Autolysed	Fresh	Autolysed	Fresh	Autolysed
Colour of slurry before fractionation	Pale yellow	Black	Pale red	Very dark brown	Pale red	Dark brown	Pale yellow	Light brown
% Availability of lysine in PAW extract	87	90	86	89	83	85	89	83
Total N (% dry matter of tuber)	1.9	1.9	1.7	1.8	1.7	1.7	1.4	1.4
mg/100 g tuber fresh wt								
Total N	437	469	399	400	382	398	387	405
Chlorogenic acid in aqueous phase	25.9	1.3	14.8	3.0	25.6	1.0	30.5	1.5
N of free glutamine in aqueous phase	31.69	29.34	36.57	31.48	27.12	24.49	12.00	13.99
N of free glutamic acid in aqueous phase	4.92	0.0	7.62	4.16	6.25	0.0	7.83	0.0
N of free 4-aminobutyric acid in aqueous phase	3.78	9.25	5.36	10.56	2.28	8.91	2.29	9.64
N of tyrosine in aqueous phase	3.41	0.0	3.15	0.72	1.21	0.25	0.40	0.0
N of cystine in PAW extract	2.54	1.50	1.17	1.08	1.63	1.70	3.15	2.48
N of cystine in residue	0.0	0.17	0.0	0.09	0.06	0.08	0.02	0.02
N of methionine in aqueous phase	1.37	1.07	1.53	1.38	0.79	0.67	0.10	0.06
N of methionine in PAW extract	1.89	1.04	1.61	0.79	1.95	1.50	2.50	2.91
N of methionine in residue	0.14	0.60	0.09	0.16	0.12	0.20	0.17	0.12
Total amino acid residues in residue	115.9	346.3	106.4	269.1	136.1	187.5	159.6	180.6

balanced by the gain in 4-aminobutyric acid (taking the difference in molecular weights into account) and indicates the presence of a glutamic acid decarboxylase, as previously reported by Schales and Schales.[17] The loss of tyrosine was in accordance with previous work.[9,10]

3.3.2. Hydrolysates

The general trend in all varieties was a loss of amino acid on autolysis from the PAW fractions and a gain in the residue fractions, in agreement with the nitrogen-distribution results. The overall recovery of amino acids was 90–110% after autolysis except for tyrosine, cystine and methionine. The tyrosine loss was due to the loss of free tyrosine. The loss of the sulphur amino acids may be due to their reactions with *o*-semiquinone radicals or *o*-quinones produced by oxidation of *o*-diphenols.[2,4,18]

3.4. Chlorogenic acid, colour and available lysine

The results for chlorogenic acid, colour and available lysine are presented in Table 2. The loss of chlorogenic acid does not suggest any correlation with colour development but a correlation between loss of free tyrosine and colour development is apparent.

There was no overall significant change in the available-lysine content of the PAW fractions after autolysis. It was thought that lysine in the residue might have a lower availability and, in view of the increase in the residue fractions after autolysis, this might have an important nutritional consequence. A test was made on the residues from Orion (the variety showing the largest increase of lysine in the residues after autolysis). However, there was no change in the availability of the lysine and, at 86%, there is no evidence that residue lysine is less available than PAW lysine.

4. Conclusions

The three main reactions demonstrated during the aerobic autolysis of potato slurries are: α-decarboxylation of glutamic acid; hydrolysis of protein; and browning with concomitant destruction of chlorogenic acid and free tyrosine. The protein-hydrolysis reaction appears to be independent of variety but the browning reaction, as judged by colour and absolute loss of free tyrosine, is clearly variety-dependent. One of the results of the browning reaction is that amino acids, either in the free state or as peptides, become increasingly bound in the unextractable residue fraction, as is shown in Figures 1 and 2.

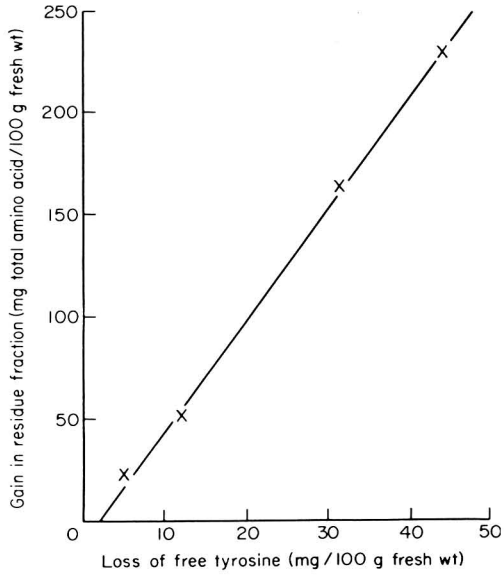

Figure 2. Correlation of free-tyrosine loss with gain in total of amino acid residues obtained by hydrolysis of the residue after extraction. $r = 0.99$.

The nutritional consequences of the browning reaction, as indicated by lysine availability, may well not be serious. If tyrosine rather than chlorogenic acid is the main source of quinonoids, as in the varieties other than Golden Wonder, it may be that these tend to polymerise through dopachrome to melanins directly, without coupling to the proteins. Polyphenols other than dopa may therefore be the quinonoid precursors which are the more damaging to the lysine residues of plant proteins.[4] Substantial losses of the sulphur amino acids occurred with all four varieties. Such reactions would be particularly serious if they occurred during processing or storage of legume-seed proteins.

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Influence of Ethylene on the Ripening of Stored Apples

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When preclimacteric apples were stored in air at 3.3°C rapid ethylene production usually began after 5 to 10 days. Treatment with ethylene caused earlier softening of apples, especially at the highest concentrations tested, 1–2 ml/litre. No effects on soluble polyuronide or peel chlorophyll levels were observed. It was not possible to induce earlier softening or compositional changes by ethylene treatment of apples stored in 2% O_2:98% N_2 at 3.3°C. Ethylene removal, by permanganate, delayed softening and increase in soluble polyuronide in preclimacteric, but not in post-climacteric, apples, stored in 2% O_2:98% N_2 at 3.3°C. Peel chlorophyll degradation was slow and unaffected by ethylene removal or fruit maturity. Loss of galactose residues from the cell wall was similar at high and low ethylene concentrations.

