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Energy Considerations in the Use of Herbicides[a]

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The total energy contents of a number of herbicides have been determined by a computer program. A comparison is made on the basis of the figures obtained between energy usages in carrying out weed-control operations mechanically and chemically, and in carrying out conventional and minimum tillage methods of cultivation.

Herbicides are essentially used as substitutes for human and mechanical labour, that is, to carry out by chemical means weeding and other cultivation operations. It has been demonstrated by investment appraisal studies that this can result in significant financial savings as a result of the reduced labour requirements. For example, in the USA it has been estimated that, in 1974, it required 50 man-hours to grow a hectare of cotton compared with 300 man-hours in 1954.[1] The financial gains from increased yields consequent on use of herbicides are well documented, for example, Hurtig estimated $58 million increase in the value of crops harvested on 10 million hectares in Canada treated with herbicides in 1960 at a cost of $8 million.[2]

From a cost-benefit viewpoint the farmer may see a number of other advantages in using herbicides such as an increase in convenience, greater leisure for himself, economy of increasingly scarce and expensive manpower, avoidance of extra cultivation operations at inconvenient times, freeing of land for other purposes, improvements in crop appearance, etc. However, in this paper, attention will be concentrated on a comparison between the total energy used in carrying out a cultivation operation chemically and that used in carrying it out mechanically.

The farmer may see a direct saving in diesel fuel in performing a herbicide spraying operation rather than another cultivation operation which is more demanding on tractor time and power. The question that needs to be asked is whether, from a world energy viewpoint, this saving is outweighed by the energy which goes into manufacture of the herbicide. It has been demonstrated in previous publications that the great increases in agricultural and horticultural yields which have been achieved during the past 30 years have involved utilisation of ever-increasing amounts of energy and it has been suggested that, on an energy input–output basis, this may be subject to the law of diminishing returns.[3,4] Are then the benefits to the farmer of herbicide usage gained only by utilising greater amounts of the world's fossil fuel resources?

To answer this question it is necessary to estimate the total amount of fossil fuels which are used in manufacture of the herbicide. Energy is used whenever material is processed or transported so that, at each stage of manufacture, there is an energy input which is carried on to the next stage. The final product has indirect energy inputs from all the materials used and direct energy inputs from heat and electricity used in processing.

A computer program has been developed at Mond Division of Imperial Chemical Industries Ltd which sums up all these energy inputs for a given chemical compound provided that an outline flow sheet for all stages of manufacture back to the primary raw materials can be constructed. In most cases this can be done either from information available within the Company or from data published in the chemical or patent literature.

[a] Presented at a joint meeting, "Energy in Agriculture", on 21 October 1974, organised jointly by the Agriculture and Pesticides Groups, Society of Chemical Industry.

Materials which are not fuels, such as sodium chloride, are assumed to have no energy content in their natural state, and energy input for them starts when they are mined. Fuels, which are the sources of carbon in the final product, have an inherent energy content in their natural state and to this is added the energy used in winning and transporting them. Direct energy inputs of heat and electricity are calculated on the basis that steam is generated in a package boiler at 80% thermal efficiency, a typical figure for industry, and that electricity is generated by the UK Central Generating Board at 31.0% thermal efficiency, the 1974 value. The details are shown in Tables 1 and 2 respectively. The total energy input to obtain natural gas equivalent to 1 joule is 1.032 joules as shown

Table 1. Energy input for steam generation

	1.03×10^4 N m^{-2} Satd	1.90×10^6 N m^{-2} 300°C	5.17×10^6 N m^{-2} 400°C
Energy from 16°C (J/kg)	2.64×10^6	2.95×10^6	3.12×10^6
Heat input at 80% efficiency (J/kg)	3.29×10^6	3.69×10^6	3.90×10^6
Capital (£/kg/S)	2.29×10^{-4}	2.64×10^{-4}	3.62×10^{-4}
Depreciation 15 years (2.88×10^7 S/year) (£/kg)	5.29×10^{-5}	6.10×10^{-5}	8.38×10^{-5}
Energy input for capital (see Table 4) (J/kg)	7.2×10^3	8.3×10^3	11.4×10^3
Total energy input for steam used (J/kg)	3.3×10^6	3.7×10^6	3.9×10^6

Prepared from Imperial Chemical Industries Ltd data, 1974.

Table 2. Energy input for electricity generation

Thermal efficiency of generation	31.0%
Average loss of energy in coal production	3.5%
Average loss of electricity in transmission	2.0%
Average loss of electricity in industrial distribution	2.0%
Corrected efficiency of generation $30.0 \times 0.96/1.035$	28.7%
Energy input as fuel for electricity used 1/0.287	3.48 J/J
Total cost, excluding fuel, to CEGB and Area Boards	8.96×10^{-10} £/J
Total energy input, excluding fuel (see Table 4)	1.48×10^{-1} J/J
Total energy input for electricity used $3.48 + 0.15$	3.63 J/J

Prepared from Central Electricity Generating Board data, 1972–3.

in Table 3. The energy input associated with use of capital or labour was estimated as an average figure by dividing total UK energy consumption by the Gross Domestic Product as shown in Table 4. These figures can be applied to fixed costs and also, at an assumed depreciation rate, to capital

Table 3. Energy input for natural gas production

Cost of natural gas purchases	1.08×10^{-10} £/J
Average cost of transmission and distribution to industry	0.87×10^{-10} £/J
Total cost	1.95×10^{-10} £/J
Energy equivalent to costs (see Table 4)	3.2×10^{-2} J/J
Total energy input for gas used	1.032 J/J

Prepared from British Gas Corporation data, 1972–3.

Table 4. Energy input for fixed costs

Total UK energy consumption	8.85×10^{18} J
UK gross domestic product 1972	5.38×10^{10} £
UK gross domestic product 1974	6.51×10^{10} £
Energy input for fixed costs 1972	1.65×10^8 J/£
Energy input for fixed costs 1974	1.36×10^8 J/£

invested but their lack of preciseness is unimportant as they make only a very small contribution to the final answer. Likewise, energy used in transportation of intermediates between various manufacturing sites is ignored because experience of Company processes for which accurate details are known show that their contribution to the total energy figures is insignificant.

This program is similar in its assumptions to that used by Imperial Chemical Industries Ltd Agricultural Division to estimate the total energy inputs for various fertilisers, the figures for which have been published by them.[5]

Table 5 shows the total energy inputs calculated on the foregoing assumptions for a number of well-known herbicides. The accuracy of the calculations obviously depends on the accuracy and detail of the flowsheets used as a basis but it is thought that, in general, it is about $\pm 15\%$. The figures shown in Table 5 are for 100% active ingredient, naked *ex* Works.

Table 5. Energy input for manufacture of herbicides

	GJ/t ($t = 10^3$ kg)					
	Naphtha	Fuel oil	Nat. gas	Electricity	Steam	Total
MCPA[a]	53.3	12.6	12.0	27.5	22.3	130
Diuron[a]	92.3	5.2	63.1	85.6	28.3	270
Atrazine[a]	43.2	14.4	68.8	37.2	24.7	190
Trifluralin[a]	56.4	7.9	12.8	57.7	16.1	150
Paraquat[a]	76.1	4.0	68.4	141.6	169.3	460
Nitram fertiliser[b]						25
Urea fertiliser[b]						35
NKP fertiliser[b] 17.17.17						18
Inherent energy of coal						25

[a] 100% active ingredient, naked *ex* Works.
[b] Packed and bagged. Data from reference 5.

The total energy content of herbicides is about ten times that of fertilisers. However, as a typical rate of application for a fertiliser for cereals is 250 kg/ha (2 cwt/acre) and for a herbicide (e.g. MCPA in cereals) is 0.75 kg/ha (2/3 lb/acre) the total energy input for one application of a herbicide (at 200 GJ/t) is about 1/30 that for one application of a fertiliser (at 20 GJ/t).

A typical value for the amount of energy used in carrying out a mechanical weeding operation is 0.56 GJ/ha (1.5 gal/acre of diesel fuel), and for a typical spraying operation is 0.056 GJ/ha (0.15 gal/acre of diesel fuel). A comparison of the total energy input in carrying out a mechanical weeding with the total energy inputs for carrying it out with herbicides, assuming typical rates of application, is shown in Table 6. Of course, in practice, for weed control throughout a whole season, the number of herbicide applications needed may differ from the number of mechanical weeding operations, the rates of application of herbicide may differ from crop to crop, and the amount of mechanical energy needed may depend on the nature of the terrain and weather conditions. Also if the herbicide can be applied pre-emergently in conjunction with a sowing operation then the mechanical energy of spraying will be saved. Nevertheless, it should be realised that, in general, at least two mechanical weeding operations are required to achieve the effect of one chemical treatment. However, the data in this paper will enable the reader to make a fair approximation for any particular case in which he is interested.

Table 7 shows a comparison of energy inputs into mechanical and chemical weeding in forestry. This is based on the use of a Massey Ferguson 165 tractor travelling at 0.56 m/s ($1\frac{1}{4}$ m.p.h.) in a

Table 6. Energy used in mechanical and chemical weeding

Method	Fuel (litres/ha)	Energy (GJ/ha)	Total
Mechanical	16.9	0.56	
Chemical	1.7	0.06	0.16
Herbicide (MCPA 0.75 kg/ha = 0.67 lb/acre)		0.10	
Chemical	1.7	0.06	0.68
Herbicide (Diuron 2.3 kg/ha = 2.0 lb/acre)		0.62	
Chemical	1.7	0.06	0.27
Herbicide (Atrazine 1.13 kg/ha = 1.0 lb/acre)		0.21	
Chemical	1.7	0.06	0.23
Herbicide (Trifluralin 1.13 kg/ha = 1.0 lb/acre)		0.17	

Table 7. Comparison of mechanical and chemical weeding in forestry

	Mechanical	Chemical
Area treated per day	2.6 ha	13.0 ha
Energy used per 8 h day by tractor	3.48 GJ	3.48 GJ
Energy used per ha	1.34 GJ	0.27 GJ
Energy in 2,4,5-T used (3.5 kg/ha = 3.1 lb/acre)		0.47 GJ
Total energy for two mechanical weedings	2.68 GJ/ha	—
Total energy for one chemical weeding	—	0.74 GJ/ha
Annual energy saving for chemical weeding	—	1.94 GJ/ha (= 13 gal/ha of diesel fuel)

Energy content of 2,4,5-T = 135 GJ/t.
Other data supplied by UK Forestry Commission.

new forestry plantation with a row spacing of 2.1 m. The mechanical weed control achieved 2.6 ha/day and the chemical weed control 13.0 ha/day. The average fuel consumption of the MF165 tractor was 1.27×10^5 J/s (3.0 gal/h). The herbicide used was 2,4,5-T at 3.5 kg/ha active ingredient. The reason why chemical treatment is so much more economical on tractor usage is that the mechanical operation can cope with only one row at a time, i.e. 2.1 m working width, whereas a mist blower on the tractor gives weed control with the herbicide of a 10 m strip for each pass of the tractor. Furthermore, it is usual to have to carry out two mechanical weed control operations per year, but only one chemical treatment.

An interesting case for comparison is the use of paraquat in minimum tillage and direct drilling operations as an alternative to conventional ploughing and cultivation. An APAS/NIAE Farm Mechanisation Study quotes diesel fuel consumption figures for a range of large farm tractors operating on both light and heavy soils.[6] Taking an average figure for a medium soil the amounts of diesel fuel consumed for conventional ploughing and cultivation and direct drilling respectively are shown in Table 8. To the direct drilling figure has been added the energy content of the paraquat used to give a total energy input.

Finney has calculated fuel consumption based on horsepower hours per acre needed on three cultivation systems included in joint Plant Protection Ltd/ADAS trials in East Anglia.[7] The four sites (two winter wheat and two spring wheat) were on clay loam soils and the assumption was made

Table 8. Energy used in conventional cultivation and direct drilling

Ploughing and cultivating			Direct drilling		
Operation	Fuel (litres/ha)	Energy (GJ/ha)	Operation	Fuel (litres/ha)	Energy (GJ/ha)
Ploughing	22.5	0.75	Spraying	1.7	0.06
Heavy cultivating (1 × 2)	22.5	0.75	Drilling	11.2	0.38
Light harrowing	5.6	0.19	Harrowing	5.6	0.19
Drilling	11.2	0.38			
Light harrowing	5.6	0.19	Paraquat (0.84 kg/ha = 0.75 lb/acre)		0.39
Total		2.26	Total		1.02

Table 9. Energy used in various cultivation systems

Cultivation system	Fuel (litres/ha)	Energy (GJ/ha)
Traditional cultivation	46.7	1.56
Reduced cultivation	32.7	1.09
Paraquat (0.56 kg/ha = 0.5 lb/acre)		0.26
Total		1.35
Direct drilling	7.4	0.25
Paraquat (0.84 kg/ha = 0.75 lb/acre)		0.39
Total		0.64

that 12 horsepower hours require 1 gal of diesel fuel. The Bettinson drill was used for direct drilling. The results are given in Table 9.

A study of the cultivation of maize was made at the University of Illinois using conventional and no-till cultivation methods.[8] The total energy requirements for planting, cultivating and harvesting the crop are shown in Table 10.

Table 10. Energy used in conventional cultivation and no tillage

Cultivation system	Fuel (litres/ha)	Energy (GJ/h)
Conventional	61.8	2.06
No-tillage	20.2	0.68
Paraquat (0.84 kg/ha = 0.75 lb/acre)		0.39
Total		1.07

The results from these three trials suggest that direct drilling and minimum tillage techniques save about 1.0 GJ/ha of total energy. In conventional farming units this is equivalent to a saving of about 2.7 gal/acre of diesel fuel, assuming 1 gallon diesel fuel is equivalent to 1.52×10^8 J.

However, there are many uncertainties when calculating total energy inputs into direct drilled crops, for example, their fertiliser requirements have not been clearly defined. The results of the simplified calculations in this paper should therefore be interpreted with some caution.

The world acreages of temperate cereals and maize have been given by the Food and Agricultural Organisation of the United Nations for 1964 as follows:[9]

Wheat	2.08×10^8 ha	$(5.15 \times 10^8$ acres)
Barley	7.0×10^7 ha	$(1.76 \times 10^8$ acres)
Oats	3.3×10^7 ha	$(8.2 \times 10^7$ acres)
Rye	2.6×10^7 ha	$(6.5 \times 10^7$ acres)
Maize	1.25×10^8 ha	$(3.10 \times 10^8$ acres)
Total	4.63×10^8 ha	$(1/15 \times 10^9$ acres)

Hypothetically, if direct drilling or minimum tillage techniques were applied to all these crops, the total world saving of diesel fuel might be about 3×10^9 gal, which is about one-tenth of the total annual imports of crude oil into the UK. It will be realised that the actual input of energy into paraquat manufacture is not, in fact, all as fuel oil but, to a very considerable extent, as natural gas and coal.

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Release of Exchangeable and Non-exchangeable Magnesium from Nigerian Soils on Cropping with Maize or Chemical Extraction

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A greenhouse experiment with maize (*Zea Mays* L.) using 12 representative soils of Western Nigeria indicated that the rate of release of non-exchangeable Mg was too slow to meet crop requirements. Total Mg removed by four successive crops of maize (y) was well correlated with initial exchangeable Mg (x) ($y=6.34+1.06\,x$, $r=0.996$***) suggesting that neutral N NH_4OAc-extractable Mg is the best measure of available Mg in these soils even under intensive cropping. The slow release of non-exchangeable-extractable Mg in the soils may be due to low percentages of silt and clay and their mineralogy.

Total and non-exchangeable Mg extracted by acidified N NH_4OAc (pH 1.0) in 15 min at a soil extractant ratio of 1:5 correlated best with the total and non-exchangeable Mg removed by cropping ($r=0.944$*** and 0.734*** respectively). The use of such an acid extractant on these soils is, however, open to question in view of the relatively large amounts of non-exchangeable Mg extracted by it compared with that extracted by cropping.

Incipient Mg deficiency symptoms appeared in two-week-old plants when they contained 0.11–0.15% Mg. Persistent deficiency symptoms were associated with 0.1% Mg in the plants, 21–22 parts/10^6 exchangeable Mg and 5% (or less) Mg saturation of CEC.

1. Introduction

Because of their poor buffering capacities and small organic matter contents, the soils of Western Nigeria are potentially poor in most essential nutrients including Mg. A bush fallow system of farming has in the past helped to restore depleted nutrient reserves in the top soils but after a decade of cultivation, sporadic Mg deficiency symptoms have been reported on maize on a few Government farm settlements. It seems therefore that with increasingly intensive farming, Mg deficiency could become a serious problem.

Although plants obtain the bulk of their Mg requirements from the exchangeable and water-soluble forms, they are also reported to tap non-exchangeable sources. In order to evaluate the amount of Mg potentially available, the ability of local soils to release non-exchangeable Mg for plant use must be determined. This investigation aimed to assess the release of exchangeable and non-exchangeable Mg from some Western Nigeria soils by exhaustive cropping with maize in the greenhouse and by chemical extraction.

Use of exhaustive cropping to measure the Mg supplying powers of soils has been reported in Europe and America but not in Nigeria. Salmon and Arnold[1] and Michael and Schilling[2] reported

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only small releases of non-exchangeable Mg when some English and German soils were intensively cropped to ryegrass and potatoes respectively. Rice and Kamprath[3] and Christensen and Doll,[4] however, reported considerable releases in some American soils where the vermiculite and montmorillonite contents were high. In field studies with some Danish soils Henriksen[5] found that most of the non-exchangeable Mg extracted by plants came from the clay and silt fractions. Magnesium is associated with the 2:1 type silicate clays as either part of the crystal lattice of micas, montmorillonites, illites or in the interlayers of chlorites and vermiculites. The pattern of Mg release may therefore be expected to differ considerably between soils containing differing amounts of these clay minerals. In soils of low buffering capacity, H released from the root surfaces during cation exchange may be utilised in releasing Mg from the non-exchangeable sites and in such a situation, crop response to Mg fertilisation may not be obvious even when the exchangeable Mg level is apparently low.[3]

The exhaustive cropping technique as a means of estimating Mg supplying potential of soils, though reasonably reliable, is highly time-consuming and rather expensive. Some workers have therefore tried chemical procedures as possible alternatives. Rice and Kamprath[3] employed acidified N NaOAc and the 0.05 N HCl extraction recommended by Graham *et al.*[6] is reported to be capable of extracting some non-exchangeable but plant-available Mg. A chemical method that could replace the greenhouse exhaustive cropping technique without undue loss of accuracy would obviously be preferred.