Removal of ethylene from apples stored in 5% CO_2:16% O_2:79% N_2 at 3.3°C did not affect their ripening, but delayed softening of apples stored in 9% CO_2:12% O_2:79% N_2 at 3.3°C.

1. Introduction

When it was discovered that some fruits produce ethylene and that ethylene can accelerate ripening, it was natural to suppose that presence of ethylene in storage chambers would lead to earlier deterioration of stored fruit. Early experiments[1] in which ripe and unripe apples were stored together confirmed this supposition. However, later workers found little practical benefit in attempts to remove ethylene from apple stores[2,3] and, correspondingly, negligible loss of quality if ethylene was allowed to accumulate.[4] In addition, ethylene treatment does not affect the respiration rate of apples at 3°C; and it came to be thought that the presence of ethylene had no effect on the ripening of apples during storage.[5]

Burg and Burg[6] have suggested that high CO_2 and low O_2 concentrations employed in fruit storage delay ripening because they interfere with the binding of ethylene to its acceptor protein. These authors later acknowledged[7] that in commercial apple stores enough ethylene would be present to overcome competitive binding of CO_2 to its acceptor site. Mapson[8] suggested that low oxygen concentrations might delay fruit ripening by maintaining "conditions in the tissue such that initiation of an accelerated rate of ethylene production is prevented", and by limiting the rate of ethylene synthesis, as well as raising the threshold of sensitivity to ethylene. Storage at subatmospheric pressures[9] allows faster diffusion of ethylene from fruit tissue, and, apparently because of this, extends the storage life of apples.[10]

Several workers[11–14] have shown recently that removal of ethylene from controlled atmosphere stores results in apples being firmer. In one instance[14] the increased firmness was associated with lower soluble polyuronide levels, and an attempt was made to relate textural measurements to cell wall structure. Attempts to remove ethylene should be more successful under controlled atmosphere conditions than in air storage, because rates of synthesis would be lower at low O_2 concentrations and ethylene action would be inhibited at high CO_2 and low O_2 concentrations.

In the present paper the influence of addition and removal of ethylene in various storage atmospheres is described in terms of changes in cell wall composition, firmness and peel chlorophyll content of various varieties of apples. It has to be remembered that in ethylene addition experiments

control apples can be affected by endogenous ethylene, and that ethylene removal is never completely effective. The experiments are not conducted with apples in the presence or absence of ethylene, but in the presence or absence of added ethylene or an ethylene remover.

2. Experimental

2.1. Source of apples

For ethylene addition experiments Cox's Orange Pippin apples were harvested from 10-year-old trees on M 7 rootstocks, 15 September 1971, James Grieve apples from 7-year-old trees on M 26 rootstocks, 15 August 1972 and Tydeman's Late Orange from 16-year-old trees on M 7 rootstocks, 12 October 1972. In each case the apples were preclimacteric; they were sorted into 15 apple samples and transferred to the appropriate storage conditions within 24 h of harvest.

For ethylene removal experiments Cox's Orange Pippin apples were harvested on four dates at 10 or 11 day intervals from 7 September 1973, from 12-year-old trees on M 7 rootstocks, and Bramley's Seedling apples were harvested on 27 September 1973, from approximately 50-year-old trees on seedling rootstocks. Apples were sorted into samples of 15 and transferred to appropriate storage containers at 3.3°C within 8 h of harvest.

2.2. Ethylene treatment

Fruit samples were stored in groups of 3 or 4 (45 or 60 apples), so that there were approximately 6 kg in each 25 litre storage container. All of these containers were stored at 3.3°C, and each was supplied with a flow of gas of the appropriate composition at 5 litres/h. Air was drawn from outside the laboratory and passed over calcium permanganate impregnated vermiculite to remove traces of ethylene. When 2% O_2:98% N_2 (subsequently referred to as 2% O_2) was required nitrogen was mixed with this air. Ethylene was admitted to the gas stream using peristaltic pumps, whose motor speeds and pump sizes were adjusted to give the desired concentration.

2.3. Ethylene removal

All fruit was stored in a room at 3.3°C, isolated from large quantities of fruit, and containing a u.v. ethylene scrubber,[15] so that background levels were less than 0.05 μl/litre. A group of apple samples representing one treatment for a given harvest date was allocated a 50 litre storage container. For storage in 2% O_2 a tray of hydrated lime was included in each container, which was sealed with a sheet of silicone rubber impregnated fabric, permeable to oxygen[16] and held in place by a 5 cm wide ring cut from a motor car inner tube. Oxygen concentration in the vessel was measured with a paramagnetic analyser (Type OA 137, Servomex Controls Ltd, Crowborough, England) and was controlled by varying the area of the silicone sheet exposed using a mask of "clingfilm", adhering to the sheet through a thin film of glycerol. CO_2 concentrations in the containers were occasionally checked by gas chromatography and never exceeded 0.2%. The oxygen content of the atmosphere in the containers was adjusted to 2%, on first sealing and after subsequent openings to remove samples, by dilution with N_2.

High CO_2 conditions were achieved by controlled leaks of air into similar containers with metal lids. It was assumed that CO_2 level was the difference between the measured O_2 content of these containers and the O_2 content of air.

Ethylene was removed by including in the storage vessel about 150 g of granular vermiculite, impregnated with a saturated $KMnO_4$ solution, and dried.

2.4. Analytical methods

Ethylene was separated and estimated by gas chromatography on a 1.0 M column of Porapak T (Waters Associates Inc.) at 40°C with a 50:50 N_2: H_2 mixture as carrier, and flame ionisation detector.

Fruit firmness was measured with a penetrometer (Effe.gi, Ravenna, Italy) with an 8 mm plunger.