2. Experimental

2.1. Greenhouse cropping technique

Twelve surface soils (eight from intensively cropped areas and four from fallows) were collected from selected areas in Western Nigeria (see Table 1), air-dried, sieved (< 2 mm), and triplicate 4-kg samples weighed out in plastic buckets and treated with the following rates of salts (reagent grade):

Table 1. Soils used (all are well-drained loamy soils)

Site	Soil description
1 Ogbomosho (A) 2 Iwo (F) 3 Ogbomosho (A)	Derived from undifferentiated basement complex, under savannah conditions, classified as luvisols, ferruginous tropical soils, or alfilsols
4 Moor Plantation (A) 5 IITA (F) 6 Odeda (A)	Derived from undifferentiated basement complex under forest vegetation. Classified as luvisols, ferruginous tropical soils, or ultisols
7 Ikenne (A) 8 Agege (A) 9 Ikenne (F)	Derived from sedimentary sandstones, under forest vegetation and humid climate; classified as nitosols—ferralitic soils or oxisols
10 Abeokuta (A) 11 Aiyetoro (A) 12 Aiyetoro (F)	Derived from sedimentary sandstones under savannah conditions, classified as cutric nitosols—ferrallitic soils or oxisols

A, cropped; F, fallow.

300 parts/10^6 N (split) as $(NH_4)_2SO_4$, 100 parts/10^6 K as KH_2PO_4, 120 parts/10^6 P as KH_2PO_4 and NaH_2PO_4 and a micronutrient mixture of 10 parts/10^6 Zn as $ZnSO_4 \cdot 7H_2O$, 5 parts/10^6 Mn as $MnCl_2 \cdot 2H_2O$, 5 parts/10^6 Cu as $CuSO_4 \cdot 5H_2O$ and 3 parts/10^6 Fe as ferric tartrate. Supplemental macronutrient applications were made at each of the three subsequent plantings at the following rates: 75 parts/10^6 K, 100 parts/10^6 P, 250 parts/10^6 N. The micronutrients were reapplied only at the third planting.

The total nutrient salt needed for each cropping was dissolved in distilled water and made up to a volume that would suffice for all the buckets and thoroughly mixed with the soils before they were

watered for planting. Soils with pH of less than 6.0 were limed with pure $CaCO_3$ to raise the pH to about 6.5. All soils were watered to field capacity and incubated for one week after which 15 approximately uniform-sized maize seeds (IITA Composite A × B) were planted in each bucket. The seedlings were thinned to eight per bucket a week later. The buckets were arranged in a completely randomised design and re-randomised weekly throughout the experiment.

The plants were watered at least once daily. The amount of water needed for each bucket was approximated by a random sampling of about one-fourth of the total number of pots and weighing them to determine the amount of water loss and this amount was then added (as deionised water) to bring the soils to field capacity.

Each cropping lasted 40 days and at the end of each harvest, the buckets were emptied, the soils air-dried, sieved, treated with nutrients, returned into the appropriate buckets, watered again to field capacity and then re-seeded to maize. The harvested maize shoots were oven-dried at 70°C, ground with a Wiley Mill (to pass through a 40-mesh screen) and analysed for Mg.

The total Mg removed by four successive croppings was the sum of the different amounts removed at the various croppings. The amount removed from the non-exchangeable sources was determined by subtracting the depression in the initial exchangeable level from the total quantity removed by the four crops.

2.2. Analytical procedures

2.2.1. Soils

The pH of the soils was determined with a Beckman pH meter using 1:1 soil:water and 1:2 soil:0.01 M $CaCl_2$ suspensions after 1 h equilibration. Organic matter was estimated by the chromic acid wet combustion procedure described by Allison.[7] A multiplication factor of 1.724 was used in converting per cent carbon to per cent organic matter. Exchangeable Ca and K were displaced by the neutral N NH_4OAc leaching procedure of Chapman,[8] and determined with the "EEL" flame photometer. CEC was determined on the leached samples by further leaching the samples with iso-propyl alcohol, distilling the adsorbed NH_4^+ into a boric acid mixed indicator and titrating with 0.1 N HCl.[9] Exchangeable Mg was extracted by two successive shakings of a 2-g sample at a soil:extractant ratio of 1:25 for 1 h and filtering. Total Mg was obtained by decomposing the soils with HF and $HClO_4$ as described by Pratt.[10] Mg in the various extracts was estimated by the Perkin Elmer model 403 atomic absorption spectrophotometer after treatment with 1500 parts/10^6 lanthanum to suppress interferences.

2.2.2. Chemical extraction of non-exchangeable magnesium

Four 2-g samples of each soil were shaken with acidified N NH_4OAc (pH 1.0) for 5, 15, 30 and 45 min respectively at a soil:extractant ratio of 1:25 and filtered. The non-exchangeable Mg extracted at each shaking was the difference between the amount extracted by the acidified NH_4OAc and that extracted with neutral N NH_4OAc.

2.2.3. X-ray diffraction analysis of the clay fractions

Each of the soil samples was buffered with NaOAc (pH 4.8), treated with H_2O_2 to remove organic matter,[11] and then dispersed at pH 8.9 with NaOH and sodium citrate–sodium dithionite to remove free Fe, Al, Mn and Si.[12] The clay fractions were separated at <2 nm, Mg-saturated, glycerol-soluted, K-saturated and heated to 350–500°C. The X-ray diffraction was carried out using a Phillips–Norelco diffraction unit with a Cu–K α-radiation and Ni-filter.

2.2.4. Plant tissue analysis

Two grams of the milled maize tops from each treatment (soil) was wet-ashed with a HNO_3–$HClO_4$–H_2SO_4 mixture and the Mg in the diluted digest was determined by atomic absorption spectrophotometry.

3. Results and discussion

A summary of the chemical and physical properties of the soils prior to liming and fertiliser treatments is presented in Table 2.

Table 2. Some chemical and physical properties of the soils

Soil	pH H_2O	pH $CaCl_2$	OM (%)	Silt (%)	Clay (%)	Exchangeable cations Total Mg	Mg (parts/10^6)	K (parts/10^6)	Ca (parts/10^6)	CEC (mEq/100 g)	Mg (% saturation of CEC)	% of total Mg exchangeable
1	5.70	5.40	0.90	10	6	879.6	84.0	136.9	566.2	2.80	25.0	9.5
2	5.80	5.40	0.80	14	6	874.8	52.8	136.9	549.6	2.70	16.3	6.0
3	6.00	5.70	1.54	14	6	1029.6	130.8	168.1	967.2	4.90	22.2	12.7
4	6.00	5.70	1.47	10	10	910.8	114.0	144.7	756.0	3.90	24.4	12.6
5	6.71	6.50	2.30	17	12	1149.6	169.2	187.7	1689.6	8.50	16.6	14.7
6	6.10	5.60	2.00	15	8	1569.6	169.2	246.3	864.0	6.00	23.5	10.7
7	5.20	4 90	1.63	18	8	899.6	110.5	152.5	588.0	4.70	19.6	15.7
8	5.35	5.10	1.60	17	12	1200.0	114.0	140.8	612.1	5.10	18.6	9.6
9	5.00	4.70	1.94	15	7	1210.0	108.0	136.9	609.6	4.20	21.4	8.1
10	5.60	5.31	0.93	10	8	650.4	105.6	156.3	751.2	3.90	22.6	16.2
11	5.30	5.02	3.72	10	16	1684.8	358.8	168.1	1284.0	12.00	19.1	16.3
12	5.70	5.42	5.32	12	16	2169.6	332.4	164.2	1370.4	12.80	21.6	15 3

OM, Organic matter (organic C × 1.724).

3.1. Exchangeable magnesium status and crop performance in the greenhouse

The dry matter yields and the Mg uptake data for each of the four croppings are presented in Table 3 whilst Table 4 shows the change in exchangeable Mg, the total Mg removed from each soil and the Mg released from the non-exchangeable sources by cropping and by chemical extraction.

The data in Table 3 show a general decline in %Mg in maize tissue, total Mg uptake and dry matter yields with successive harvests. It would appear that the dry matter yields were directly

Table 3. Dry matter (DM) yields (g/pot), Mg content of maize tops and Mg uptake (mg/kg soil) at each harvest

Soil	1st crop DM	1st crop % Mg	1st crop Uptake	2nd crop DM	2nd crop % Mg	2nd crop Uptake	3rd crop DM	3rd crop % Mg	3rd crop Uptake	4th crop DM	4th crop % Mg	4th crop Uptake	Total uptake
1	33.50	0.550	46.06	26.59	0.316	21.00	37.54	0.050	4.50	16.86	0.088	3.71	75.00
2	22.00	0.355	19.50	20.00	0.303	15.00	37.89	0.063	5.75	14.28	0.073	2.50	43.25
3	68.50	0.482	82.50	25.09	0.423	26.50	54.58	0.066	9.00	19.11	0.072	3.25	121.25
4	67.50	0.442	74.50	19.28	0.390	18.75	45.12	0.068	7.50	21.95	0.072	3.75	104.50
5	65.50	0.527	86.25	36.54	0.525	47.75	46.84	0.175	20.25	33.90	0.066	5.50	159.75
6	73.62	0.487	89.50	20.82	0.635	33.00	58.96	0.164	24.00	43.14	0.065	6.50	153.00
7	40.50	0.455	46.00	17.21	0.480	20.50	35.85	0.166	14.88	33.32	0.060	4.75	98.00
8	63.50	0.475	75.25	17.70	0.300	13.28	41.00	0.154	15.75	23.37	0.055	3.00	107.25
9	45.50	0.480	54.40	25.89	0.309	20.00	33.95	0.182	15.25	22.17	0.063	3.25	93.00
10	55.00	0.430	59.00	23.15	0.293	21.00	44.75	0.106	11.75	14.35	0.065	2.25	94.00
11	63.50	0.667	105.75	36.53	0.484	44.00	39.49	0.464	45.75	57.93	0.310	44.90	236.00
12	67.50	0.822	138.50	52.07	0.466	60.25	48.50	0.544	67.00	57.07	0.337	48.00	313.75

Table 4. Changes in exchangeable Mg in 12 Nigerian soils after four croppings of maize in the greenhouse, total magnesium removed from non-exchangeable form by cropping and by acidified N NH_4OAc (15 min)—mg/kg soil

Soil	Exchangeable Mg			Total Mg removed		Mg released from non-exchangeable form		Crop released Mg as % total uptake	Balance of exchangeable Mg (after 4th crop) as % of intake level
	Before cropping	After 4th cropping	Decrease during cropping	By cropping	By extraction	By cropping	By extraction		
1	83.8	12.5	71.3	75.0	93.8	3.8	10.0	5.0	16.1
2	52.5	12.1	40.5	43.3	72.0	2.8	19.5	6.5	23.0
3	130.5	13.8	116.8	121.3	157.0	4.5	26.5	3.7	10.5
4	115.0	15.0	100.0	104.5	156.0	4.5	41.0	4.4	12.0
5	169.4	16.0	153.4	159.8	223.4	6.4	54.1	4.6	9.5
6	168.6	21.5	147.1	153.0	213.3	5.4	44.7	3.5	12.3
7	110.0	16.3	93.8	98.0	139.1	4.3	29.1	4.3	14.8
8	115.0	11.3	103.8	107.3	139.1	3.5	24.1	3.3	9.8
9	107.5	19.4	88.1	93.0	134.4	4.9	26.9	5.3	18.0
10	105.2	13.2	92.0	94.0	148.4	2.0	43.4	2.1	12.2
11	275.0	50.0	225.0	236.0	312.5	11.0	37.5	4.7	18.2
12	332.5	33.8	298.8	313.8	407.9	15.0	75.4	4.8	10.2

related to Mg content of the maize tops and that the latter was in turn directly related to the exchangeable Mg level. The flush in the third harvest resulted from a higher plant population used in that particular cropping. At the end of the fourth cropping, about 86% of the initial exchangeable Mg in each soil had been used up. Per cent Mg in plant fell from 0.5 in the first to about 0.06 in the fourth harvest while total uptake fell from 76 mg to 11 mg per bucket respectively. Only about 4–6% of the total Mg that was taken up by plants during the four croppings conceivably came from the non-exchangeable sources, and taking into account the small amounts that might have been added in the maize seeds it is obvious that only a very negligible amount of Mg was extracted by plants from the non-exchangeable sources of the soils. This suggests that the level of exchangeable Mg in these soils is probably the best predictor of their Mg supplying powers.

The relationships among exchangeable Mg before and after cropping, non-exchangeable Mg removed chemically and by cropping, total Mg before cropping and total Mg removed by cropping are shown by the correlation coefficients in Table 5. Total Mg uptake correlated highly significantly

Table 5. Correlation coefficient among amounts of Mg extracted from the 12 soils by chemical methods and by cropping in the greenhouse

	Exchangeable Mg before cropping (r)	Non-exchangeable Mg removed by cropping (r)	Total Mg removed by cropping (r)
Exchangeable Mg before cropping	—	0.953***	0.982***
Exchangeable Mg after cropping	0.841***	0.813***	0.793***
Non-exchangeable Mg released by acidified N NH_4OAc	0.773***	0.734***	0.800***
Total Mg before cropping	0.879***	—	0.887***

*** Significant at 0.01% level.

with the initial and final exchangeable Mg values ($r = 0.982^{***}$ and 0.793^{***}, respectively); 99% of the variation in the Mg uptake was thus dependent on variation in the exchangeable Mg level. The high correlations between the non-exchangeable Mg removed by cropping and the initial and final exchangeable values ($r = 0.953^{***}$ and 0.813^{***}, respectively) suggest that the non-exchangeable Mg released by cropping is proportional to the exchangeable Mg soil contents. These results further strengthen the view that exchangeable Mg is the best predictor of Mg availability in the soils studied.

3.2. Exchangeable magnesium levels, % magnesium in plant tissue, and the appearance of magnesium deficiency symptoms

Table 6 shows the levels of exchangeable Mg in soils at the beginning of each crop. The soils which had the lowest initial exchangeable Mg contents (nos 1, 2, 3 and 4) were the first to show Mg deficiency (symptoms appeared during the third cropping) and by the fourth cropping, all the soils

Table 6. Exchangeable Mg levels at the beginning of each crop and the Mg saturation CEC when deficiency symptoms appeared

Soil	Exchangeable Mg (parts/10^6)				Mg saturation of CEC at which deficiency symptom appeared
	1st crop	2nd crop	3rd crop	4th crop	
1	83.8	37.8	16.6	12.0	4.9
2	52.5	33.0	18.1	12.3	5.5
3	130.5	48.1	21.4	12.3	3.6
4	115.0	40.3	21.0	13.0	4.3
5	169.4	83.2	35.4	16.2	1.6
6	168.8	79.5	46.5	21.5	3.0
7	110.0	64.0	23.0	19.3	3.5
8	115.0	40.0	26.9	11.1	1.8
9	107.5	53.1	33.0	17.8	3.5
10	105.2	46.0	25.0	13.1	2.8
11	275.0	170.0	126.2	80.5	—
12	332.5	194.0	133.6	57.2	—

except nos 11 and 12 had become deficient in Mg. It would appear, from these results, that Mg deficiency might be expected in these soils when the exchangeable Mg level is down to about 21–22 parts/10^6 and the Mg saturation of CEC is about 5% or less.

Incipient Mg deficiency symptoms which were associated with plant Mg content of about 0.11–0.15% in the harvest following the appearance of the symptoms, were observed during the third cropping on plants grown on soils nos 8 and 10 about 10–14 days after planting. Persistent deficiency symptoms which were associated with $\leq 0.09\%$ Mg in plant tissue appeared on plants grown on soils nos 1, 2, 3 and 4 during the third cropping.

These findings corroborate the results of other investigators.[14–16]

3.3. Magnesium availability as characterised by chemical extraction

Extraction of a nutrient by a strongly acid solution might provide a quick and convenient means of approximating the long-term supplying capacity of soils provided the amount extracted correlates reasonably well with plant uptake. Such chemical extractions are, however, usually neither entirely complete at a certain time interval nor exactly identical for different acid concentrations or soil: solution ratios.[17] In practice, therefore, the acid concentration, time of contact, and soil:solution ratios are compromises selected for soil fertility investigations.

In this study, N NH_4OAc acidified with concentrated HCl to pH 1.0 was used to extract non-exchangeable Mg (Table 7). Correlations between the amounts of total Mg extracted at various time intervals and the total amounts removed by cropping show no differences between the various

Table 7. Non-exchangeable Mg extracted (parts/10^6) with acidified NH_4OAc as affected by period of soil/extractant contact

Soil	5 min	15 min	30 min	45 min
1	3.0	10.0	16.3	16.3
2	5.0	19.5	31.4	49.5
3	12.5	26.5	69.4	65.3
4	5.0	41.0	59.0	70.3
5	40.6	54.1	65.9	67.7
6	13.9	44.6	54.5	59.4
7	20.0	29.1	27.5	33.7
8	22.5	24.1	26.1	32.9
9	20.0	26.9	40.4	41.3
10	18.6	43.4	41.4	42.9
11	25.0	37.5	48.0	62.4
12	37.5	75.4	81.0	81.5

periods of soil/solution contact. Non-exchangeable Mg removed by cropping correlated best with that extracted by shaking for 15 min ($r=0.734$***) (Table 8) but accounts for only 54% of the variation. Also, non-exchangeable Mg removed by cropping was much less than the amount extracted by acidified NH_4OAc in 15 min. It is therefore questionable whether such a procedure is of any practical value.