Chlorophyll and carotenoid were estimated spectrophotometrically in extracts of fruit peel.[17]

Soluble polyuronide content of apples in ethylene addition experiments was estimated colorimetrically in ethanol precipitates of aqueous extracts.[17] In ethylene removal experiments, soluble polyuronide was extracted by disintegrating wedges cut from opposite sides of each apple in an equal weight of 2% trichloroacetic acid solution and filtering under suction on Whatman No 541 paper. A portion of the filtrate (2.0 ml) was applied to a column (40 × 2.0 cm) of Sephadex G-25 equilibrated with 0.1 M-NaCl, 0.1% chlorbutol solution as eluent. After the void volume had emerged, 20 ml was collected in a 25 ml volumetric flask containing 5 ml 0.1 M-NaOH. The polyuronide content of this solution was estimated by an automated colorimetric procedure.[18] Cell wall material was precipitated from the suspension of disintegrated apple in trichloroacetic acid solution by mixing 20 ml of this with 80 ml acetone. After filtration on sintered glass the residue was washed with acetone and allowed to air-dry. Portions (*ca* 5 mg) of this air-dried material were allowed to dissolve in 0.1 ml 72% (w/w) H_2SO_4 for 48 h and then diluted with 3 ml H_2O. Hydrolysis was completed by autoclaving at 121 °C and the products were neutralised with $BaCO_3$. After centrifugation the supernatant was freeze-dried over P_2O_5; neutral sugars were then separated and estimated by g.l.c. of their trimethyl silyl ethers.[18]

3. Results

3.1. Effects of added ethylene on apples ripening in air

When Cox's Orange Pippin apples treated with 800 μl/litre ethylene in air were compared with control fruit, stored in air, there was little difference between the trends in firmness, soluble polyuronide (Figure 1) and peel chlorophyll (Figure 2). Ethylene production by control fruit reached 1.0 μl/kg/h after 8 days storage.

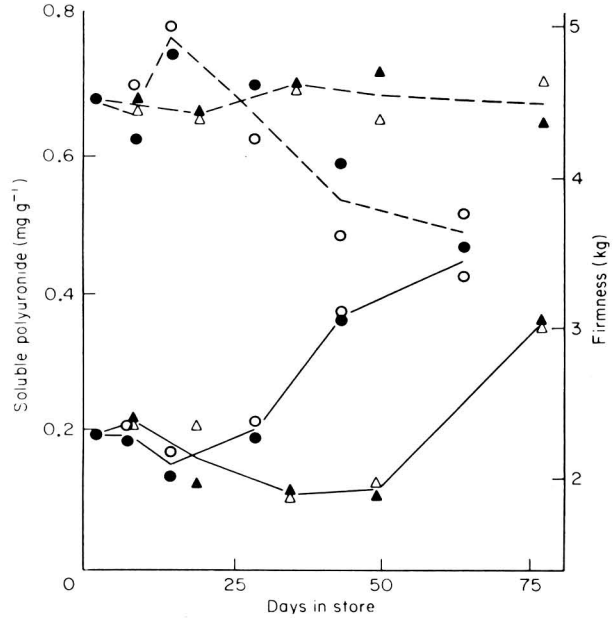

Figure 1. Fruit firmness and concentrations of soluble polyuronide in Cox's Orange Pippin apples during storage in air and 2% O_2:98% N_2 at 3.3 °C with and without added ethylene. Solid lines (soluble polyuronide) and broken lines (firmness) join mean values for fruit stored in air with (○) and without (●) ethylene, and for fruit stored in 2% O_2 with (△) and without (▲) ethylene.

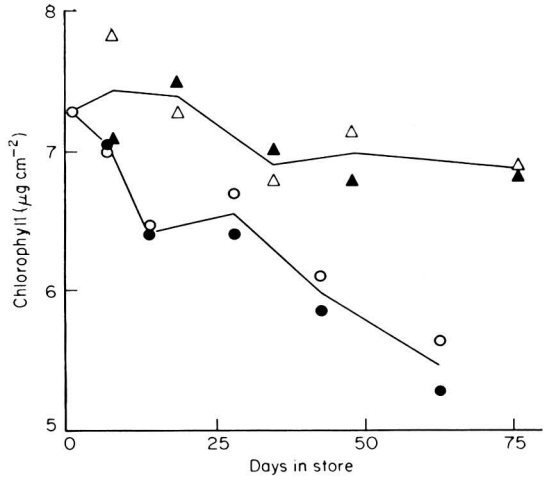

Figure 2. Peel chlorophyll concentrations in Cox's Orange Pippin apples during storage in air and 2% O_2:98% N_2 at 3.3°C with and without added ethylene. Lines join mean values for fruit stored in air with (○) and without (●) ethylene, and fruit stored in 2% O_2 with (△) and without (▲) ethylene.

Effects of ethylene on chlorophyll loss and softening were investigated further with two varieties of apples stored for up to 50 days in a range of ethylene concentrations at 3.3°C. In one experiment with the variety James Grieve ethylene production by control apples reached 1.0 μl/kg/h after 5 days storage and in the other experiment with Tydeman's Late Orange after 10 days storage. In both experiments fruit firmness remained constant for an initial period of 10 to 20 days, after which it apparently declined linearly. Results for 20 to 50 days were analysed to obtain values for mean firmness and regression of firmness against time for ethylene treatments and controls. Table 1 shows values for the experiment with Tydeman's Late Orange apples; similar values were obtained in the experiment with James Grieve. Ethylene treated fruit was usually softer than controls, but only at the highest concentration was the difference highly significant ($P < 0.01$) in both experiments. The regression coefficients indicate that rates of softening were similar in all treatments, so the difference in mean firmness was probably a result of different times of initiation of softening. Differences in firmness were not associated with different mean levels or rates of release of soluble polyuronide in the Tydeman's Late Orange experiment (Table 1).

Unlike firmness and soluble polyuronide, the trend in peel chlorophyll contents did not show an inflexion at any time during storage, so data obtained throughout the experiment were analysed. Mean chlorophyll contents were similar for control and ethylene treated fruit in both experiments. In the James Grieve experiment, however, rates of loss of chlorophyll apparently increased with ethylene concentration, though the effect was significant ($P < 0.05$) only at the highest concentration. Rates of chlorophyll loss in Tydeman's Late Orange apples were similar for all treatments (Table 1).