Table 8. Correlation coefficients for the various amounts of non-exchangeable Mg extracted by shaking with acidified N NH_4OAc and the corresponding amounts removed by cropping

Non-exchangeable Mg extracted chemically	Non-exchangeable Mg removed by cropping		
	r^2	r	y
5 min	0.395	0.629*	$6.495+2.092\,x$
15 min	0.539	0.734**	$15.335+3.563\,x$
30 min	0.364	0.604*	$27.451+3.361\,x$
45 min	0.366	0.605*	$33.343+3.177\,x$

* Significant at 5%; ** significant at 1%.

X-ray analyses showed that the clay fractions of these soils were predominantly kaolinitic (>50%) with only minor amounts of quartz, feldspars, micas, and some 2:1–2:2 interstratified layers. The slow release of non-exchangeable Mg may therefore be due to low percentages of silt and clay and their mineralogy.[3–5]

The results of this investigation suggest that available Mg in the soils studied can be depleted rather rapidly under intensive cultivation. The rate of release of non-exchangeable Mg seems rather too slow to have any practical value. The implication of this from the long-term fertility point of of view is that the available Mg in these soils may be easily exhausted as farming becomes increasingly intensive; Mg deficiency may thus pose a serious practical problem in the near future especially when other nutrients, particularly K, are used in adequate amounts.

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Yield, Nitrate Levels and Sensory Properties of Spinach as Influenced by Organic and Mineral Nitrogen Fertiliser Levels

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Spinach was fertilised at two levels of nitrogen, 140 kg/ha (x) and 420 kg/ha ($3x$), using split applications of ammonium sulphate and dried blood as nitrogen sources. A single application of ammonium sulphate (x) 19 days prior to harvest was also tested. A no-nitrogen control was also employed. The x rate of mineral nitrogen produced statistically the same yield as the $3x$ organic source but at a lower tissue nitrate level. The $3x$ mineral fertiliser produced both the highest yield and nitrate concentration. Excluding late fertiliser application, nitrate levels were closely related to yield and dependent upon rate of nitrogen availability from each of the two sources. Late application of mineral nitrogen did not increase yield over a no-nitrogen application control, but resulted in an eight-fold increase in tissue nitrate. Triangle taste panels demonstrated significant differences in raw, cooked, and frozen samples when comparing the no-nitrogen application against both the $3x$ organic and mineral nitrogen applications and the late mineral application. No significant sensory differences resulted between organic and mineral fertilisers at either application level. Colour measurements revealed that higher nitrogen application darkened colour. Organic sources gave darker colours than corresponding mineral fertiliser levels. Late application of mineral nitrogen resulted in the darkest colour. Gas–liquid chromatography headspace scans demonstrated that increasing nitrogen rates from x to $3x$ approximately doubled total peak area. No qualitative differences were noted. Late application of mineral fertiliser resulted in a headspace scan similar to those obtained at the $3x$ nitrogen rates.

1. Introduction

In an effort to maximise yields it is common practice to apply large quantities of nitrogen fertiliser. In the case of leafy vegetables such as spinach it is also a common practice to apply additional N as sidedressings several weeks before harvest. However, one of the problems that may arise from the excessive use of a nitrogen fertiliser is the accumulation of nitrate in the edible tissue.[1–7] This in turn can lead to toxicity problems[8, 9] and detinning of cans in processed products.[10]

Besides excessive fertilisation, other factors such as light intensity[3] and duration,[3, 11] temperature,[12] variability among cultivars,[12–15] storage conditions[16] and time of sampling[17] can also influence nitrate accumulation.

Recently, increased attention has been devoted to the utilisation of organic fertilisers as substitutes for the commonly used mineral fertilisers. With regard to organic fertilisers, certain nutritional and flavour superiority claims have been made. Therefore, this study was undertaken to investigate

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the possible influence of fertiliser source on spinach yield, nitrate levels and sensory properties including colour and flavour measured both by panel and analytical techniques.

2. Materials and methods

2.1. Field experiment

Savoyed leaf spinach (*Spinacia oleracea* L. cv. Longstanding Bloomsdale) was seeded in a Del Norte sandy loam containing 0.6% organic matter and 24 parts/10^6 NO_3-N (0–15 cm) on 24 March 1972 at the San Luis Valley Research Center near Center, Colorado, a semi-arid region at an altitude of 2334 m. Row spacing was 33 cm on beds with centres 102 cm apart. During seeding, all plots were sidebanded with 50 kg of phosphorus per hectare as treble super phosphate. At this time, nitrogen-bearing minerals, undecomposed finely divided dried blood (14.5% N) and ammonium sulphate, were mixed with the phosphorus for sidebanding. Soil and bloodmeal analysis indicated that P, K, Zn and Fe need not be added with the ammonium sulphate treatment in order to legitimise the organic nitrogen–mineral nitrogen comparisons. The nitrogen sources were each applied at planting at half of the final rates of 140 (x) and 420 kg ($3x$) of nitrogen/ha. The remaining nitrogen was applied 34 days prior to harvest. Another treatment consisted of only one application of 140 kg of nitrogen/ha as ammonium sulphate, sidebanded 19 days prior to harvest. Sixty-two days from seeding, each succeeding replication was harvested in its entirety during a period from 10 to 12 a.m. None of the plants had produced seed stalks at this time. All fresh edible tissue including blades and petioles exclusive of crown leaf tissue was obtained as a measure of yield and also for nitrate determination.

A randomised complete block design was employed. There were six treatments including a no-nitrogen control and four replications of each. Plots for harvest each consisted of the two inside rows of two beds 6.1 m long. All plots were furrow irrigated throughout the growing period.

2.2. Tissue nitrates

The nitrate analysis procedure was essentially that of Cantliffe *et al.*[18] but resin was not used. Nitrate ion activity was measured in the 0.025 M $Al_2(SO_4)_3 \cdot 16\ H_2O + 10\ \mu g/ml\ NO_3$-N extracting solution with an Orion 92-07 nitrate ion activity electrode and a Corning Ag/AgCl reference electrode with ceramic junction together with an Orion Model 801 Digital Millivolt Meter. Approximately 450 g of fresh tissue selected as representative of that harvested from each plot was held overnight at 1.7°C washed with distilled water, dried at 65°C in a forced draft oven and ground to pass a 40 mesh screen. Aliquots (400 mg) of each tissue sample were used for the nitrate determination.

2.3. Processing

Freshly harvested spinach was chilled overnight in a 7.2°C cooler. Approximately 2.3 kg of leaves selected as being representative of each treatment were washed in cold running water. The leaves were then torn into uniform pieces approximately 2.5 cm in diameter. Samples at this stage were evaluated raw by the sensory panel. Portions of each sample were also blanched and frozen in 450 g units for later evaluation. Cooked samples were prepared by dropping 450 g units of the raw or frozen product into 471 ml of boiling water and heated for 10 min. The products were then drained and served to the panel.

2.4. Sensory evaluation

A panel consisting of 20 female and 5 male college-age students having training in sensory evaluation methods was used. A total of 45 triangle tests covering all of the possible comparisons among all treatments was performed. To compensate for any possible visible colour differences among samples, all evaluations were done in red-light equipped booths. Rinse water was provided for panel use. Approximately 50 g units of each sample were presented and care was taken to ensure that all cooked samples were cooked, drained and served under the same conditions.

Sensory evaluation of the 15 randomly presented raw samples was done the day after harvest, freshly cooked samples 2 days after harvest and frozen sample evaluation was done 1 week after harvest so as to minimise the possible formation of 2-pyrrolidone-5 carboxylic acid and its possible influence on flavour.[19]

2.5. Colour measurements

The colour of approximately 50 g of three representative samples each of raw, cooked, and frozen (then cooked) spinach was measured using a Hunter Lab Model D-25 Color and Color-Difference Meter equipped with a green standard No. 6416.

2.6. Gas–liquid chromatography headspace analysis

Direct g.l.c. headspace analyses were performed on 1 g samples of raw spinach by the method described previously.[20] Individual peak areas were quantitated using a Hewlett Packard Model 3370B Automatic Integrator. These individual values were then totalled to obtain a total peak area value for each sample. No effort was made to identify individual peaks during this phase of the study. However, owing to the procedure used it is postulated that the compounds observed are alcohols and carbonyls common to most vegetable products.

3. Results and discussion

3.1. Yield

The yield data collected from each plot were averaged and converted to metric tons per hectare. These data are summarised in Figure 1. Both fertiliser types gave significantly higher yields than the no-fertiliser application control. Also, the mineral fertiliser was significantly superior in yield to the organic fertiliser to such an extent that the x level of mineral nitrogen had statistically the same yield as the $3x$ level of organic nitrogen. At the $3x$ nitrogen level, the yield from the mineral nitrogen source was approximately 23% higher than that of the organic nitrogen source. This is in general agreement with results reported by other workers.[21] Peavy and Greig found that mineral fertiliser outperformed the organic, but not significantly so during the first crop of spinach in 42 days. However, in their study, feedlot manure was used as the organic nitrogen source which undoubtedly contained some mineralised nitrogen readily available to the plant; whereas, in our study, undecomposed dried bloodmeal was employed. Thus, for short season crops, the use of bloodmeal as the nitrogen source may not be advisable owing to the relatively slow mineralisation of nitrogen.

Data from Figure 1 also clearly demonstrate that the application of mineral nitrogen approximately $2\frac{1}{2}$ weeks before harvest does little in the way of increasing total yield when compared to the earlier split application of nitrogen.

3.2. Nitrate levels

As mentioned in the Introduction, nitrogen fertiliser application has a definite influence on nitrate accumulation in the plant. This is dramatically illustrated in Figure 1. It can be seen that, excluding the near harvest application, nitrate levels were closely related to the level of nitrogen application and dependent upon nitrogen availability from each of the two sources. The reduction, although not significant, observed in nitrate concentration at the $3x$ level of organic nitrogen application can be explained as the result of the slower availability of the organic fertiliser nitrogen. The dramatic data, Figure 1, are those illustrating the influence of a later mineral nitrogen application which results in approximately an eight-fold increase in dry nitrate tissue over the no-nitrogen control. Thus, from a yield and economic standpoint as well as undesirable tissue nitrate levels, the practice of late fertiliser application does not appear advisable.

3.3. Sensory evaluation

The sensory panel triangle test results are summarised in Table 1. As can be seen, no significant difference in flavour was detected between the no-nitrogen control and the lower nitrogen level (x)

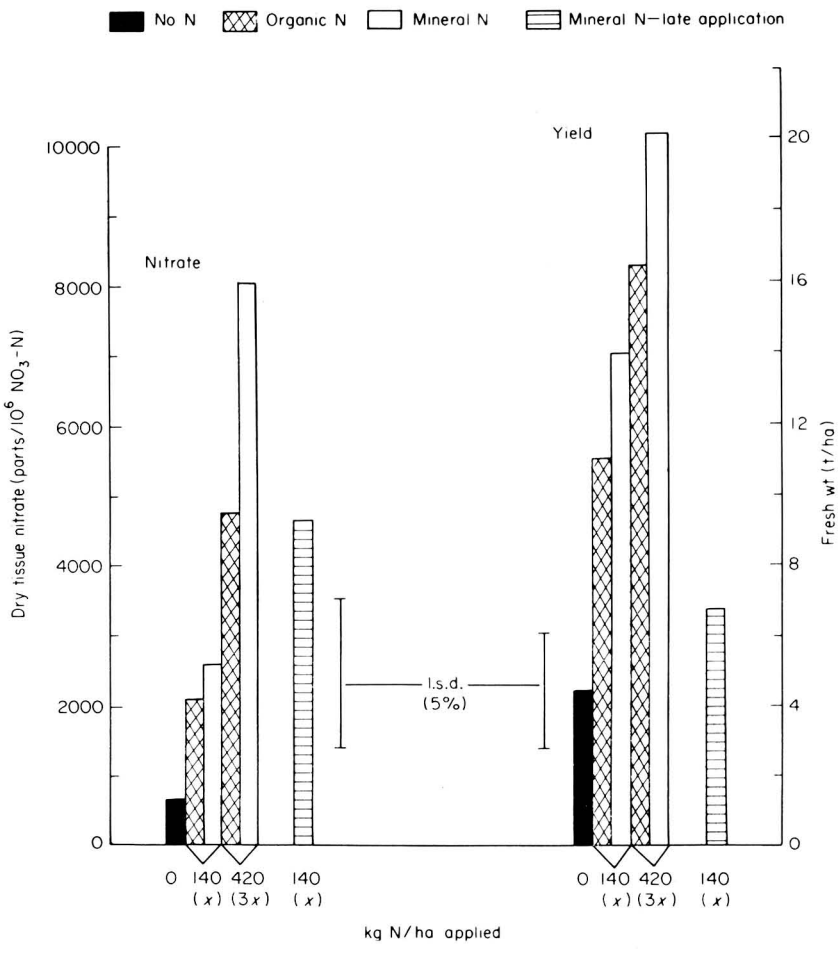

Figure 1. Effect of mineral and organic nitrogen application on spinach nitrates and yield.

of either mineral or organic fertiliser source for any of the spinach forms evaluated. However, highly significant flavour differences were noted between the no-nitrogen control and the higher nitrogen fertiliser level ($3x$) independent of nitrogen source. Interestingly, significant differences in flavour were also detected between the no-nitrogen control and the near harvest mineral fertiliser application samples. However, all other variables involving fertiliser types and levels showed no significant differences in flavour.

Since it is generally conceded that fertilisation of some nature, whether it be from a mineral or organic source, is required for adequate production, it would appear from these data that no flavour difference can be detected when a mineral fertiliser source is compared against an organic fertiliser source and perhaps different conclusions would be obtained if other organic sources were evaluated.

3.4. Colour

The primary advantage cited for the near harvest application of fertiliser to spinach is the darkening effect that apparently results in a more acceptable product. As can be seen in Table 2, this practice

Table 1. Spinach sensory data summary

Treatment	No. correct responses out of 25		
	Raw	Cooked	Frozen
No nitrogen *vs* *x* mineral	12	11	12
No nitrogen *vs* 3*x* mineral	18***	22***	19***
No nitrogen *vs* *x* organic	9	11	8
No nitrogen *vs* 3*x* organic	15**	19***	17***
No nitrogen *vs* late *x* mineral	14**	14**	13*
x mineral *vs* 3*x* mineral	8	10	10
x mineral *vs* *x* organic	11	11	12
x mineral *vs* 3*x* organic	10	8	11
x mineral *vs* late *x* mineral	9	9	9
3*x* mineral *vs* *x* organic	6	8	8
3*x* mineral *vs* 3*x* organic	12	12	11
3*x* mineral *vs* late *x* mineral	4	5	7
x organic *vs* 3*x* organic	7	5	6
x organic *vs* late *x* mineral	8	10	10
3*x* organic *vs* late *x* mineral	6	8	5

* $P=0.05$; ** $P=0.01$; *** $P=0.001$.

Table 2. Spinach colour data summary[a]

Treatment	L^b		
	Raw	Cooked	Frozen
No nitrogen	20.0	20.8	20.6
x mineral	19.7	20.1	20.0
3*x* mineral	18.6	19.2	19.0
x organic	18.5	18.9	18.9
3*x* organic	17.4	17.7	17.6
Late *x* mineral	17.2	17.5	17.5

[a] Hunter Lab Model D-25 Color and Color Difference Meter Green Standard No. 6416 (*L*, 61.0; *a*, −15.9; *b*, 7.0).
[b] Total lightness: 100 = white; 0 = black.

does have a definite darkening effect. However, it should be noted that the 3*x* level of inorganic fertiliser had statistically the same colour as the near harvest application of mineral fertiliser. As would be expected, the no-nitrogen control was significantly the lightest in colour of all three spinach forms evaluated. Interestingly, at each of the two fertiliser levels (*x* and 3*x*) the organic nitrogen source resulted in a significantly darker product than the corresponding mineral nitrogen source. Perhaps there were differences in chlorophyll content as influenced by the type of fertiliser, although in an earlier study,[21] it was reported that the fertiliser source had little or no influence on chlorophyll content. However, as mentioned previously, a different organic fertiliser source was used in that study.[21]

3.5. Gas-liquid chromatography headspace analysis

Assuming that generally total volatiles can be correlated with overall flavour intensity, the data in Table 3 are presented for discussion. Using the no-nitrogen control as the base, there was no obvious difference between it and the *x* rate of nitrogen application independent of fertiliser source. These

Table 3. Raw spinach g.l.c. headspace data

Treatment	Total peak area (cm^2)
No nitrogen	426
x mineral	514
$3x$ mineral	981
x organic	503
$3x$ organic	986
Late x mineral	962

data correspond to the sensory results reported in Table 1. However, there was an approximate doubling of total g.l.c. detectable volatiles at the higher ($3x$) fertilisation level as compared to the control. The late mineral fertilisation also resulted in the doubling of total volatiles. These were the same treatments that the panel determined as being significantly different in flavour. There was no obvious difference in total peak area for different fertiliser sources at a given level of application. Thus, these data indicate that flavour superiority claims made for organically fertilised foods may not be valid when dried blood is used. It should be noted that no qualitative differences were noted in the chromatograms for any of the treatments.

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Effect of the Application of Cow Slurry to Grassland on the Composition of the Soil Atmosphere

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The effect of a heavy application (550 tonnes/ha) of unamended cow slurry to grassland in early spring (March) on the composition of the soil atmosphere was examined by analysing the gaseous phase under adjacent treated and untreated areas for a 12-month period. Restricted aeration and products of intense reducing activity were observed in the slurry layer for 3 weeks, and in the underlying surface soil for several months, after the application; oxygen contents of the atmosphere to a depth of 80 cm were decreased and carbon dioxide contents increased, for 12 months. Although the slurry and soil never became completely anaerobic (minimum oxygen contents observed were 8% (v/v) in the slurry and 3.5% in the soil), high methane concentrations (6% in the slurry and 1.7% in the soil) indicated that a large proportion of the slurry and surface soil volumes were occupied by anaerobic volumes shortly after the application. Other hydrocarbon gases (ethane, ethylene, propane) were evolved, but did not accumulate to concentrations greater than 7 parts/10^6.

Nitrous oxide evolution indicated a pattern of gaseous N loss from the soil and slurry, presumably due to denitrification. Major evolution occurred in the slurry in May, June and July, and in the surface soil at the 10-cm depth in July–August. Nitrous oxide was detected continuously in the winter, and in concentrations as high as 680 parts/10^6, at a depth of 40 cm below the soil surface of the treated grassland after nitrate had been leached to this depth by autumn and winter rains. Nitrous oxide was detected continuously at 80 cm below the soil surface in spring and early summer, and again in the following winter, under treated as well as untreated areas.

The observed consequences of the slurry application are attributed to physical effects in restricting aeration, in association with the effect of the organic substrates on soil respiratory activity.

1. Introduction

Excreta from housed animals has traditionally been combined with straw and spread on to agricultural land in the form of farmyard manure. Modern developments have led to the practice of spreading the mixture of dung and urine, either unamended (slurry) or diluted with water (gulle), on to the soil surface. Modest dressings improve plant growth,[1,2] but the deleterious effects of heavy applications are poorly understood and characterisation of the changes in soil physical, chemical and biological properties is needed. Since heavy dressings of wet organic material may be expected to induce anaerobic conditions, resulting in the production of toxic gases and loss of nitrogen by denitrification, the present study was undertaken to examine effects on the composition of the soil atmosphere. Other associated studies have examined the effects on soil moisture relations, nitrate accumulation and leaching patterns,[3] and changes in faunal populations[4] and microbial activity.[5]

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2. Experimental

Cow slurry was spread on to grassland in early spring and the composition of the gas phase in the slurry, in the underlying soil, and in soil of an adjacent untreated area, was monitored over the following 12 months. The slurry remained as a surface layer for over 6 months before it was cultivated into the underlying soil, and a new pasture sown.

2.1. Experimental site and agronomic technique

The experiment was situated on the Hurst soil series,[6] a non-calcareous gley soil developed from loamy drift overlying river-terrace gravel, at Arborfield Hall Farm, National Institute for Research in Dairying, Shinfield, near Reading. The pasture on the site was a 2-year-old stand of short-rotation ryegrass (*Lolium multiflorum*, Lam.).

The two experimental plots, each 18.3 × 3.5 m, were situated 3 m apart with the long axes parallel. Tile drains were situated 75 cm below the boundary of each plot. Soil gas reservoirs and neutron moisture meter access tubes were located in a 5 m long × 2.5 m wide area in each plot; other areas were reserved for soil sampling, biological observations, and soil physical measurements.

The slurry was applied to the grassland on 28 March 1972. Almost all of the ryegrass was killed by the application of slurry but a few plants survived and grew during the summer. In late summer, further establishment of pasture occurred from wind-blown seed on to the slurry. In the autumn (16–23 October), the slurry layer was thoroughly intermixed with the surface 10-cm depth of soil during several cultivations with a Howard "Gem" rotary hoe. The control plot was cultivated to a depth of 10 cm at the same time. Short-rotation ryegrass seed was sown by hand, the soil surface lightly raked and then rolled with a garden roller.

The pasture was periodically cut and removed.

2.1.1. Slurry application

The slurry was obtained from a storage pit into which the fresh excreta from cows had been added at 2–3 day intervals during the previous 10 weeks. It contained 15.6% oven dry (at 105°C) material, 0.35% N, 0.16% P and 0.34% K (all percentages on a w/w of moist material basis).

The unamended slurry was spread at the rate of 550 tonnes/ha to a central strip 3.2 m wide, running the length of the plot, using a conventional rear-delivery slurry-spreader ("Salopian Slurribuggy", Salopian–Kenneth Hudson Ltd, Press, Shrops.) fitted with a canopy above the spreader to ensure a uniform deposition rate.

2.2. Composition of the gaseous phase

The soil atmosphere was sampled using permanently installed diffusion-equilibrium reservoirs. Each reservoir provided a cavity for a pool of gaseous phase that was in equilibrium with gaseous phase in the soil pores thus allowing a representative sample of the soil atmosphere to be obtained via a low dead-space tube connected to a sealed sampling point at the soil surface. The installation and sampling technique is described by Burford and Stefanson.[7]

2.2.1. Diffusion-equilibrium reservoirs

The reservoirs used were modified from those described previously by constructing the reservoir body (approx. 7 ml internal capacity) of aluminium alloy (pressed into an inverted saucer shape) and crimping a base of expanded aluminium alloy mesh on to the bottom of the reservoir. Two 5BA nuts were used to attach the reservoir to the threaded end of the fine-bore (0.8 mm i.d.) copper connecting tube.

2.2.2. Reservoir installations

Reservoirs were installed in the soil at depths of 5, 10, 15, 20, 30, 40, 60 and 80 cm prior to the slurry application. These were positioned in each strip according to a Latin Square randomisation (8 depths × 8 replicates) with the rows and columns being sited along the long and short axes of each

plot. Reservoirs were installed in the slurry layer immediately after the application, at depths of 0, 2 and 4 cm above the soil–slurry interface; these reservoirs were attached to a sheet of expanded aluminium to prevent movement in the semifluid slurry. Reservoirs sited above the 20 cm soil depth were removed prior to the cultivation in October, and those from the 5, 10, 15 cm depth were reinstalled immediately after sowing the pasture.

2.2.3. Sampling

The soil atmosphere was sampled at the 10, 40 and 80 cm depths in the soil, and 2 cm above the soil surface in the slurry, at intervals of 1–60 days. A complete profile sampling on both plots was made on 26 April 1972. Samples (5 ml) of the gaseous phase were obtained via the gas sampling point with 5 ml capacity, all-glass, hypodermic syringes, that had been lubricated with Edwards High-Vacuum silicone grease.

2.2.4. Gas analysis

The N_2, (O_2 and Ar), N_2O, CO_2 and light hydrocarbon (methane, ethane, ethylene and propane) content of each sample was determined by a gas chromatographic method. A 4.5 ml sample was injected into the helium carrier (115 ml/min flow rate) which was diverted after the injection point by a splitter (1:2.8 split) to two column systems operated in parallel. Carrier from one splitter exit flowed (at 30 ml/min) through a 145 cm long × 3 mm i.d. stainless steel column, filled with 60–80 mesh Porasil B (Pechniney-Saint-Gobain, France) and operated at 25°C, for separation of methane, ethane, ethylene and propane[8,9] and subsequent detection of these gases by a Pye-Unicam flame ionisation detector. The parallel system (85 ml/min flow from the second splitter outlet) consisted of two columns and two katharometer detectors connected in series for the separation and detection of ($Ar+O_2$), N_2, N_2O and CO_2; this system was modified from that previously described[10] by use of a separate katharometer for each column, and the omission of the third column for an Ar–O_2 separation. A standard katharometer fitted to the Pye-Unicam Model 104 gas chromatograph was used for detection of N_2O and CO_2; ($Ar+O_2$) and N_2 were sensed with a Taylor Servomex detector (MK 158) and controller (g.c. 197). Power inputs to the katharometers were 300 mA (Pye-Unicam) and 9 V (Taylor Servomex). All output signals were recorded on standard potentiometric recorders having a 10 mV range (f.s.d.) for hydrocarbon analyses and a 1 mV range for the remaining gases.

Calibration curves relating peak height or peak area with amount (μl at s.t.p.) of each component were constructed by recording responses of the g.c. system to injections of mixtures of N_2O, CO_2 and the hydrocarbons in air. Peak areas, obtained by means of a disc integrator, were used only for the ($Ar+O_2$) and N_2 peaks. The volume of each component in a gas sample was obtained from the calibration curves, and the percentage composition was calculated by standard summation technique. Oxygen contents were calculated by deducting the estimated Ar concentration from the ($Ar+O_2$) concentration; Ar concentration was estimated as % N_2/83.3. The maximum error in oxygen concentration involved for likely deviations of N_2/Ar ratios from 83.3 was small (< 0.2%).

2.3. Soil and meteorological data

Meteorological data and soil analytical data (including soil moisture and nitrate contents) are reported separately.[3]

3. Results

3.1. Temporal distributions

3.1.1. Oxygen

Figure 1 shows that the slurry markedly decreased the oxygen content of the gaseous phase in the soil at all profile depths examined during the 12 months following application.

In the slurry layer, the oxygen concentration was as low as 8% (v/v) during the two weeks after the application. After suddenly increasing in late April, it was always greater than 19%.

The oxygen concentration at the 10 cm depth in the soil underneath the slurry decreased to less

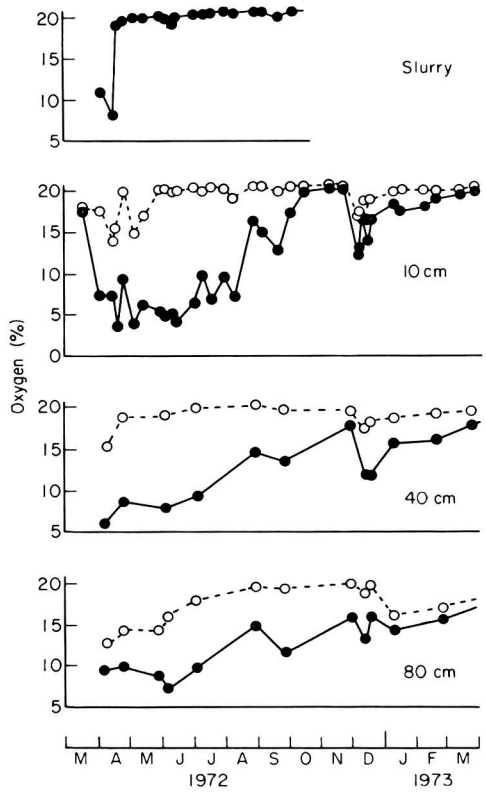

Figure 1. Oxygen content of the gaseous phase under slurry-treated (●——●) and untreated (○- - -○) grassland.

than 10% within a few days of the application, and remained below 10% for the following 4 months (until mid-August). The minimum mean concentration detected was 3.5%. With drying of the soil during the summer, the concentration generally increased to a value of 19.0% immediately prior to the cultivation in October.

In contrast to the extreme values recorded under the slurry-treated area, the oxygen concentration at 10 cm under the control plot was always greater than 13%; after drying of the surface soil in May, it was greater than 19.0% until October. After cultivation, differences between the concentrations under the treated and untreated plots were much smaller than previously, and under the slurry plot the concentration did not drop below 13% during the only major wet period (in December) of the dry 1972/73 winter.

Changes in concentrations with time were generally slower below 10 cm than in the surface layer. At 40 cm, the concentration under both the treated and the untreated plot increased gradually until the profile was rewetted to field capacity 8 months later. The marked differential of 9–11% O_2 (v/v) between the two treatments in the 2 months after the application gradually decreased to about 2–3% O_2 (v/v) during the following autumn and winter. At 80 cm under the slurry, the minimum concentration was not observed until more than 2 months after the application but, from this time onwards, it generally increased and the differential between concentrations under slurry-treated and control plots generally decreased.

3.1.2. Carbon dioxide

Carbon dioxide concentrations (Figure 2) were always higher at a given depth under the slurry-treated area than at the corresponding depth under the control area.

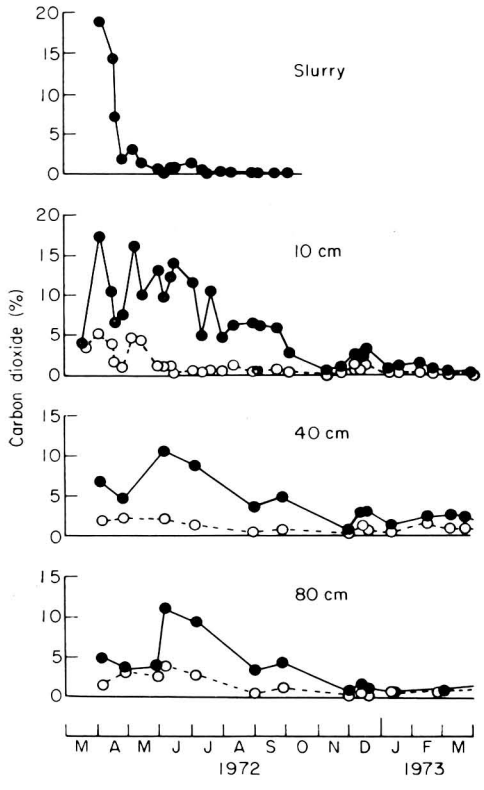

Figure 2. Carbon dioxide content of the gaseous phase under slurry-treated (●——●) and untreated (○- - -○) grassland.

The concentration in the slurry was very high (19%) shortly after the application, but decreased rapidly during April. It was always less than 2% from May onwards.

At 10 cm under the slurry-treated plot, the concentration varied between 4.5% (v/v) and 17% during the first 6 months after the application, but generally decreased with time elapsed since the application; it was usually greater than 10% for the first 3 months (April–June), and usually greater than 6% for the next 3 months (July–September). Under the control plot, the concentration decreased with drying of soil in early summer and was always less than 1% over the period June to October. After cultivation in October, the concentration at 10 cm under each plot was low, and increased to only 4% under the slurry plot during the wet period in December.

At lower depths under the control plot, carbon dioxide concentrations were almost constant at about 2% at 40 cm and 4% at 80 cm during the April–June period, then gradually declined until December. The concentrations under the slurry plot at 40 and 80 cm were highest (>10%) in June, and thereafter declined until they were lowest (<1%) in late autumn. Only small increases occurred during the winter. Differences between subsoil concentrations under treated and untreated areas were greatest in June (>7%) and generally decreased after that time: they were usually less than 2% at 40 cm and barely perceptible at 80 cm in the winter.

3.1.3. Methane and other hydrocarbon gases

High concentrations of methane were found both in the slurry and the soil (Figure 3). In the slurry, the concentration was as high as 6% in the 2-week period following the application, but significant evolution continued only for a further 4 weeks. In the underlying soil, methane was detected continuously for the first 6 months, although the highest concentrations (>0.5%) were found only

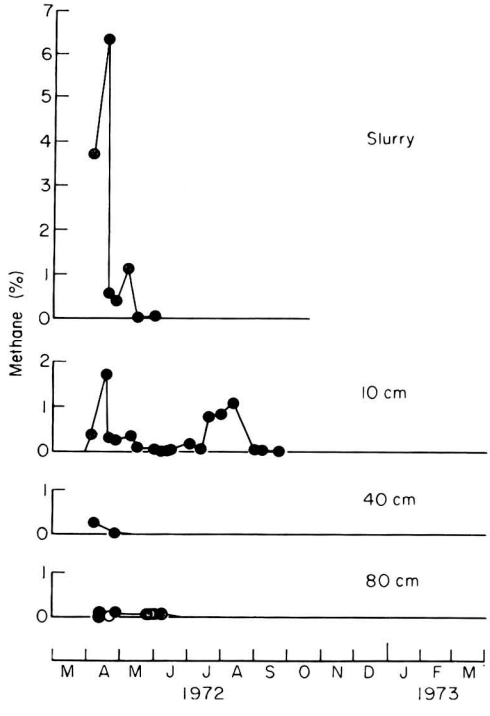

Figure 3. Methane content of the gaseous phase under slurry-treated (●——●) and untreated (○- - -○) grassland.

in April, and again in July–August. The concentration at 40 cm and 80 cm was always less than that at 10 cm and decreased with time from the highest level found (*ca* 0.3%) shortly after the slurry application. Significant methane evolution could not be detected under the control plot, although traces (up to 400 parts/10^6) were found in one reservoir (at 80 cm) in April and May.

Ethane and propane were detected continuously in the surface soil and slurry for several months after the application (Figures 4 and 5); highest levels occurred in the slurry shortly after spreading, but the concentrations found (up to 7 parts/10^6) were much smaller than the highest methane concentrations (62 000 parts/10^6). The concentrations in the slurry declined rapidly after the first 2 weeks, and only traces were detected from mid-May onwards.

In the soil at 10 cm under the slurry, the concentrations generally declined from shortly after the application until the end of July. Some ephemeral evolution probably occurred during the summer period, but this was not firmly established owing to large contamination at this time (although not at other times) of samples by mains gas leakages in the laboratory. Evolution in the following winter (after cultivation) was confined to a brief period in December.

Ethylene concentrations, and the pattern of evolution, were very similar to those for ethane and propane; the highest ethylene concentrations recorded were 2.8 parts/10^6 in the slurry and 1.8 parts/10^6 in the soil (at 10 cm).

Traces of ethane, ethylene and propane were detected at 10 cm under the control plot, but the concentrations found were always much smaller than those under the slurry treatment. The similar low concentrations of these hydrocarbons under both the slurry-treated and the control areas at 40 cm and 80 cm were obviously not related to the slurry application.

3.1.4. Nitrous oxide

Nitrous oxide was detected throughout the profile of the slurry-treated plot on many occasions, but detectable occurrences under the control plot were limited to one small peak at 10 cm (in April) and longer periods at 80 cm in May–June and December–January (Figure 6).

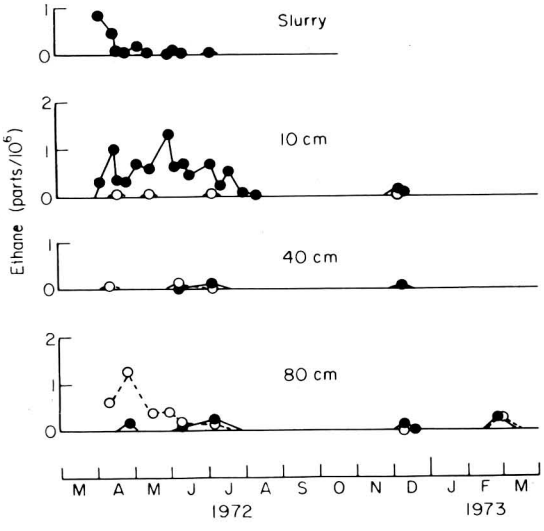

Figure 4. Ethane content of the gaseous phase under slurry-treated (●——●) and untreated (○- - -○) grassland.