3.2. Effects of added ethylene on apples ripening in 2% O_2

As shown in Figures 1 and 2 storage in 2% O_2:98% N_2 had the expected effects of delaying ripening by comparison with apples stored in air. Ethylene production by fruit in 2% O_2 reached 1.0 μl/kg/h after 33 days compared with 8 days for air stored fruit. However ethylene treatment (800 μl/litre) did not reverse the effects of low oxygen on ripening processes (Figures 1 and 2).

3.3. Ethylene removal experiments with fruit in 2% O_2

When Cox's Orange Pippin apples were stored in a self generated atmosphere containing 2% O_2, ethylene accumulated rapidly, reaching final concentrations of several hundred μl/litre (Table 2).

Table 1. Softening and compositional changes in Tydeman's Late Orange apples stored in air at 3.3 °C with various concentrations of ethylene

	Added ethylene conc. (μl/litre)				
	0	0.8	5.4	406	1409
Mean firmness (kg)	5.10 (0.051)	5.10 (0.049)	5.01 (0.069)	5.01 (0.088)	4.83 (0.054)
Regression of firmness on time (kg/day)	−0.055 (0.0045)	−0.060 (0.0044)	−0.053 (0.0062)	−0.049 (0.0078)	−0.043 (0.0048)
Degrees of freedom	15	7	7	7	7
Mean soluble polyuronide content (μg/g)	461 (22)	426 (12)	418 (23)	398 (19)	450 (28)
Regression of soluble polyuronide content on time (μg/g/day)	13.7 (1.97)	12.7 (1.06)	13.0 (2.02)	11.7 (1.66)	14.0 (2.52)
Degrees of freedom	7	7	7	7	7
Mean chlorophyll content of peel (μg/cm²)	7.75 (0.097)	7.66 (0.072)	7.65 (0.115)	7.18 (0.135)	7.52 (0.113)
Regression of chlorophyll content on time (μg/cm²/day)	−0.046 (0.0066)	−0.049 (0.0049)	−0.046 (0.0079)	−0.037 (0.0093)	−0.058 (0.0077)
Degrees of freedom	15	7	7	7	7

Standard errors are shown in parentheses. The mean storage period was 35 days in analysis of firmness and soluble polyuronide data and 28 days in analysis of chlorophyl data.

Table 2. Ethylene accumulation in storage vessels containing Cox's Orange Pippin apples with a self generated atmosphere of 2% O_2:98% N_2

Picking date	Control		Permanganate scrubber	
	Days to 1.0 μl/litre	Mean conc. (μl/litre)	Days to 1.0 μl/litre	Mean conc. (μl/litre)
7 September	13	295	28	6.7
17 September	11	182	33	2.9
27 September	8	142	33	3.9
8 October	0	149	3	7.2

After sealing, the time taken to reach a given concentration decreased as picking was delayed. Inclusion of permanganate impregnated vermiculite in the vessels extended this time considerably, except for fruit from the last pick. The average concentrations of ethylene were much reduced by this treatment.

The increase in the concentration of soluble polyuronide was delayed by 2% O_2, and further delayed by the presence of the permanganate scrubber, except for fruit from the last pick (Figure 3). Continuous softening was observed in fruit from all treatments throughout the experiment; fruit stored in the presence of permanganate was usually slightly firmer than controls. To eliminate

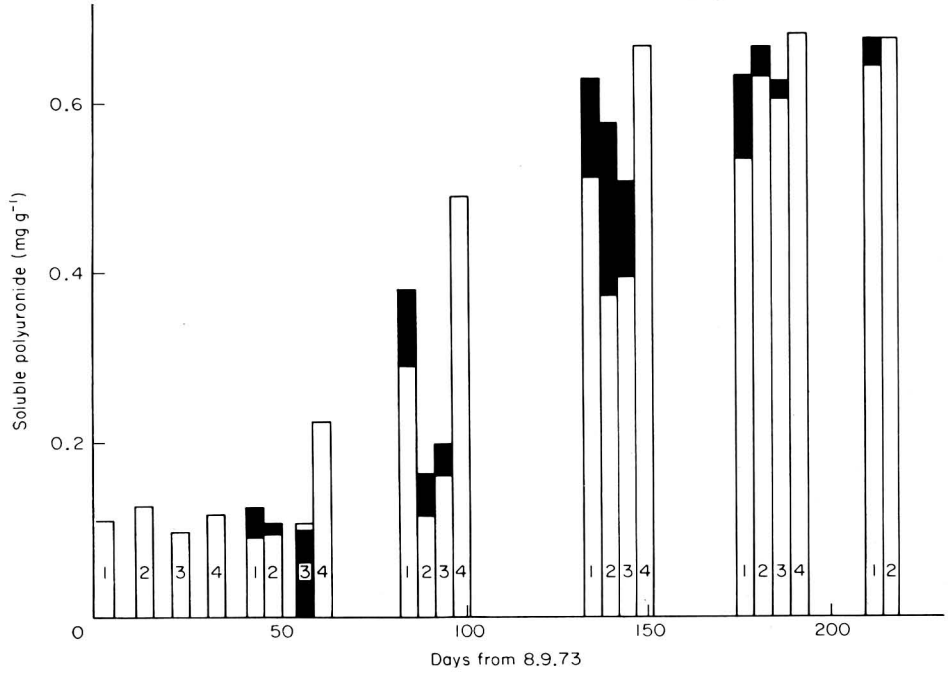

Figure 3. Soluble polyuronide concentrations in Cox's Orange Pippin apples picked on various dates during storage in 2% O_2:98% N_2 at 3.3°C with and without permanganate to remove ethylene. Numerals in columns refer to successive picks. Columns for permanganate treated (white) and control fruit (black) are superimposed, so that higher soluble polyuronide contents in controls show as black topped columns and higher contents in permanganate treated as white topped columns.

variability, results for galactan content of cell walls, which decrease during ripening, were expressed as a ratio to xylose, which remains constant.[19] Galactose content decreased between picking dates, and during subsequent storage in 2% O_2 with and without permanganate at similar rates (Figure 4). Peel chlorophyll content decreased rapidly between picking dates, but chlorophyll loss during storage in 2% O_2 was slow and unaffected by ethylene removal (Figure 5).

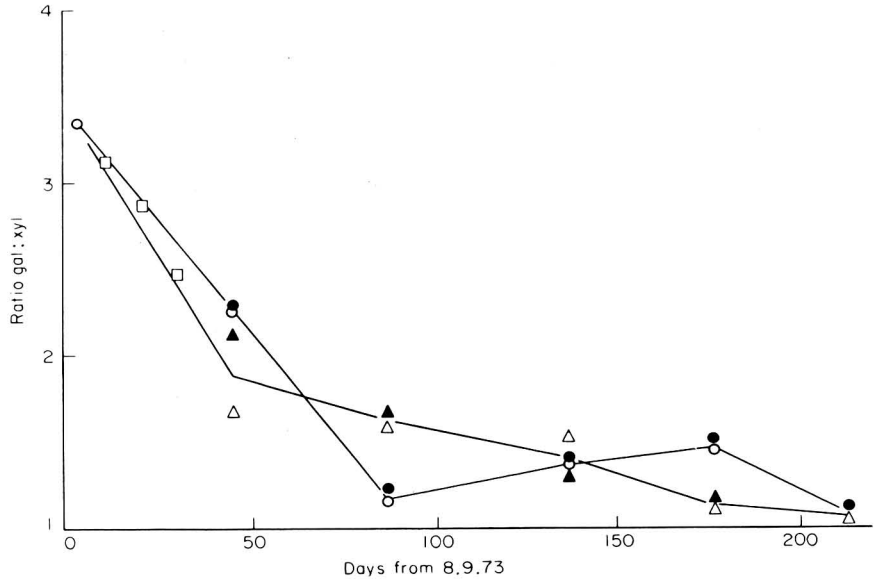

Figure 4. Ratios of galactose to xylose residues in cell walls of Cox's Orange Pippin apples, picked on various dates, during storage in 2% O_2:98% N_2 or 5% CO_2:16% O_2:79% N_2 at 3.3°C with and without permanganate to remove ethylene. Fruit picked 7.9.73; ○, control fruit in 2% O_2; ●, permanganate treated fruit in 2% O_2; △, control fruit in 5% CO_2; ▲, permanganate treated fruit in 5% CO_2. □, Fruit picked on later dates.

3.4. Ethylene removal experiments with high CO_2 concentrations

Cox's Orange Pippin apples picked 7 September were stored in 5% CO_2:16% O_2 with and without permanganate. Ethylene reached 1.0 μl/litre after 10 days in controls and 20 days in permanganate treated, and average concentrations of 135 μl/litre in controls and 4.7 μl/litre in permanganate treated were recorded. No differences between treatments, in firmness, soluble polyuronide content, chlorophyll content or galactose:xylose ratio in cell walls (Figure 4) were observed. In Bramley's Seedling apples stored in 9% CO_2:12% O_2 with and without permanganate, peel chlorophyll contents decreased similarly and soluble polyuronide remained at similarly low levels. However, fruit stored in the presence of permanganate was firmer late in storage.

4. Discussion

4.1. Effects of added ethylene

It is now generally accepted, that the climacteric rise in respiration of apples is initiated when an endogenous ripening inhibitor falls to a level at which the fruit becomes sensitive to the low concentration of ethylene present in preclimacteric tissue.[20] Coincident with the climacteric, ethylene production increases logarithmically and other ripening processes are initiated or accelerated. The significance for the fruit of rapid ethylene production is unclear, though it might lead to abscission of attached fruit;[21] when detached apples are stored in an enclosed space, rapid ethylene production

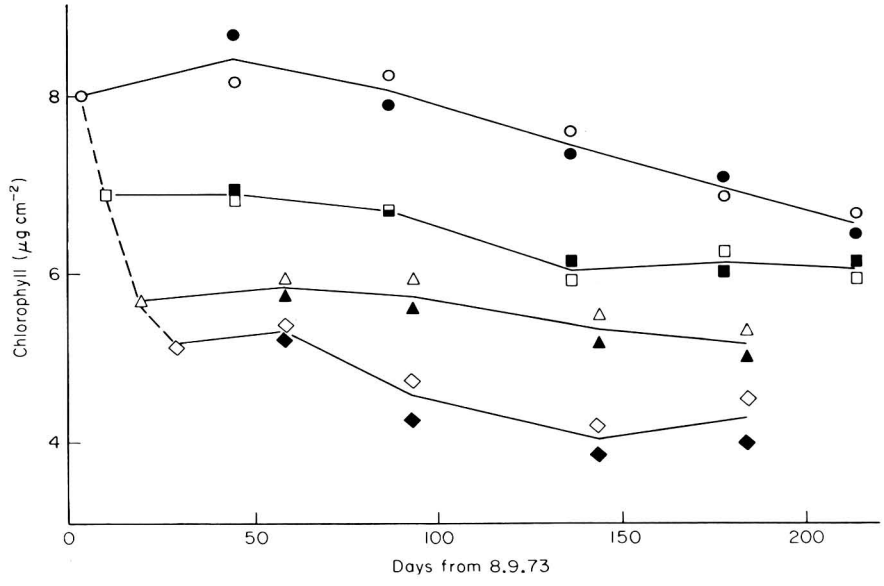

Figure 5. Peel chlorophyll contents in Cox's Orange Pippin apples, picked on various dates, during storage in 2% O_2:98% N_2 at 3.3°C with and without permanganate to remove ethylene. Fruit picked 7.9.73: ○, control; ●, permanganate treated; 17.9.73: □, control; ■, permanganate treated; 27.9.73: △, control; ▲, permanganate treated; 8.10.73: ◇, control; ◆, permanganate treated. Broken line shows chlorophyll loss "on tree".

by individual apples would be expected to cause the initiation of ripening in other fruits in the population.[22] When ethylene is externally applied to such a population, the greatest effect that could be observed would be the difference between immediate initiation of ripening and the time of endogenous initiation. In the above work a rate of ethylene production of 1.0 μl/kg/h was chosen as the point at which it could be said, unequivocally, that production was rising logarithmically. The time taken to reach this point varied from 5 to 10 days for apples stored in air, so 5 to 10 days effect would be the maximum that could be expected from externally applied ethylene.