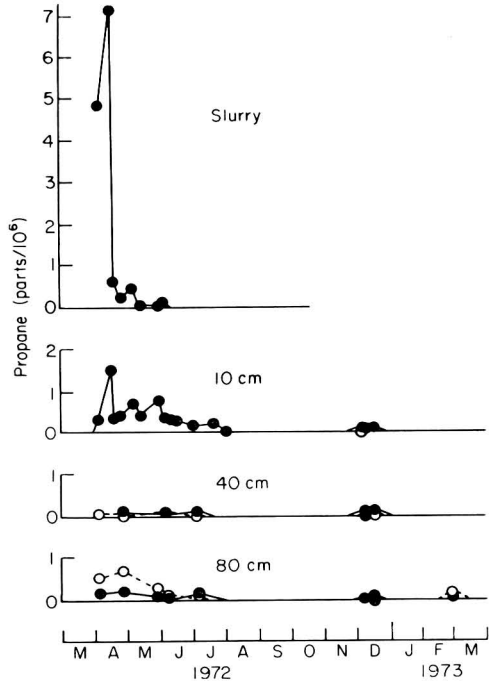

Figure 5. Propane content of the gaseous phase under slurry-treated (●——●) and untreated (○- - -○) grassland.

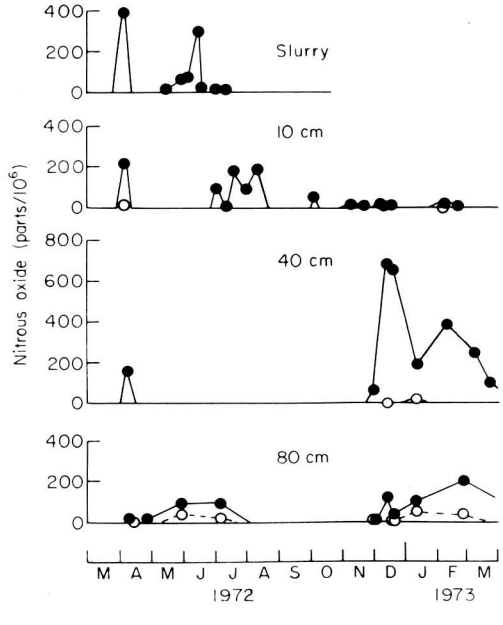

Figure 6. Nitrous oxide content of the gaseous phase under slurry-treated (●——●) and untreated (○- - -○) grassland.

Nitrous oxide was found throughout the soil profile of the slurry-treated plot immediately after the application. The brief occurrence at depths of 40 cm and shallower indicated a very short period of production.

Evolution recommenced in the slurry layer in late May; this continued into early July. Spasmodic significant evolution occurred at 10 cm in the soil under the slurry after falls of rain in June and July; minor peaks were subsequently recorded in summer, autumn and winter. After the return of the soil profile to field capacity and leaching of nitrate from the soil surface deeper into the profile in autumn and winter, nitrous oxide was found continuously at 40 and 80 cm.

3.2. Profile distributions

The data from the temporal distributions indicated that highest concentrations of products known (or suspected) to be evolved as a result of anaerobic respiration occurred close to the soil–slurry interface. Distribution of the gases in the profiles of the treated and untreated plots was therefore determined on 26 April 1972.

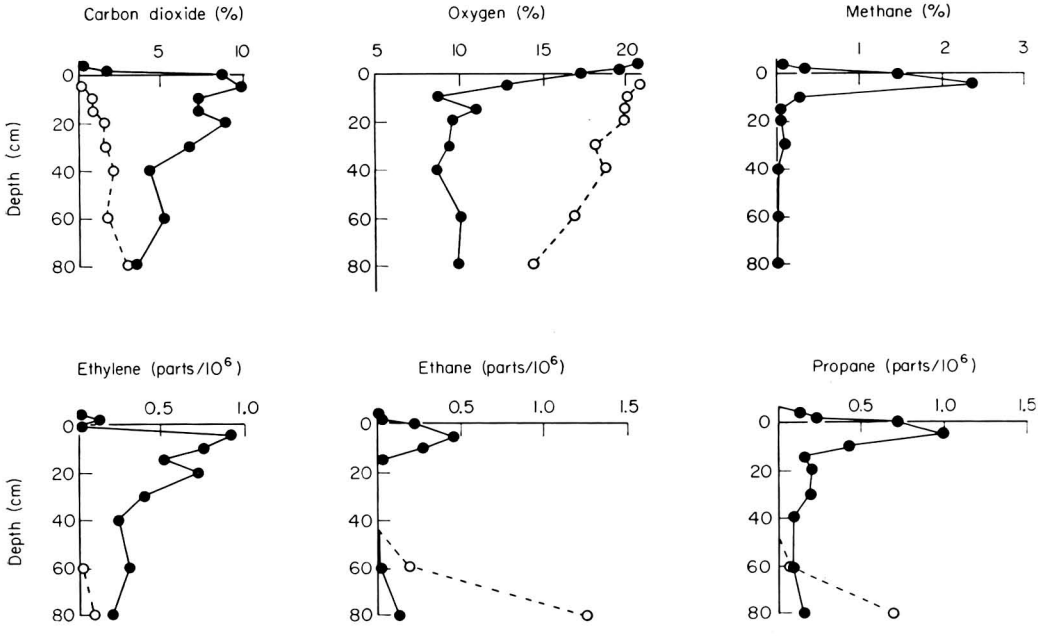

Figure 7. Profile distributions of carbon dioxide, oxygen, methane, ethylene, ethane and propane under slurry-treated (●——●) and untreated (○- - -○) grassland on 26 April 1972.

The results (Figure 7) show that under the treated area, the highest concentrations of methane, ethane, ethylene and propane occurred at about 5 cm below the soil–slurry interface. Concentration gradients were very steep between this indicated major source (at about 5 cm) and the atmosphere above; concentrations of methane and ethane diminished rapidly with depth below 5 cm, but ethylene and propane were detected throughout the soil profile.

Oxygen and carbon dioxide concentrations under the control plot show the normally expected concentration gradients,[11] from values most divergent from normal air composition at depth in the soil, to values less divergent as the soil surface is approached.

Under the treated area, the oxygen distribution shows a very steep gradient from *ca* 10% at 10 cm to less than 1% in the slurry, and possibly a slight gradient with increasing soil depth. The carbon dioxide distribution indicates diffusion of carbon dioxide away from the maximum concentration of *ca* 10% at 5–10 cm towards both the terrestrial atmosphere and the subsoil.

Comparison of profile and temporal distribution data indicates that the soil under the treated plot at about 10 cm contained, on average, minimum O_2 and maximum CO_2 concentrations in the profile for several months after the slurry application. A similar effect may also have occurred under the control plot shortly after rainfall when CO_2 is maximal and O_2 minimal at 10 cm, but there was not sufficient sampling at depth in the soil to establish this satisfactorily.

Standard errors for oxygen (1.5% under the control plot and 3.6% under the treated plot) and carbon dioxide (1.0% and 3.4%) concentrations in the soil profiles indicate a satisfactory level of precision. The hydrocarbons showed the high point-to-point heterogeneity commonly observed with products of anaerobic activity in agricultural soils,[7,12] but use of the Mann-Whitney *U*-test[13] established that the hydrocarbon gases evolved in the surface layers were due to the slurry application and that there were no significant differences between the concentrations detected at 80 cm under the two plots.

4. Discussion

Previous work has indicated that the application of animal excreta to soil stimulated anaerobic activity in the soil;[14,15] the current study shows the changes occurring in the soil profile following a single heavy application of excreta as a discrete layer on the soil surface, and free of complications due to poaching[14] or repeated applications. The effects were detected for 12 months, but the creation of a zone of high anaerobic activity close to the soil–slurry interface is of particular significance.

4.1. Anaerobic microsites

The production of nitrous oxide and methane by biological reduction requires anaerobic conditions and low redox potentials.[16–18] Detection of significant concentrations of these gases in the soil and slurry, which never became wholly anaerobic (as mean oxygen contents never fell below 3.5%), shows that production occurred at anaerobic volumes within the slurry or soil matrix. Carbon dioxide may be evolved in addition to methane under anaerobic conditions.[19,20] The methane concentrations were as high as $\frac{1}{6}$ to $\frac{1}{3}$ of the carbon dioxide concentrations. Therefore, a significant proportion of the slurry and surface soil must have contained intense reducing activity in the first 2 months after the application, and a larger volume would have been in a less reduced but still anaerobic state.

The formation of anaerobic microsites in soil has been discussed in earlier work,[21–23] and a recent estimate[24] indicates that only a minor development of anaerobic microsites is likely within the moderately well-aerated matrix of agricultural soils (unamended with organic materials). The application of slurry to grassland has resulted in the contrasting situation where anaerobic volumes of soil may predominate for a period.

4.2. Aeration, substrates and anaerobic activity

The high water content of the slurry restricted aeration in the soil, but the slurry layer did not provide a complete seal above the soil for a long period; oxygen contents in the slurry were low only for the first 3 weeks (Figure 1). Water was lost from the slurry by drainage into the underlying soil and by evaporation, which was promoted (and aeration improved) by the extensive network of tunnels created in the slurry in April by the burrowing larvae of a dung-fly, *Scatophaga* spp. (identified by B. Pain, NIRD). Although the improved aeration resulted in high oxygen contents (> 18%) thereafter, it did not inhibit the anaerobic activity at microsites that resulted in the formation of methane in April–May and nitrous oxide in May–July.

Aeration in the soil was restricted also by the increased water content due to the mulching effect of the slurry,[3] and additionally by the blocking of pores, by particulate organic matter which would have also provided substrates for microbial respiration.[25,26] Extractions made 6 weeks after spreading (Burford, unpublished data) showed that significant concentrations of water-soluble organic matter were found to a depth of 10 cm below the soil surface, but none could be detected below 20 cm; it is presumed that the movement of particulate matter was confined to a much shallower depth. The location of the zone of intense reducing activity close to the soil surface may be attributed to the combination of the concentration of organic substrates at this depth, and to the physical impedance to gas movement caused by the high soil moisture content and the blocking of pores by particulate organic matter.

The improved aeration resulting from drying of the soil over summer and the removal of organic material blocking pores, and the decreasing rate of respiration as the more readily decomposable substrates were consumed, would have all contributed to the diminution of anaerobic activity with time. A further improvement in aeration resulted from the cultivation preceding the sowing of the new pasture in October; the oxygen and carbon dioxide contents of the slurry-treated plot did not reach the extreme concentrations observed earlier. Russell and Appleyard[27] earlier suggested that superior aeration on cultivated dung-treated areas partly overcame the effect on soil atmosphere composition of higher soil respiration due to the dung.

4.3. Carbon mineralisation

Carbon dioxide was always the major gaseous-C product, although methane accounted for a significant proportion of the carbonaceous gases evolved while aeration was restricted.

The relative concentrations of the hydrocarbon gases are in agreement with those observed in incubation studies using soil[28] or faecal[29] substrates; these studies showed that only small concentrations of ethane, ethylene, and propane accumulate even under favourable conditions (of added organic substrates and restricted aeration), and that methane may accumulate to much higher concentrations than the other hydrocarbons. Even where specific stimulatory substrates for ethylene production were added to a soil with known ethylene producing ability,[30] ethylene accumulated to only 3 parts/10^6 in the gas phase above the culture.

The distributions of the hydrocarbon gases in the profile (Figure 7), particularly below the 5 cm soil depth, indicate differences in evolution and transport of the gases in the profile, but utilisation[31] by microbial populations may also be involved.

4.4. Nitrate-N accumulation and gaseous-N losses

The brief initial occurrence of nitrous oxide in the slurry and in the soil at 10 and 40 cm in April (Figure 6) indicated rapid denitrification of the nitrate-N initially present in the slurry (49 parts/10^6) and the surface soil (5 parts/10^6); lack of continued evolution in April and May was probably due to a lack of nitrate substrate which did not accumulate in the surface soil for several months after the application, probably as a result of inhibitory substances in the slurry[32] or restricted aeration limiting nitrification. The accumulation of nitrate-N firstly in the slurry layer and subsequently in an increasing depth of the surface soil[3] is attributed to a corresponding increase in the depth of well-aerated soil with time.

Nitrous oxide evolution in the slurry in May–June, and surface soil in July–August, was associated with high moisture contents following rainfall[3] and may have resulted from denitrification by one of three possible routes: (a) simultaneous denitrification and nitrification, via mineralisation of nitrate at an aerobic microsite and diffusion of nitrate in the aqueous phase to an anaerobic microsite,[33,34] (b) change in the aeration status of a site by an increase in water content (following rainfall) creating an anaerobic microsite from an aerobic one; or (c) leaching of nitrate from an aerobic surface layer to an anaerobic microsite in a less well-aerated subsurface layer. The relative importance of these routes is not known but the zone rich in anaerobic microsites below the soil surface obviously provided a mechanism for reducing the accumulation of nitrate in the subsoil by leaching from the surface zone of accumulation.

The soil was moistened to field capacity in November and December, and water movement down

the profile leached most of the nitrate into the subsoil. The continuous detection of nitrous oxide at 40 cm for several months afterwards is in agreement with previous work[35,36] showing that subsoil production of nitrous oxide may be more continuous than the usual ephemeral production in the surface soil; this was attributed to less rapid changes in moisture content (and therefore aeration) with depth, but nitrate concentration may also have a significant effect on evolution.[35]

Rates of N_2O-N losses are not easily assessed from concentrations of N_2O in the soil atmosphere,[7] and preliminary studies have indicated difficulties in measuring the air-filled porosity of the soil at this site. Gaseous losses may have been greatest from the more porous layers close to the soil surface (particularly the slurry) than from the deeper soil layers, where nitrous oxide may have accumulated to high concentrations because diffusion to the surface was retarded by low volume of air-filled pore spaces. Further studies are necessary to determine the magnitudes of the N losses as N_2O, and also the concurrent losses as molecular N_2.

4.5. Plant growth

The death of almost all the pasture plants on the treated plot following the application may be attributed to the exclusion of light by the 5 cm depth of slurry. Conditions under the slurry continued to be unfavourable for plant growth for 4 months after the application: the detection of methane showed that there were always some microsites capable of sufficiently intense reduction for the production of compounds toxic to plants, e.g. butyric acid, sulphides,[25] H_2S[37] and divalent manganese.[38] Ethylene was continuously present in the surface layers for several months, but it is not yet possible to predict the effects on plant growth since both inhibitory[39,40] and stimulatory effects[41,42] have been reported.

The slurry application was typical of dressings applied to sacrifice areas not required immediately for pasture production. The rate applied provided N, P and K greatly exceeding seasonal pasture requirements; it was over 5-fold greater than the optimum dressing for maximum pasture growth in the year of application under the current experimental conditions.[43] Further work is necessary to define the interaction between beneficial effects (e.g. nutrient supplies) of slurry on plant growth and the effects that are deleterious.

5. Conclusions

Although the organic materials in the slurry promoted anaerobic activity in the soil, they did not at any time result in a completely anaerobic layer or horizon in the drained, normally well-aerated soil at the experimental site. Cultivation and establishment of pasture was possible within a few months of the application; disposal of slurry by heavy applications on this soil series is therefore possible provided that pollution of groundwaters can be kept within acceptable limits. However, more severe effects on soil properties may occur with lighter applications to poorly drained, heavier textured soils. If a soil aeration problem is likely to result from an application of unamended slurry, it may be desirable to treat the slurry prior to application e.g. by prior aeration and digestion, flocculation,[44] or composting with straw.[45]

Models have been developed for predicting the rates of application on to agricultural land.[46] Maximum rates are calculated by estimating the maximum nutrient and moisture loadings that can be applied without polluting streams or groundwaters with organic materials or nutrients (particularly nitrates). Consideration of a further possible constraint on disposal rates, that of the effects of organic loadings on plant growth and nutrient transformations, is necessary.

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A Simplified Procedure for Fractionating Plant Materials

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Sequential extraction of plant materials with methanol–chloroform–water and phenol–acetic acid–water mixtures gave good yields of water-soluble low-molecular-mass substances, lipids, proteins and polysaccharides in separate fractions unlaboriously and with little chemical damage. Results with potato tubers (*Solanum tuberosum* L.), leafy shoots of lucerne (*Medicago sativa* L.) and an oilseed (*Pentaclethra macrophylla* Benth.) are presented.

1. Introduction

Since phenol–acetic acid–water mixtures were introduced, several years ago, for selective extraction of bulk protein from plant materials,[1–3] they have often been used for such isolations;[4–10] failures may be attributed either to working with excessively dried materials[2,11] (cf. reference 12) or to insufficient disruption of cell walls.[13] The extracted residues have proved useful for studies of their polysaccharides[14–19] and of the associated hydroxyproline-rich "cell-wall proteins".[20–23] For a general review on extraction of proteins from leaves, see reference 24.

In the procedures originally described,[1–3] low-molecular mass water-soluble substances were initially eliminated either by plasmolysis according to Chibnall,[25] which in effect uses the cell-walls as dialysis sacs, or by extraction with aqueous trichloroacetic acid. Lipid materials were then eliminated either by extraction with ethanol, acetone, etc., or by electrophoresis of the protein away from the non-migrating lipids.

In recent years we have formed a good impression (cf. reference 26) of the procedure of Bligh and Dyer,[27] which uses monophasic methanol–chloroform–water (MCW) for simultaneous extraction of low-molecular-mass water-soluble constituents and of lipids. This seems first to have been used on plant materials by Bieleski and colleagues.[28,29] We find that, after such an extraction, good yields of protein can be obtained by further extraction of the residue with phenol–acetic acid–water (PAW). In the present paper we describe application of the new procedure to potato tubers (*Solanum tuberosum* L.), leafy shoots of lucerne (*Medicago sativa* L.) and to endosperm of a West African oilseed (*Pentaclethra macrophylla* Benth.). A somewhat similar sequential extraction has been recommended by Sutherland and Wilkinson[30] for use with microorganisms.

2. Experimental

2.1. Materials and sampling

2.1.1. Potato

This was cv. Orion.[31,32]

2.1.2. Lucerne

This was grown in open ground near Watton, Norfolk. The subaereal parts of the plants were cut off from the roots and brought to the laboratory in a polyethylene bag. Working up took place within 1 h of harvesting. Young leaves and tips were used.