At 3.3°C rates of softening, chlorophyll loss and other ripening processes are slow. Taking account of the standard errors of the estimations involved, the minimum effects ($P<0.05$) on the timing of these changes that could be detected would be 2 or 3 days for fruit firmness, 5 days for soluble polyuronide, or 8 to 10 days for peel chlorophyll. Except for firmness these times are of the same order as the maximum expected effect.

When apples at 12°C are treated with ethylene, up to 8 days effect on the time of initiation of the climacteric can be observed.[23] There is little information on the effects of ethylene on other ripening processes in apples at similar temperatures, though the general assumption is that they are similarly affected. Below about 7°C the respiration of apples does not exhibit the climacteric pattern; since there is no respiratory response to endogenous ethylene, a response to applied ethylene is not to be expected. Early work[24] suggested that treatment with emanations from ripe fruit would cause fruit to soften more at 0°C or above, while later authors[4] failed to detect such effects at 0, 7, or 18°C. Confirmation of these findings would undermine the whole concept of the role of ethylene in apple ripening. However, this confusion probably arises from the difficulties of detecting effects of ethylene.

In the present work effects of ethylene on the softening of apples at 3.3°C were observed. It was not possible to detect effects on soluble polyuronide and effects on chlorophyll loss were inconsistent. Surprisingly the effects on firmness were most marked at the highest ethylene levels employed, that is above 1 ml/litre; it is generally found that maximal responses by plant tissues are observed at

10 μl/litre.[6, 25] Possibly, these effects on fruit firmness result from action of ethylene at some other cellular site than its hypothetical acceptor protein.

Failure of ethylene to overcome the effects of 2% O_2 on apple ripening implies that oxygen concentration affects something, other than ethylene synthesis or the binding of ethylene to its acceptor, which prevents the fruit from responding to ethylene, whether it is applied or endogenous. In time the inhibitory effect is overcome and the apples are able to respond to ethylene.

In practical terms, Gerhardt and Siegelman[4] were correct in maintaining that the effects on unripe apples of storage with ripe apples had been exaggerated. The effects of added ethylene reported above are not commercially significant.

4.2. Effects of ethylene removal

Effects on stored apples of the presence of calcium permanganate impregnated vermiculite in storage vessels probably result from ethylene removal, since this material does not remove other fruit products such as esters from the atmosphere.

It has been suggested that effects of ethylene removal from apple storage atmospheres are due to maintenance of minimal initial concentrations, rather than the reduction in average concentration throughout storage.[14] This is inconsistent with the observation that, early in storage in controlled atmosphere conditions, apples are insensitive to applied ethylene. If this is correct, when the fruit becomes sensitive it responds to the level of ethylene present at that time. The main effect, observed on preclimacteric Cox's Orange Pippin stored in 2% O_2, was to delay the increase in soluble polyuronide. This was not accompanied by such a substantial delay in softening, as was observed previously with Tydeman's Late Orange apples in 2% O_2.[14] Ethylene accumulated rapidly in storage vessels containing fruit from the last pick of Cox's Orange Pippin suggesting that it had reached the climacteric. It is not surprising that ethylene removal had little or no effect on the timing of the increase in soluble polyuronide in this fruit.

Softening need not reflect changes in any one component of the cell wall. It is commonly observed that soluble polyuronide content does not increase in Bramley's Seedling in 9% CO_2, though the apples soften.[26] Presumably another change in wall composition is responsible for this softening. Since it is shown above to be sensitive to ethylene removal, the compositional change is unlikely to be loss of galactose residues, which in Cox's Orange Pippin is not affected by this treatment.

The effectiveness of ethylene removal probably depends upon a collaborative effect of controlled atmosphere conditions. Cox's Orange Pippin cannot be stored in more than 5% CO_2 without damage and this concentration seems not to be sufficient to give the required collaborative effect. Bramley's Seedling can tolerate higher CO_2 levels and ethylene removal is effective with 9% CO_2 for this variety.

The effects of ethylene removal on fruit ripening which have been observed are not commercially significant. It is possible, however, that development of techniques for more effective removal of ethylene, or prevention of ethylene action will lead to useful apple storage treatments.

Acknowledgements

Thanks are due to Mrs J. T. Cockburn and J. B. Watt for technical assistance.

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Agriculture Group Symposium
Components of Yield

The following are summaries of papers presented at a symposium held on 18 November 1975. The reports so published are entirely the responsibility of the authors and in no way reflect the views of the Editorial Board of the Journal of the Science of Food and Agriculture.

Structural Components of Yield in Forage Grasses

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The yield of the grass crop is the amount of harvestable shoot dry matter which can be readily digested by the ruminant animal and is therefore affected by the growth rate of the crop and its chemical and structural composition. Genetic differences in leaf, plant and crop structure influence both these aspects of yield.

Leaf mesophyll is the most nutritious fraction for the animal. The best quality grass leaf, although not necessarily the most productive, has an inherently high proportion of mesophyll. Vascular bundles and epidermis are relatively indigestible. Rates of digestion and digestibility of different anatomical fractions can be demonstrated using a cellulolytic enzyme technique on leaf sections.[1] A low proportion of cellulose, which is only slowly digested, is associated with thin primary cell walls, rapid feed intake by the animal[2] and an ability to withstand drought, but, because of leaf laxness, might lead to poorer light utilisation at high leaf area indices (LAI).

Leaves with many small mesophyll cells usually have rapid rates of photosynthesis of unit leaf tissue,[3] but contain high proportions of cell wall and can contribute to low critical LAI in the sward,[4] since small cells are generally contained in small leaves.

Epidermal structure can affect yield through water use. Improved yield in drought has been achieved in *Lolium* by selecting for few stomata and for reduced epidermal ridging.[5,6] In *Dactylis*, larger epidermal papilla cells are associated with reduced transpiration and drought avoidance.[7]

In the crop, long-leaved, erect, genotypes can have greater maximum yields than short-leaved types, particularly in the first year of growth.[8] This is often associated with higher critical LAI,[4] but there are also differences in assimilate allocation. In *Lolium*, short-leaved genotypes have more tillers, less root and much less leaf water-soluble carbohydrate than long-leaved genotypes, and often lack winter hardiness.