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2.1.3. Pentaclethra

The plants were growing on farms in Nigeria. After dehiscence of the ripe pods, the ejected seeds were collected as normally, and air-freighted in paper cartons to England. They arrived at this Institute approximately 3 weeks after collection and were immediately stored at −40°C until worked up. Only the endosperms were used.[34]

2.2. Trituration

2.2.1. Potato

Tubers (100 g fresh wt) were quickly chopped into small pieces using an "Autochop" (Zyliss, from William Levene Ltd), dropped into liquid nitrogen in a 500 ml polypropylene centrifuge pot and triturated for 1–2 min with an Ultra-Turrax colloid mill (Janke and Kunkel Kg., Staufen i. Br., West Germany).

2.2.2. Lucerne

Leaves and tips (100 g fresh wt) were quickly weighed out in 20 g lots, dropped into liquid nitrogen and triturated as above.

2.2.3. Pentaclethra

Endosperms (100 g fresh wt) were weighed out, cut into small pieces, dropped into liquid nitrogen in a mortar and ground to a fine powder, which was transferred to a 500 ml centrifuge pot.

2.3. Extraction

The powders were slurried in methanol–chloroform (2:1 by vol., approx. 300 ml). The actual volume used depended on the water content of the plant material, as an overall MCW mixture of 2:1:0.8 (by vol.) was aimed at.[27] Oxygen-free nitrogen (OFN) was bubbled through the slurry until it came to nearly room temperature. The pot was then capped and centrifuged at 1150 rev/min for 15 min in a MSE Major centrifuge in a +1°C room. The supernatant was decanted off (filtered, if necessary, to remove floating material, which was returned to the pot) and the residue slurried in 250 ml MCW (2:1:0.8 by vol.), flushed with OFN, capped and centrifuged. This procedure was repeated using a further two portions of MCW (150 ml). The residue was extracted in the same manner with 150 ml PAW (1:1:1, w/v/v) to remove the MCW. It was then slurried in another 150 ml PAW, flushed with OFN, capped and stood at +1°C overnight to allow the protein time to swell and dissolve before removing the supernatant and extracting with two further portions of PAW (100 ml). This residue was thoroughly washed with ethanol to remove the solvents and air-dried. All four PAW extracts were bulked.

The MCW extracts were pooled, their volume measured and sufficient chloroform and water added to give a methanol:chloroform:water ratio of 2:2:1.8 (by vol.). The lot was shaken up in a separating funnel, flushed with OFN and stood at +1°C. When clear, aqueous and chloroform phases were run off separately and stored under OFN. The bulked PAW extracts and the alcohol wash were also stored under nitrogen at +1°C.

2.4. Evaporations

These were done *in vacuo* below 40°C. Phenol-containing samples were evaporated several times with repeated additions of water.

2.5. Dry-matter determination

Dry matter was determined after storing evaporated fractions overnight in an evacuated desiccator over H_2SO_4 and NaOH. Dry matters of original plant materials were calculated by aggregating the dry matters of the derived fractions.

2.6. Nitrogen determination

This was by a micro-Kjeldahl procedure.[2]

3. Results

These are summarised in Table 1.

Table 1. Dry-matter (DM) and nitrogen balances on fractionated materials (100 g fresh wt)

Fraction	DM (g)	N (mg)	N (% of dry matter of fraction)	DM of fraction (as % of total DM)	N of fraction (as % of total N)
Potato					
Aqueous phase	2.78	213.7	7.7	12.0	48.5
Chloroform phase	0.19	3.2	1.7	0.8	0.7
PAW extract	2.08	190.8	9.2	8.9	43.3
Ethanol wash	0.01	0.4	5.2	0.03	0.1
Residue	18.16	32.7	0.2	78.2	7.4
Totals	23.21	440.8	1.9	(100)	(100)
Lucerne					
Aqueous phase	4.41	75.0	1.7	20.4	6.3
Chloroform phase	2.15	35.6	1.7	9.9	3.0
PAW extract	7.70	762.9	9.9	35.6	64.6
Ethanol wash	0.33	15.4	4.7	1.5	1.3
Residue	7.03	292.5	4.2	32.5	24.8
Totals	21.62	1181.4	5.5	(100)	(100)
Pentaclethra					
Aqueous phase	10.50	710.0	6.8	15.4	21.5
Chloroform phase	19.46	125.0	0.6	28.5	3.8
PAW extract	18.77	1877.0	10.0	27.5	56.8
Ethanol wash	1.39	53.0	3.8	2.0	1.6
Residue	18.07	542.0	3.0	26.5	16.4
Totals	68.19	3307.0	4.9	(100)	(100)

4. Discussion

The "bulk-protein" fractions (PAW extracts) from these diverse materials all contained N, 9–10% of dry matter of fractions (Table 1). Those from potato have been studied further by Davies and Laird,[31,32] those from lucerne by Van Sumere and colleagues[33] and those from *Pentaclethra* by Mbadiwe.[34] Their ultraviolet spectra, measured in alkali after evaporation to dryness (2.4), were suggestive of contamination by nucleic acids etc. and by aromatic materials. Dialysis in cellophan of the "bulk protein", still dissolved in PAW, against 70% (by vol.) aqueous acetic acid has proved useful for further purification.[35,36] Little N was lost to the diffusate and resulting preparations had N, 13–15% of dry matter. They should prove useful for nutritional evaluations of the protein without major interference by other constituents of the plant. With lucerne, the diffusate was rich in materials absorbing in the range 260–350 nm; with potato, the diffusate had little ultraviolet absorption. "Bulk proteins" may be further freed, at least in part, from nucleic acids by partition between phenolic and aqueous phases[3,30,37] and from other contaminants, especially neutral or acidic ones, by electrophoresis in phenol-rich media.[3,38] Resulting preparations should prove suitable for detailed studies of the extent to which minor components such as carbohydrates, amino sugars, lipids,[39] aromatic acids[33,40] etc. occur covalently linked to proteins.

The sequential-extraction procedure here outlined has the further advantage of recovering most of the lipids and of the water-soluble low-molecular-mass substances (including most of the non-protein N) in the chloroform and aqueous phases respectively. The final residues, mainly polysaccharide in nature, should prove just as useful for chemical and nutritional studies as those obtained by equally mild, but more direct procedures.

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Determination of S in Extracted Leaf Protein

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The oxygen-combustion technique for converting organic S to sulphur oxides, absorbing these in hydrogen peroxide, and determining the sulphate formed by measuring the amount of chloranilic acid it releases from an excess of barium chloranilate, was found to give satisfactory recoveries of S from methionine and cystine. This method for determining S was then applied to extracted leaf protein concentrates, almost all of whose S is derived from methionine and cyst(e)ine.

1. Introduction

Arising from the difficulties of determining the cyst(e)ine content of extracted leaf protein[1–3] it was decided to approach the problem obliquely by first determining the total S content of the preparations.

There are many methods, both chemical and physical, for measuring S in organic compounds, but the type and amount of sample, as well as equipment available, limit the choice. Most chemical methods involve two stages: oxidation of the sample and measurement of the sulphate formed. That developed by Schöniger[1,5] for converting the S in organic compounds to sulphate by combusting the sample in oxygen and absorbing the resultant sulphur oxides in hydrogen peroxide has been used with many sorts of samples, including plant material[6–8] and foodstuffs.[9–13] Its reported advantages over the more widely used wet oxidation methods are that methionine is fully recoverable from plant material and from compounds containing much methionine;[6,14] further, any volatile S-containing compounds are not lost.[6,7] It seemed suitable for these leaf protein fractions because (a) the S content of whole dried foliage has been successfully determined using this technique;[6–8] (b) about half the S present in extracted leaf protein is there as methionine;[1,2] and (c) only small amounts are needed for combustion. Sulphate was determined by modifying the colorimetric method of Bertolacini and Barney.[15] This was chosen in preference to the more sensitive reduction methods because a simple technique was required to meet the needs of workers in some overseas laboratories where no specialised equipment existed.

2. Experimental

2.1. Materials

Cystine and methionine (BDH Chemicals Ltd) had N contents of 11.21 and 9.18% (*ex* bottle) respectively: their S contents were calculated relative to the found N.

Lucerne leaf proteins: their preparation is described elsewhere:[3] all were well washed with distilled and deionised water before freeze-drying. Subsamples of one of these preparations, a chloroplast-containing fraction made by heating the extract to 55°C, were extracted with various solvents to remove lipid wholly or partially.

Commercially supplied barium chloranilate is seldom free from chloranilic acid (from which it is made) and needs to be well washed with water: the acid-free salt is dried by washing with ethanol and diethyl ether, in that order. That supplied by Hopkin and Williams required minimal washing. Other reagents were of AnalaR grade.

Cigarette paper (Rizla, green pack) trimmed of the gummed edge, was used as the sample carrier: allowance was made for its S content (26.7 μg/100 mg) when calculating results.

2.2. Combustion apparatus

That described by Lysyj and Zarembo[16] was used, but a spiral coil made from 130 mm rhodium-platinum (20:80) wire, diameter 0.5 mm, was substituted for platinum (grade 4) wire after persistent melting of the latter during combustion. The alloy (m.p. 1900°C) was more resistant to melting.

2.3. Combustion procedure

The sample, in its paper carrier, is inserted into the wire coil, ignited and plunged into the 500 ml oxygen-filled conical flask. The assembly is left in place for 1 h and shaken occasionally to assist absorption of the sulphur oxides by 15 ml 1% (v/v) hydrogen peroxide.[14] From 40 to 100 mg leaf protein, amount depending on protein content, but estimated to contain from 300 to 700 μg S was taken for combustion; weights much in excess of 100 mg failed to combust completely.

2.4. Removal of cations from combustion solution

The contents of the combustion flask (followed by two water washes) were passed through a 50 × 12 mm column of Dowex 50 H^+ resin to remove cations, many of which form insoluble chloranilates[15] and would, if left, interfere with the subsequent determination of the sulphate. All eluates were boiled to remove excess hydrogen peroxide:[6, 16] those from leaf protein samples were concentrated to 1–2 ml and those from cystine or methionine made up to a known volume with distilled water. (NB: Eluates must not be allowed to go to dryness as this affects the recovery of S.[13])

2.5. Determination of sulphate

The concentrated eluate from leaf protein samples, or a known volume of cystine or methionine eluate, not to exceed 5 ml in either case, was transferred into a 10 ml graduated glass-stoppered tube. Alcoholic acetate buffer (5 ml) pH 5.1,[10] was added to each tube and the contents well mixed before making up to 10 ml with water. Approximately 40 mg barium chloranilate was added to each tube and the contents shaken for 10 min. The precipitated barium sulphate and the excess chloranilate were removed by centrifuging (1000 × g for 10 min) and the choranilic acid released measured spectrophotometrically at 530 nm. The total S content of each tube was calculated from a standard curve prepared in the same way using a solution of potassium sulphate (the relationship between chloranilic acid released and concentration of sulphate ion is linear up to 300 μg SO_4/ml or 100 μg S/ml) and the result converted to S as percentage of dry matter for each sample. Tubes were read against a blank prepared from distilled water and the reagents.

2.6. Nitrogen determinations

Total N was determined by a micro-Kjeldahl procedure using a copper–selenium catalyst (9 K_2SO_4: 1 $CuSO_4$:0.02 SeO_2 by wt).

3. Results

The S:N ratio calculated from the found N and S contents of cystine and methionine agreed with the theoretical figures (Table 1) indicating a good recovery of S from these compounds using the method outlined. The amount of S found, and the S:N ratio in some lucerne leaf proteins are given in Table 2. Although the S content rose with increasing protein content the S:N ratio remained in the range 0.064–0.072, averaging 0.067 for all 28 samples examined. Removal of the lipid by various solvents from a chloroplast-containing preparation with a large lipid content (approx. 35% of dry matter) also led to a rise in N content, but again without altering the S:N ratio in the protein concentrate (Table 3).

Table 1. S and N contents of methionine and cystine: and a comparison of their theoretical and found S:N ratios

		%S	%N[a]	S:N
Methionine	Theoretical	21.45	9.38	2.287
	Ex-bottle	21.45±0.541 (average of 20 determinations)	9.18	2.337
Cystine	Theoretical	26.63	11.65	2.286
	Ex-bottle	25.70±0.110 (average of 10 determinations)	11.21	2.293

[a] %N average of 3 determinations.

Table 2. S and N contents of, and S:N ratio in, some lucerne leaf proteins

Lucerne leaf protein	S[a] (% of DM)	N[a] (% of DM)	S:N
Unfractionated			
Precipitated: 80°C	0.61	9.53	0.064
TCA[b]	0.61	9.09	0.067
"*Chloroplastic*" *fraction*			
Centrifugate: 1000 ×*g*	0.37	5.78	0.064
Precipitated: 55°C	0.56	8.08	0.069
"*Cytoplasmic*" *fraction*			
From 1000 ×*g* supernatant:			
Precipitated: 80°C	0.88	13.53	0.065
TCA[b]	0.83	12.18	0.068
From 55°C supernatant:			
Precipitated: 80°C	0.89	13.43	0.065
TCA[b]	0.86	13.38	0.064

[a] All S and N results are the average of three determinations each.
[b] Trichloroacetic acid.

Table 3. Effect of removing lipid from a chloroplast-containing lucerne leaf protein fraction on the S and N content (as percentage of DM) and on the S:N ratio

Solvent treatment	%S[a]	%N[a]	S:N
Nil	0.56	8.09	0.069
2:1 (v/v) Chloroform:methanol	0.73	11.15	0.066
3:1 (v/v) Ethanol:diethyl ether	0.69	10.68	0.065
Propan-2-ol	0.73	11.08	0.066

[a] All S and N results are the average of three determinations each.

4. Discussion

As a technique the oxygen-combustion method for converting the S in extracted leaf protein to sulphate is quick and accurate. It has the added advantage that, for homogeneous samples, only small quantities are needed, which is useful when material is limited. Further, the method requires no elaborate apparatus or special skills beyond a certain deftness. With care, reproducible results are obtained when the sulphate is determined by measuring the amount of chloranilic acid it releases from an excess of barium chloranilate. Although many sulphate determinations can be done simultaneously this part of the procedure is cumbersome because interfering cations must first be removed.[15] Also because of the small amount of S in extracted leaf protein (<1% of dry matter) the whole of the reaction solution from the combustion has to be used for the final step. Combining

the oxygen-combustion technique with a more sensitive method for determining sulphate, for example, by reducing it to hydrogen sulphide,[17,18] would be advantageous,[6] but in the absence of specialised apparatus the technique described here is useful.

The results obtained show that there is a relatively constant S:N ratio in all extracted leaf protein concentrates, irrespective of protein content. Removing lipid has no effect on this ratio suggesting that all the S (excepting any associated with the very small amount of sulpho-lipid)[19] is contained in the protein or protein-carbohydrate fraction. Since preparations containing upward of 80% protein contain little carbohydrate it is not unreasonable to suppose that almost all the S is contributed by the methionine and cyst(e)ine in the protein.

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Relationship Between Total N, Total S and the S-containing Amino Acids in Extracted Leaf Protein

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The total S:total N ratio in both unfractionated and fractionated proteins extracted from the leaves of lucerne and lupin has been determined. The results were similar to those found with 80% ethanol-insoluble residues from leaves of lucerne and some other species. Methionine-S accounted for 38–43% of the total S. If methionine-S is deducted from the total S content, and the difference expressed as cystine, it appears that all preparations contained from 2.1–2.8 (as % of protein content)—more than had been previously supposed. When compared with the FAO reference protein none of the preparations are deficient in total S-containing amino acids, although the problems associated with unavailability remain. Calculating cystine by this method is not suggested as a substitute for direct determination, where this is practicable, but may be a useful approach when cyst(e)ine is present only in small amounts in a complex protein concentrate and is liable to be destroyed during acid hydrolysis.

1. Introduction

Although the ratio of total S:total N in whole foliage has been extensively studied because of its potential use in assessing S-deficiency in a crop[1–3] there are fewer reported results of this ratio in the protein of leaves and most of these have been obtained using the alcohol-insoluble leaf residue[1,2,4–7] rather than the extracted leaf protein.[8–12] Dijkshoorn and van Wijk[2] reviewing this field comment on the variability of the S:N ratios in some older papers, but as many of these results were calculated from cystine and methionine analyses done before ion-exchange chromatography was introduced the reliability of such ratios is doubtful.

Methionine is the limiting nutritionally essential amino acid in extracted leaf proteins: when it is added to diets containing the unfractionated protein, nutritive value improves.[13–15] On the other hand, chloroplast-free preparations (with a similar methionine content)[16] have a biological value approaching that of casein, indicating that lack of availability of methionine, rather than its absolute amount, is responsible for the apparent deficiency.

When assessing the potential nutritive value of proteins the total S-containing amino acid content is more often used than the content of methionine alone. With extracted leaf protein this presents certain difficulties, for while methionine can be measured reliably, the published figures for cystine show wide variations irrespective of whether cystine was determined directly or as cysteic acid after a pre-hydrolysis performic acid oxidation.[17] This is partly due to the small amount of cyst(e)ine in relation to the other amino acids (from 1 to 2% of the total amino acid content) but also results from the destruction of cyst(e)ine during hydrolysis of samples containing non-protein material. A few total S and cystine-S determinations have been made on proteins extracted from the leaves of different species,[8–12] but the most recent analyses were on atypical preparations having a large ash and small protein content.[12] The results were given solely as ratios against the N content of the preparations.

The decision to analyse some well-washed extracted leaf protein preparations (i.e. free from inorganic sulphate) for total S was made because of uncertainty about their cyst(e)ine content. The

difference between total S and methionine-S has been calculated as cystine, and it is suggested that this provides a more reliable estimate of this amino acid in extracted leaf proteins than direct analysis.

2. Experimental

2.1. Extraction of protein

Unfractionated and fractionated proteins made from three cuts of lupin (*Lupinus albus* cv. Agricultural white) leaves of increasing age, and from three separate crops of lucerne (*Medicago sativa*) were used for this work. The preparation of the lupin extracts and the fractionation procedures have been described elsewhere.[16] Lucerne extracts were prepared using the IBP pulper[18] and press,[19] and the fractionation procedure was the same as that used with the lupin extracts. All preparations were well-washed, using distilled and deionised water, and freeze-dried.

2.2. Analyses

Nitrogen was determined by a micro-Kjeldahl procedure using a copper–selenium catalyst (9 K_2SO_4: 1 $CuSO_4$:0.02 SeO_2 by wt).

Methionine and cystine were determined directly (i.e. without performic acid oxidation) on an acid hydrolysate of the protein. The sample (5–10 mg) was mixed with constant boiling HCl (1.0 ml for each 2.0 mg sample wt), sealed *in vacuo* after degassing the HCl, and kept at 110°C for 18 h.

The hydrolysates were taken to dryness under reduced pressure in a rotary evaporator, freed from HCl, and redissolved in 0.01 N HCl containing norleucine (0.1 mM) as a standard. The amino acids were resolved on a single-column Technicon automatic analyser, using a gradient described by Burns *et al.*[20]

Total S was measured by combusting the protein (from 40–100 mg sample according to protein content) in oxygen and absorbing the sulphur oxides produced in hydrogen peroxide: the sulphate produced was determined by measuring the amount of chloranilic acid released from an excess of barium chloranilate at 530 nm.[21]

3. Results

The ratio of total N:total S in the extracted leaf proteins examined here ranged from 0.064 to 0.072 (average 0.067) for lucerne (Table 1) and from 0.058 to 0.069 (average 0.067) for lupin (Table 2), showing a similarity between different fractions made from the same species as well as between preparations made from different species.

Expressed as a percentage of total S, the methionine-S content of all preparations ranged from 38 to 43% (Tables 1 and 2) which is probably not unreasonable allowing for variability between species,[16] the possible presence of other coprecipitable S-containing compounds such as lipids, losses during protein hydrolysis, and the unavoidable magnification of errors with individual amino acids present only in small amounts. Given that the preparations are washed free from inorganic sulphate, which is essential if this determination is to work, the remaining S ought to be due solely to cyst(e)ine if no other S-containing compounds are present. Deducting methionine-S from the total S content, and expressing the difference (57–62%) as cystine, gives a range of 2.24–2.78 and 2.00–2.67 (as % of protein content) for lucerne and lupin respectively (Tables 1 and 2).

From these calculated results it would appear that extracted leaf protein contains more cyst(e)ine than the 1–2% which has usually been accepted. The progressive destruction of cyst(e)ine during acid hydrolysis as the amount of non-protein material in the preparation increases is seen in Tables 1 and 2: the only direct determination of cyst(e)ine which resembles the calculated amounts are those on hydrolysates of cytoplasmic fractions containing around 85% protein.

4. Discussion

The ratio of total S:total N in extracted lucerne and lupin leaf proteins (averaging 0.067 and 0.063 respectively) is similar to that found in unfractionated protein extracted from Sudan grass

Table 1. The total S:total N ratio in some extracted lucerne leaf proteins and a comparison of their "found" and "calculated" cystine contents

	Protein content N × 6.0 (% of DM)	Total S (% of DM)	S:N	Total S (% of protein content)	Met-S[a] (% of protein content)	Cys-S[a] (% of protein content)	Met-S (% of total S)	Cystine	
								Found[a] (% of protein content)	Calculated[b] (% of protein content)
Unfractionated (80°C ppt)									
Batch 1	54.8	0.64	0.070	1.18	0.47	0.35	39.6	1.33	2.67
Batch 3	57.2	0.61	0.064	1.06	0.43	0.40	40.2	1.49	2.39
Unfractionated (TCA ppt)									
Batch 1	54.4	0.60	0.066	1.10	0.45	0.22	40.9	0.82	2.44
Batch 3	54.5	0.61	0.067	1.12	0.43	0.37	38.3	1.39	2.59
Chloroplastic (made by centrifuging)									
Batch 1	43.1	0.49	0.068	1.13	0.47	0.32	41.3	1.18	2.49
Batch 3	34.7	0.37	0.064	1.06	0.47	0.25	41.2	0.94	2.35
Chloroplastic (55°C ppt)									
Batch 1	46.7	0.54	0.069	1.15	0.46	0.29	39.8	1.10	2.59
Batch 3	48.5	0.56	0.069	1.15	0.48	0.29	42.0	1.09	2.50
Cytoplasmic (from centrifuged supernatant)									
Batch 1: TCA ppt	70.4	0.84	0.072	1.19	0.49	0.56	41.2	2.11	2.63
Batch 2: TCA ppt	80.3	0.86	0.064	1.07	0.42	0.44	39.3	1.63	2.45
Batch 2: 80°C ppt	80.6	0.89	0.066	1.11	0.44	0.56	40.1	2.11	2.49
Batch 3: TCA ppt	71.9	0.86	0.072	1.20	0.46	0.57	38.3	2.15	2.78
Cytoplasmic (from 55°C ppt supernatant)									
Batch 1: TCA ppt	71.6	0.85	0.071	1.19	0.51	0.53	42.9	2.00	2.55
Batch 2: TCA ppt	78.2	0.83	0.064	1.06	0.46	0.46	43.6	1.71	2.24
Batch 3: TCA ppt	73.1	0.83	0.068	1.13	0.47	0.44	41.2	1.65	2.49
Batch 3: 80°C ppt	81.2	0.88	0.065	1.08	0.47	0.58	43.2	2.18	2.30

[a] From total amino acid analysis.
[b] Calculated from difference between total S and met-S.

(*Andropogon sudanensis*) (0.065),[2,11] and with protein remaining in the alcohol-insoluble residue from leaves of wheat (0.066),[1,4] sugarbeet (0.060),[1,5] ryegrass (0.062)[1,6] and lucerne (0.059–0.063).[1,7] The small differences between the ratios found with protein from different species could be accounted for by differences in methionine content. This amino acid is one of three (the others being aspartic acid and alanine) whose percentage in leaf protein varies according to species.[16] The observed differences are small, but coordinate analysis has shown that differences between species are most highly correlated with methionine content. The S due to any sulpholipid that might be present has not been taken into account in these calculations: it could in any case only be a negligible amount as about 90% of the total lipid in leaf protein concentrates consists of unsaturated and saturated straight chain fatty acids[22,23] and defatted samples have an unchanged S:N ratio.[21]

The similarity of the S:N ratio in different types of preparations contradicts the observations of Barrien and Wood[24] and Hanson *et al.*[11] who concluded that a chloroplastic fraction extracted from *A. sudanensis* contained a S-rich protein. Recent amino acid analyses on similarly prepared leaf protein fractions have failed to detect any major difference in S-containing amino acid content.[16,25–27] The results of Subba Rau *et al.*[12] are also open to question: from their reported

Table 2. The total S:total N ratio in some extracted lupin leaf proteins and a comparison of their "found" and "calculated" cystine contents

	Protein content: N×6.0 (% of DM)	Total S (% of DM)	S:N	Total S (% of protein content)	Met-S[a] (% of protein content)	Cys-S[a] (% of protein content)	Met-S (% of total S)	Cystine	
								Found[a] (% of protein content)	Calculated[b] (% of protein content)
Unfractionated (80°C ppt)									
3rd age cut	66.2	0.67	0.061	1.01	0.42	0.40	41.9	1.51	2.20
Unfractionated (TCA ppt)									
1st age cut	49.6	0.51	0.062	1.02	0.40	0.31	39.0	1.18	2.34
2nd age cut	59.5	0.58	0.059	0.98	0.38	0.30	38.5	1.14	2.26
3rd age cut	66.8	0.68	0.061	1.02	0.42	0.28	41.3	1.06	2.25
Chloroplastic (made by centrifuging)									
1st age cut	31.1	0.32	0.062	1.03	0.43	0.19	42.2	0.70	2.23
2nd age cut	40.3	0.41	0.061	1.01	0.46	0.22	45.2	0.83	2.07
3rd age cut	48.4	0.47	0.058	0.97	0.44	0.20	44.9	0.75	2.00
Chloroplastic (43°C ppt)									
1st age cut	27.1	0.28	0.062	1.03	0.45	tr	43.6	tr	2.18
2nd age cut	40.9	0.40	0.059	0.97	0.42	0.29	43.5	1.10	2.05
3rd age cut	50.9	0.51	0.060	1.01	0.44	0.29	43.7	1.09	2.12
Cytoplasmic (from centrifuged supernatant)									
1st age cut	86.5	0.91	0.063	1.05	0.45	0.32	42.3	1.18	2.28
2nd age cut	87.8	0.94	0.064	1.08	0.43	0.69	39.6	2.60	2.46
3rd age cut	85.9	0.98	0.068	1.15	0.44	0.75	38.8	2.79	2.67
Cytoplasmic (from 43°C ppt supernatant)									
1st age cut	84.4	0.97	0.069	1.15	0.49	0.43	42.4	1.62	2.48
2nd age cut	86.9	0.98	0.068	1.13	0.42	0.54	37.4	2.03	2.66
3rd age cut	89.7	0.98	0.066	1.08	0.47	0.57	42.9	2.14	2.35

[a] From total amino acid analysis.
[b] Calculated from difference between total S and met-S.
tr = trace.

organic-S:N and cystine-S:N ratios one would expect to find large differences between the S-containing amino acid content of the various preparations yet this was not confirmed by amino acid analysis of their preparations. The organic-S:N ratio in unfractionated lucerne protein (0.059) is the sole result which agrees with those quoted above.[1,4–7]

The range for the calculated cystine content of proteins from both species of leaves is similar, but on averaging the results there is less cystine in the preparations from lupin (2.29 as percentage of protein content) than in those from lucerne (2.50). These results follow the same trend as the observed average methionine contents, 1.79 and 2.00 (as percentage of protein content) for lupin and lucerne respectively,[16,17] but any relationship between cyst(e)ine content and leaf species is purely speculative at this stage.

FAO currently recommend that an ideal protein should contain 3.5% total S-amino acid content:[28] it is now reasonably certain that most extracted leaf proteins meet this recommendation although the problems associated with unavailability of both cyst(e)ine and methionine in preparations containing chloroplastic material remain.[16,17]

In conclusion, it is not suggested that this indirect determination should be substituted for a direct analysis of cyst(e)ine wherever this is possible. It is, however, a useful method of approach for complex protein concentrates containing only a small amount of cyst(e)ine liable to be wholly or partly destroyed during hydrolysis.

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Sweetness of Sucrose and Xylitol. Structural Considerations

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The sweetness of sucrose and xylitol is examined in relation to conformation and configuration. Arabitol, ribitol and the "galacto" and *O*-methyl analogues of sucrose are also examined on a comparative basis. The Shallenberger theory of sugar sweetness, which in part relates intensity of response to the degree of intramolecular hydrogen bonding, satisfactorily explains the high sweetness of these structurally dissimilar compounds.

1. Introduction

The sensation of sweetness is induced by a wide variety of non-ionised aliphatic hydroxy compounds, particularly alcohols glycols, sugars and sugar derivatives. The common structural feature of these sweet compounds has been recognised[2] as being the limiting geometrical separation of two electronegative atoms, A and B. The covalent linkage of a hydrogen atom to A creates a proton-donating AH component, B adopting a proton-accepting character. Provided the separation of A and B is between 2.5 and 4.0 Å, sweet taste initiation was considered possible due to intermolecular hydrogen bonding between the AH, B system and the receptor site. Sugar sweetness was proposed to be due to a multiplicity of hydroxyl groups in which A and B are oxygen atoms attached to adjacent carbon atoms. A substituent hydrogen atom may form an intramolecular hydrogen bond with an adjacent oxygen atom if the two oxygen atoms are in the true *cis* or eclipsed conformation. It was proposed[1] that such an intramolecular hydrogen bond might lower perceived sweetness and that the greater the degree of intramolecular hydrogen bonding, the greater the inhibition of intermolecular hydrogen bonding between the sapid unit and the receptor site.[3]

The principles of this hypothesis have been used in attempts to explain the differences in sweetness of numerous conformationally unequivocal structures, e.g., glycosides,[4] cyclohexanepolyols,[5] and deoxysugars.[6] We now report the utilisation of this theory in explaining the sweetness of sucrose and xylitol.

Sucrose and xylitol are two of the sweeter known polyhydroxy compounds having approximately equal sweetness on a weight basis. A 5.0% xylitol solution was found by earlier workers to be equal in intensity to a 5.03% solution of sucrose,[7] and 0.26 M xylitol was shown to have the same sweetness as 0.11 M sucrose, i.e., 4% solutions are equisweet.[8] Sucrose is a non-reducing disaccharide which possesses little structural similarity to xylitol, a sugar alcohol (Figure 1). This investigation is an attempt to identify, utilising sensory evaluation studies and infrared spectroscopy, the structural features of each compound responsible for its intense sweetness.

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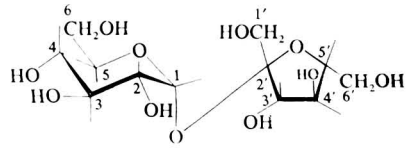
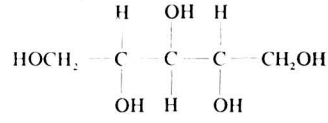


Sucrose



Xylitol

Figure 1. Sucrose and xylitol.

2. Experimental

2.1. Test materials

Sugar alcohols were purchased from British Drug Houses Ltd, Poole, Dorset. The synthesis of the sucrose derivatives is described elsewhere.[9,10] All preparations were chromatographically pure as judged by thin-layer chromatography (diethyl ether:light petroleum, 2:1; silica gel) and were characterised by their nuclear magnetic resonance and mass spectra.[10]

2.2. Taste testing

The assessors (ten in all) were selected and trained according to well established procedures.[11] This involved recognition of suprathreshold levels of the four basic tastes and ranking of different concentrations of sucrose as established by Spencer.[11] They were instructed to place a few milligrams of each test substance on the tongue and to classify it according to the following descriptions: very sweet (SS), sweet (S), trace sweet (tr), very bitter (BB), bitter (B), trace bitter (tr). Results reported are the agreement of at least 70% of assessors in duplicate tasting sessions, i.e. at least 70% of the assessors agreed with the description listed. No averaging of results was carried out.

2.3. Infrared spectra

Infrared spectra were recorded against air with a Perkin Elmer 457 Spectrophotometer, equipped with a sodium chloride prism. The powdered sugars were dried for 2 h at 5 Torr Hg and 70°C, and examined as nujol mulls.

3. Results and discussion

3.1. Sucrose

The sensory evaluation of the sucrose derivatives is reported in Table 1. Of the mono-*O*-methyl ethers tested, 6′-*O*-methyl sucrose is very sweet indicating that the C-6′ hydroxyl group has little or no effect on the overall sweetness of sucrose. The sweetness intensity of 4-*O*-methyl sucrose was determined by the assessors to be much lower than that of sucrose implying that the 4-hydroxyl group is important to sucrose sweetness. This result is not unexpected. Brown and Levy[12] carried out a precise determination of the crystal and molecular structure of sucrose by neutron diffraction. They showed that the 4-hydroxyl group is not involved in hydrogen bonding (neither intra- nor inter-molecular), therefore that hydroxyl group is free to participate in intermolecular hydrogen bonding with the receptor site. The substitution of an *O*-methyl group thus prevents that hydroxyl group from participating in such an interaction. The importance of this hydroxyl group is exemplified by considering the almost total lack of sweetness of "galacto" sucrose (Figure 2). The reduced

Table 1. Sweet compounds under test

Compound	Sweetness	Bitterness
Sucrose	SS	0
"Galacto"-sucrose (α-D-galactopyranosyl-β-D-fructofuranoside)	tr[a]	0
6′-*O*-methyl sucrose	SS	0
4-*O*-methyl sucrose	S	0[b]
6,6′-di-*O*-methyl sucrose	SS	tr
4,6′-di-*O*-methyl sucrose	S	tr
4,6-di-*O*-methyl sucrose	S[a]	tr
1′,6′-di-*O*-methyl sucrose	S[a]	tr
Xylitol	SS	0
L-(−)-Arabitol	S	0[a]
Ribitol	S[b]	0

[a] 70% agreement among panellists.
[b] 80% agreement among panellists.
All other values 90–100% agreement among panellists.

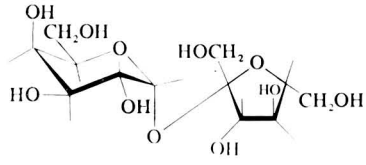


Figure 2. "Galacto"-sucrose.

sweetness intensity of D-galactose, compared with D-glucose, has been explained[1] as being due to the formation of an intramolecular hydrogen bond between the hydrogen atom of the 4-hydroxyl group of galactose and the ring oxygen atom. A similar occurrence probably accounts for the reduced sweetness of "galacto"-sucrose. This hypothesis is given substance by consideration of the hydroxyl absorption portion of the infrared spectra of sucrose and "galacto"-sucrose (Figure 3). Sharp infrared absorption bands in the region 3400–3600 cm^{-1} were assigned to free hydroxyl absorption, as has been done with diols[13] and sucrose.[14] Composite bands in the region 3200–3400 cm^{-1} were assigned to hydrogen bonded hydroxyl groups.[1] Sucrose shows a strong free hydroxyl absorption peak at 3560 cm^{-1} and a weak peak at 3530 cm^{-1}. These assignments cannot be considered unequivocal because of differing literature reports. However, "galacto"-sucrose has no strong free hydroxyl absorption peak, only a minor peak at 3520 cm^{-1} indicating that the strong peak of the sucrose spectrum is due to the unbonded 4-hydroxyl group.