Plant and sward structure continually changes as the young seedlings develop into a mature sward of high LAI, which is thereafter sequentially defoliated. Development and maintenance of mature swards can entail heavy respiratory losses.[9] These losses may be reduced by using genotypes with inherently slow rates of dark respiration as the basis of varieties.[10] Selected *Lolium* families exhibiting relatively slow respiration grow much faster in sequentially cut micro-swards than families with fast respiration. This difference does not occur in the early stages of a young developing sward.

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Photosynthesis and Yield in a Perennial Ryegrass Crop

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Harvestable crop yield is the balance between gains (mainly photosynthesis) and losses (mainly respiration, death and non-harvestable plant parts) and an attempt has been made to quantify these processes in the perennial ryegrass crop. In the field it is not yet possible to measure all of the processes directly: there are additional difficulties due to the varying nature of the environment, which reduces the precision of measurement, and in this investigation a combination of field and controlled environment studies was used.

In a fully light intercepting sward in the field in spring a typical rate of crop net photosynthesis was 7.5 g CO_2 m^{-2} (ground area) h^{-1} at 500 W m^{-2} (300–3500 nm). Daily photosynthetic yield varied widely and randomly owing to variation in radiation receipt, and systematically over the growth period owing to changes in the efficiency of the canopy. Total photosynthetic yield was 2545 g CO_2 m^{-2}. Over the same period respiration losses from the shoot in the dark amounted to 504 g CO_2 m^{-2} (20% of the photosynthetic yield). The increase in dry matter (converted for convenience to its CO_2 equivalent) was 956 g CO_2 m^{-2} (38%) of which 748 g CO_2 m^{-2} (29%) was harvestable. Neither root respiration nor death could be measured but, by difference, these processes must have accounted for the loss of 1085 g CO_2 m^{-2} (42%).

Use of simulated swards in a controlled environment (daylength 16 h) enabled a more complete carbon balance to be drawn up and, in particular, leaf death and root respiration to be determined directly. So far, data are restricted to seedling swards which differ in some important respects from established swards in the field. Nevertheless rates of crop photosynthesis and dry matter production are remarkably similar. Dark respiration losses were, however, less (shoot 11%, root 10%) probably reflecting the shorter night and the higher shoot root ratios than the field, so that harvestable yield was higher (41%).

In conclusion it appears that even under the most favourable conditions (seedling swards in a growth room) harvestable yield is unlikely to contain more than about 40% of the carbon initially fixed in net photosynthesis, or more than 30% in an established sward subject to good husbandry in the field.

Environmental Factors Influencing Cereal Yield Components

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Cereal yield is the product of three factors: ear number per unit field area, grain number per ear and mean weight per grain ($\overline{W_g}$). The final levels of these yield components depend on the processes of tillering, ear development and grain growth respectively. Unfortunately, most studies report only the final level of yield components. However, such studies have often shown that variation in $\overline{W_g}$

for a particular variety is small and that grain yield depends mainly on grain number per unit field area. Effects of environmental factors on tillering and ear development are therefore of fundamental importance in yield studies.

Environmental conditions causing fast photosynthesis and growth, such as heavy fertilisation and bright sunshine, promote both fast rates and high levels of tiller and flower production; they also increase the fraction of ear bearing tillers and grain bearing flowers. Unfavourable conditions, such as weak sunshine and extremes of temperature, before and during flowering interfere with fertilisation and ovary growth causing poor grain set and sometimes decreasing $\overline{W_g}$. During the period when the processes determining ear, flower and grain numbers overlap (i.e. from about 3 weeks before until 1 week after flowering) the effects of adverse environmental conditions on grain yield are particularly severe.

Grain growth is apparently buffered against adverse environmental conditions and this ensures a relative stability of the $\overline{W_g}$ yield component. For instance, although the duration of grain growth is decreased by high temperatures this is largely offset by a faster growth rate. Similarly, environmental factors which limit current assimilate supply for grain growth are often compensated by increased translocation of stem reserves to the grain.

Thus, while the general pattern of responses of cereal yield components to environmental factors is known, the physiological mechanisms underlying these responses remain obscure.

Effects of Shade on the Components of Yield of Apple

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Shade can be regarded as a manageable variate in apple production since orchards are not grown to give full ground cover and the pattern of light distribution within trees and hedgerows of trees can be controlled by the genetic vigour of the tree rootstock, spacing and pruning.

Yield of a tree or part of a tree is reduced by shading owing to effects on both fruit size and the number of fruits. This latter factor is influenced by shade effects on fruit bud initiation in the pre-cropping year and by effects on the proportion of fruitlets set and retained in the cropping year. Experiments involving shading for one or two seasons have shown that its influences on these separate components of yield, although possibly working through distinct mechanisms, are not independent or simply additive. They interact with each other in such a way that manipulation of shading treatments so as to minimise their direct effect on one component of yield increases their effects on other components, so tending to maintain the overall adverse effect on total yield. The influence of shade on some of the yield components may probably be regarded as a mechanism whereby the cropping level is pre-adjusted to the lowered productive potential of the shaded tree. Under these conditions of interacting components of yield a limiting factor rather than an additive approach must be employed.

Plant Density and the Yield Components for Silage and Grain Maize

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Maize in England is used mainly for silage (16 000 ha in 1974), whilst a smaller area in the south-east is grown for grain (1060 ha). Plant density and variety are two factors that predominantly affect the components of yield. When the *whole plant* is ensiled, the response of yield to plant

density is asymptotic, the optimum being a plant stand of 110 000 plants/ha, and the components of dry matter yield are approximately 50% ear, 35% stem and 15% leaf. Higher total yields of dry matter may be obtained, though at a decreasing rate, even up to 150 000 plants/ha, but the seed cost then becomes uneconomic. It has also been suggested that high densities reduce the ear content, which might increase the moisture content of the whole crop (above 75%) which leads to high silo seepage losses. However, as density is increased, although there is ear barrenness, the proportion of plant dry matter in the ear only drops by 3–4%, and the dry matter % of the whole plant only drops by some 2%, so silo seepage is not likely to increase seriously. Hence, separate breeding programmes for silage maize can include material that does not necessarily contain a high grain content at high density.

At the optimum plant density in the UK for grain maize (90 000–100 000 plants/ha), the component of yield "grain number" is usually constant at approximately 2000 grains/m^2, whatever the variety. Ideally, this will be produced by one large ear on each plant, although in some varieties more than one ear per plant may be produced and the secondary ears may delay harvesting. The relationship between grain yield and density is parabolic, and as density is raised above the optimum, so yield will decline owing to a reduction in both of the components "grains/m^2" and "individual grain weight". Increasing the yield of maize in temperate regions will depend on a more rapid establishment of leaf ground cover during our cool spring conditions, earlier flowering (in late July) to lengthen the grain-fill period, and faster rates of water loss during the later stages of maturation to give a higher dry matter content in silage and grain crops.

Components of Yield of Strawberry and Blackcurrant and the Orientation of Research

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Crops of fruit and seeds, derived from flowers, are the result of an ordered sequence of developmental stages, sometimes spaced over 2 years. The stage-by-stage evaluation of plant performance in relation to the development of components of yield becomes an exercise in critical path analysis because partial or complete failure at any component stage cannot usually be rectified later and affects final yield.

Since yield = number of fruit × individual size, the problem resolves into the identification and evaluation of developmental stages affecting numbers and those affecting size. When a flower is a necessary precursor of a fruit the numerical components are:

1. Number of sites for flower initiation (buds and branch tips) produced vegetatively
2. Number of inflorescences initiated per site
3. Number of flowers developed per inflorescence

which multiplied together produce the numerical component of potential crop. This is modified in the fruiting season by losses from various causes leading to some failure to produce ripe fruit.

The natural range of fruit sizes in strawberry and blackcurrant can be accounted for in terms of position on the inflorescence, seed number per fruit, and a development factor.[1–3] Placed on the time scale of development these give rise to a component of yield:

4. Potential size, determined at flower formation, but later modified by
5. Effectiveness of pollination, and
6. Degree of tissue development, resulting in actual yield.

Comparisons of potential and actual yield[4,5] indicate the losses arising during the fruiting season and can be used to direct research to phases where benefit seems likely to be greatest.

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Effects of Environment on the Yield of Glasshouse Roses

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The yield of the glasshouse rose can be quantified in terms of the quantity and quality of marketable blooms produced from unit area in unit time. Each bloom terminates the growth of a leafy axillary shoot and at marketing, they are cut so as to leave a few compound leaves with axillary buds whose outgrowth is then no longer inhibited by the presence of the flowering shoot. The induction of flower initiation is not under the control of any environmental variable and occurs in every shoot soon after the onset of growth. Thus the potential yield is determined principally by the effectiveness of environmental factors in stimulating the uninhibited axillary buds into growth. The environment also influences whether certain floral organs are initiated and in what number. Incomplete flower buds abscise to leave "blind" shoots, the formation of which can reduce potential yields by 50%. Bloom quality is assessed mainly on the basis of stem length which can also be modified by environment.

Enrichment of the glasshouse atmosphere with carbon dioxide (CO_2) to a level of 1000 v.p.m. increased bloom production of "Sonia" by 20%, while additional light produced one extra bloom for every 9 MJ of solar radiation-equivalents supplied. These responses were mainly the result of increased shoot production although CO_2 enrichment also reduced the incidence of blind shoots. Canopy photosynthesis is enhanced by elevated CO_2 concentrations and high light flux densities so that these responses may be mediated directly through changes in the level of assimilates. Increases in both environmental factors also reduce stem extension, possibly as a result of increasing the within-plant competition for assimilates and growth regulators.

Components of Yield—Static Contrivance or Dynamic Concept?

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It is shown from a survey of the literature of 50 years ago[1–3] that yield component analysis started with the same phenomenological assumptions as another technique which was being developed by other botanists of the same school at about the same time, namely growth analysis. It is suggested that the two techniques stand in direct line of intellectual descent from F. F. Blackman's Law of Limiting Factors. Key ideas of interaction, and uncertainty, which R. A. Fisher and others were developing, were neglected during the early years of yield analysis. Surprisingly, the equally relevant ideas coming from growth analysis were also neglected, and, for reasons which will be discussed in the paper, much more attention was paid to roots than to leaves as determinants of yield components.

Eventually the utility of yield component analysis came to be questioned on pragmatic and philosophic grounds.[4,5] A survey of the recent literature shows that some have abandoned classical yield components for physiological components of yield,[6] some have retained the classical components and introduced powerful statistical techniques to aid their analysis,[7–9] and some have extended conventional yield analysis using matrix algebra[10] and stochastic, rather than deterministic models. The assumptions implicit in each approach are examined.

The components derived from matrix yield analysis can be used as input for a stochastic model of the yield process.[11] Further development of such models may lead to an understanding of the interdependence of the yield components. Consideration of crop models raises two questions. Would yield analysis be more meaningful if yield components were formulated not as a product,

but as a sum? Does the reported stability[12] of certain yield components in the face of apparently large environmental fluctuations imply that there is a much greater "constancy of the milieu intérieur"[13] in plants than the early pioneers of yield component analysis had assumed?

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(c) Present summaries of data or the results of representative experiments rather than extensive data which can be deposited free of charge with the British Library, Boston Spa, Wetherby, Yorkshire LS23 7BQ. Never give the same data in two forms (e.g. both as a graph and as a Table) or tabulate data that can be described in a few lines of text.
(d) Avoid repetition. For example, when there is a separate Discussion section do not use it to recapitulate the results; use it to discuss their significance, their relationship to the object of the work and to the results of other workers.
(e) Distinguish clearly between original and published findings.

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(e) *Chemical nomenclature:* Use that recommended in the *Biochemical Journal* "*Suggestions to Authors*" (revised yearly) (The Biochemical Society, London).

(f) *Symbols:* Check symbols, formulae and equations with great care. Use the symbols recommended in the various parts of the *British Standard No.* 1991 *Letter Symbols, Signs and Abbreviations* (British Standards Institution, London). Use S.I. Units wherever possible; these are described in the British Standards publication No. PD5686 *The Use of S.I. Units* (B.S.I., London) and in *Chemistry and Industry* 1968, p. 396.

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