The sensory evaluation of 4,6′-di-*O*-methyl sucrose is of importance. The reduction in sweetness intensity noted must be due to substitution at C-4 as it has been shown that substitution at C-6′ has little or no effect on sweetness. 6,6′-di-*O*-methyl sucrose is very sweet and therefore the 6-hydroxyl group contributes little or nothing to the overall sweetness of sucrose. The reduced sweetness of 4,6′-di-*O*-methyl sucrose must thus be due to the 4-substituent alone. The 1′-hydroxyl group was shown to be of importance by sensory evaluation of 1′,6′-di-*O*-methyl sucrose, which was found to have reduced sweetness. This may be explained by further consideration of Brown and Levy's neutron diffraction studies.[12] They showed that the *O*-1′ hydrogen atom is intramolecularly hydrogen bonded to the C-2 oxygen atom. Thus the two monosaccharide moieties are held in a particular relative position by a strong intramolecular (inter-residue) hydrogen bond. Although there is no evidence that such a bond can persist in solution it might influence the manner of presentation of a molecule to the immediate environment of a taste bud during the process of dissolution. If a methyl group is substituted at *O*-1′, such an intramolecular hydrogen bond cannot form and the fixed geometry of the molecule will be altered. It is possible that such an alteration in the geometry of

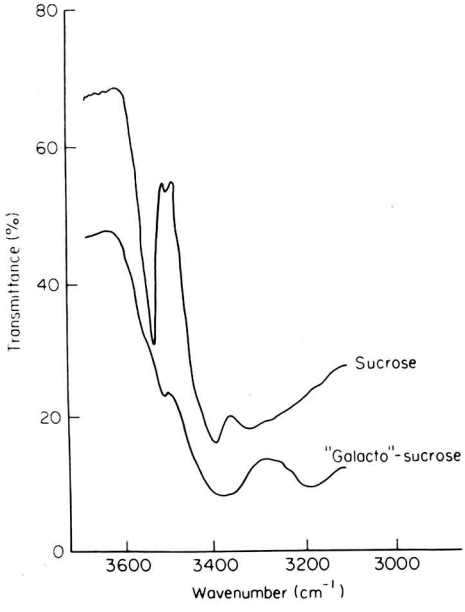


Figure 3. The hydroxyl absorption spectra of sucrose and "galacto"-sucrose.

sucrose is responsible for the drop in sweetness of the 1′-substituted compound. Two sugar alcohols were compared with xylitol, L-(−)-arabitol and ribitol. In assessing their sweetness, it was clearly shown that xylitol is much the sweeter (Table 1). On studying the structures of these pentitols (Figure 4), it is apparent that only xylitol has an all-trans arrangement of secondary hydroxyl groups. This structural feature might be responsible for its enhanced sweetness. Although there is flexibility about each carbon atom, there is a most stable, least energetically demanding conformation adopted by each molecule due to non-bonded interactions between adjacent carbon atom substituents.[15] In the case of xylitol this formation is a planar "zig-zag" conformation. Molecular models reveal that the oxygen–oxygen distance between all four pairs of oxygen atoms is 2.9–3.0 Å. Thus adjacent hydroxyl groups form gauche α-glycol groups which according to the Shallenberger theory are ideally disposed to elicit sweetness.[2] Xylitol would also be expected to show a strong free hydroxyl absorption peak on infrared spectral analysis. Figure 5 shows the hydroxyl absorption spectra of the three pentitols under test and, as predicted, xylitol shows a strong peak at 3440 cm^{-1} which may be due to free OH groups. Ribitol and arabitol show no such peaks, only composite bands due to hydrogen bonded hydroxyl groups. This suggests that the non-bonded hydroxyl groups of xylitol must be responsible for its high sweetness. In addition, the lower sweetness intensity of ribitol and arabitol must be due to the intramolecular hydrogen bonding indicated in the spectra.

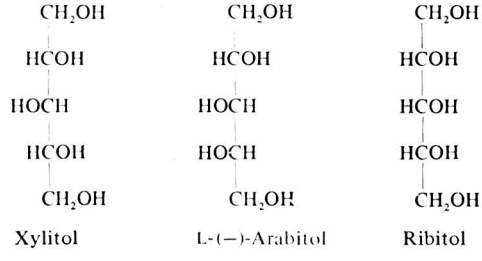


Figure 4. Xylitol, L-(−)-arabitol and ribitol.

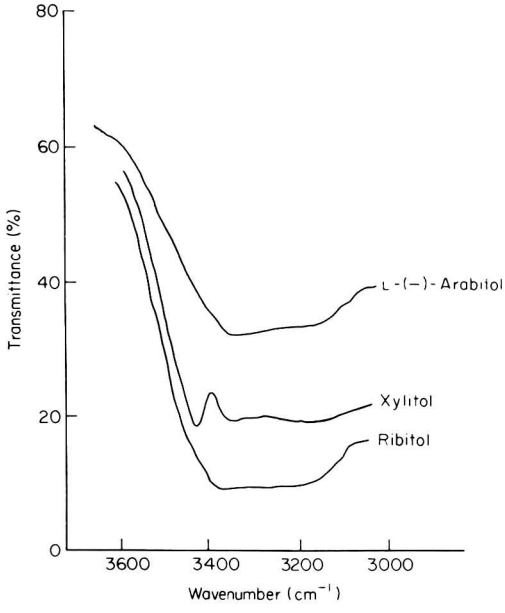


Figure 5. The hydroxyl absorption spectra of the pentitols tested.

4. Conclusions

Support for the validity of Shallenberger's hydrogen bonding theory has been found by sensory and infrared spectral evaluation of the compounds under test. The theory satisfactorily explains why sucrose and xylitol, two structurally dissimilar compounds, have intense sweetness. The feature common to sucrose and xylitol which may be responsible for their high sweetness is non-intramolecularly hydrogen bonded hydroxyl groups.

Acknowledgements

We thank the Science Research Council and Tate and Lyle Ltd for a CAPS award and Dr K. J. Parker for valuable discussions.

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2-Methoxy-3-*sec*-butylpyrazine[a]—an Important Contributor to Carrot Aroma

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A concentrate obtained by concurrent solvent extraction–steam distillation of carrots, *Daucus carota* L. gave complex chromatograms with more than 100 peaks. Attention was focused on a region of the chromatogram judged to be of sensory importance even though it contained components present only in very low concentrations. Procedures based on the use of glass porous layer open tubular (PLOT) capillary traps, a nitrogen selective detector and mass spectrometry led to the isolation and identification of 2-methoxy-3-*sec*-butylpyrazine. The possible significance of this highly potent odorant in carrot aroma is discussed.

1. Introduction

Apart from an earlier report in which a number of low-boiling alcohols, aldehydes and sulphur compounds were characterised,[1] the first systematic study of the volatile compounds of carrots was carried out by Buttery *et al.*[2] The major portion (80%) of an oil obtained by steam distillation of carrot root consisted of monoterpene hydrocarbons including α-pinene, β-pinene, camphene, myrcene, α-terpinene, sabinene, terpinolene, caryopyllene, β-bisabolene and γ-bisabolene. In the oxygenated fraction of the oil, heptanal, octanal, nonanol, 2-nonenal, 2-decenal, 2,4-decadienal, dodecanal, terpinen-4-ol, α-terpineol, bornyl acetate, carotal and myristicin were identified; a number of other compounds present in the very complex mixture were tentatively identified. While the aldehydes, especially 2-nonenal appeared to make a significant contribution to the total odour intensity of the oil, no evidence for the presence of key aroma compounds responsible for the characteristic aroma of raw or cooked carrots was found.

More recently, Heatherbell *et al.*[3] developed a gas entrainment on-column trapping procedure to study carrot volatiles and used it to examine the volatiles of both raw and processed carrots.[4] Most of the compounds previously characterised were found again, and in addition, a number of low-boiling substances such as diethyl ether, acetaldehyde, acetone, propanol, methanol and ethanol were identified. Sensory analysis performed by "sniffing" the peaks being eluted from the gas chromatographic column suggested that a number of compounds contributed to carrot aroma, e.g. acetaldehyde ("soft sweet" note), myrcene and sabinene ("green earthy" and "carrot top" notes) and terpinolene ("perfumey"). Although these compounds were of importance in the overall aroma profile they did not represent the full story, and the authors commented on the lack of evidence for other key contributions to carrot aroma. Some tentative evidence suggesting that carrots contain a highly potent aroma compound present at a concentration below the detection limits of their instrumental methods was put forward by Heatherbell and Wrolstad,[5] who succeeded in generating raw carrot aroma from essentially odourless, vacuum distilled and nitrogen purged

[a] For clarity in this paper, the methoxy substituent is assigned the 2-position in pyrazines, and is written first, irrespective of the rules of nomenclature.

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carrot substrates, by means of flavour forming enzyme preparations isolated from raw carrots. Of special interest was the fact that the chromatograms of the gas entrained headspace volatiles of the aroma-bearing enzyme treated carrot substrate were virtually identical to those of the deodorised substrate itself.

A preliminary examination[6] by a slightly modified version (recently described for work on parsnip volatiles[7]) of the gas-entrainment technique of Heatherbell *et al.*, of the headspace vapour over an aqueous extract prepared by blending blanched raw carrots, produced very similar chromatograms to those obtained by the latter workers. However, in g.c. peak "sniffing" runs one region of the chromatogram containing peaks only barely distinguishable from the background was described by four assessors as having a "raw rooty" or "raw carroty" aroma, and was considered to be the region of the chromatogram which most strongly suggested the aroma of the raw vegetable. Since the concentrations of components in this region were far too low for examination by combined gas chromatography–mass spectrometry (g.c.–m.s.) using the headspace approach, an examination of the area of sensory interest in a steam distilled oil from carrots was then carried out. The aromas, assessed by the same four assessors were qualitatively similar but more "earthy" and "sharper" than in the headspace samples. Figure 1 shows the major portion of a chromatogram of 0.5 μl of the essence on a PLOT column coated with Carbowax 20M. The region of interest,

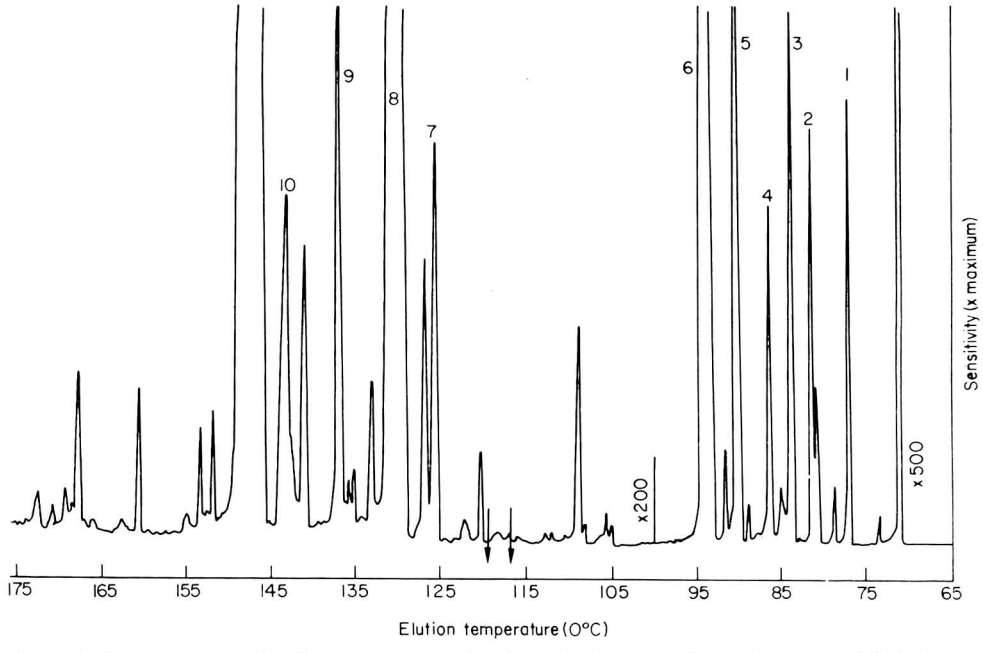

Figure 1. Chromatogram of 0.5 μl carrot essence on 60 m PLOT column coated with Carbowax 20M (Column 3); helium flow rate, 3.5 ml/min; programmed from 65°C at 1°/min. Identities of some major peaks; 1, α-pinene; 2, β-pinene; 3, myrcene; 4, limonene; 5, γ-terpinene; 6, terpinolene; 7, bornyl acetate; 8, caryophyllene; 9, terpinene-4-ol; 10, bisabolene.

eluting around 117–119°C, is indicated by the vertical arrows. These peaks are still extremely small. While it is possible to obtain mass spectra of this region with a 30 μl sample on a packed column, the spectra revealed the presence of several unresolved components and were incapable of interpretation.

Techniques, based on the use of glass PLOT capillaries containing a permanently bonded layer of a support material such as Celite 545 or alumina for the trapping and subsequent handling of

very small amounts of volatile organic compounds, have been developed in recent years. Such methods have been found to be extremely effective for obtaining structural information on sub-microgram quantitites of organic substances as are obtained on elution from capillary g.c. columns by procedures employing colour reactions,[8] as well as hydrogenation or ozonolysis[9] of the trapped compounds. The availability of the new trapping and handling methods suggested that a fresh examination of the region of apparent sensory importance to carrot aroma should be undertaken, and the isolation and characterisation of a hitherto undetected, highly potent constituent of carrot volatiles using the new techniques is described below.

2. Experimental

2.1. Isolation of steam volatile oil

Fresh carrots were washed, chopped into small sections and heated at atmospheric pressure in a continuous steam distillation apparatus of the type described by Likens and Nickerson.[10] In a typical isolation, 3 kg of carrots were heated for 2 h with 3 litres of refluxing water in a 10-litre flask and the condensed volatiles on their way back to the flask were continuously extracted with solvent from 20 ml of boiling redistilled pentane. The pentane extract, dried over anhydrous sodium sulphate, was concentrated at atmospheric pressure using a low hold-up fractionating column and progressively smaller flasks to a final volume of about 0.4 ml. The extract was transferred to a small Pyrex ampoule having a narrow capillary neck about 75 mm long, and before sealing the latter the remainder of the pentane was removed by inserting a narrow hypodermic needle into the extract and purging gently with a flow of nitrogen. The final concentrate (140 μl) was a pale yellow non-viscous oil, and while it possessed a somewhat more "cooked" aroma than the headspace isolates, the characteristic slightly sharp and pleasant aroma of carrots predominated.

2.2. Instrumentation and analysis

The gas chromatography-peak sniffing runs and also the trapping experiments were carried out on a Pye Series 104 gas chromatograph equipped with a heated flame ionisation detector (FID) and heated fraction collector outlet port. A Pye Series 104 chromatograph equipped with a heated FID and a Pye alkali flame ionisation detector (AFID) was used to analyse for nitrogen containing constituents. The two detectors were operated in parallel with the column effluent split equally between them. Combined g.c.–m.s. was performed on a Philips PV 4000 chromatograph linked by means of a jet separator to an Edwards E-60 low resolution fast scanning mass spectrometer.[11]

2.3. g.c. Columns and PLOT traps

Column 1: 2.3 m × 6.25 mm o.d. glass column packed with 10% w/w of Carbowax 20M on 84–100 mesh acid-washed silanised Chromosorb W. Nitrogen at 30 ml/min was used as carrier gas and the column was programmed from 65°C at 2°/min from injection of the sample. Trapping of the fractions of interest was carried out from 30 μl samples of carrot root oil applied to this column.
Column 2: 34 m × 0.5 mm i.d. silanised Pyrex PLOT column coated with 1.5% w/v silicone elastomer (OV-1) + 0.075% w/v Atpet 80 in dichloromethane, prepared as recently described.[12] Helium at 3 ml/min was used as the carrier gas and the column was programmed from injection at 2°/min from 65°C.
Column 3: 60 m × 0.5 mm i.d. glass PLOT column coated with 0.8% w/v Carbowax 20M in dichloromethane prepared as described previously.[12] The carrier gas was helium at 3.5 ml/min and the column was programmed from injection at 1°/min from 65°C. Unless otherwise stated the above operating conditions for each column were used throughout.

The glass PLOT capillary traps were prepared from 120–150 mesh Celite 545 with B-37 Pyrex glass as a binding agent as described.[13] Straight lengths (2 m) of the capillary were washed successively with concentrated hydrochloric acid, distilled water and Analar acetone. After drying in a stream of nitrogen they were silanised by filling with 2% v/v dimethyldichlorosilane (DMCS) in pentane, letting stand overnight and then expelling the solution by nitrogen under pressure. Finally,

the capillaries were rinsed with acetone and the solvent totally removed in a flow of nitrogen. The tubes were broken into 10 cm lengths which were used as the traps.

2.4. Trapping and handling of trapped fractions

The desired fractions were collected by splitting the effluent from the packed column and trapping on lengths of PLOT capillaries cooled by means of crushed solid carbon dioxide as shown in Figure 1.[9] A standard Pye effluent splitter giving a split ratio FID:trap of 1:10 was used in this case. In the trapping of peaks eluted from PLOT columns the arrangement was exactly as shown in ref. 9, with 80–85% of the effluent going to the trap. After trapping the PLOT capillaries, still immersed in the coolant, were immediately sealed at both ends in the flame of a microburner. The trapped fractions were subsequently analysed by injecting them on to one of the PLOT capillary columns, either with or without m.s., using the all glass capillary crushing injection device which is shown in detail in Figure 2.[9]

Prior to trapping components from either packed or capillary columns, the exact position for insertion of the traps was established in preliminary g.c.-peak sniffing runs, i.e. the regions on the chromatograms encompassed by the first appearance of the aroma of interest and its final disappearance were clearly marked on the chart, and only these areas were then trapped out.

3. Results and discussion

The portion of a chromatogram up to the very large caryophyllene peak of a 30 μl sample of the carrot essence is shown in Figure 2(a). The small broad area of peaks eluting over a 3 min period (Fraction A), described by four assessors as possessing principally "strong sharp raw rooty" and "rooty earthy carrot" notes was trapped. Analysis of the trap contents on Column 2 produced the chromatogram shown in Figure 2(b), which clearly illustrates the considerable complexity of Fraction A. While most of the peaks in Figure 2(b), including the major component, produced weak and/or ill-defined aromas, there were two regions (Fractions B and C) that were of considerable sensory significance. Fraction B gave a weak to moderate response described as "slightly sweet, fresh raw-carrot like". The peak in Fraction C possessed a strong aroma ranging in character from "astringent or sharp raw rooty vegetable" to "somewhat rooty carrot-like" on the trailing edge.

Analysis of Fraction A on the OV-1 column with the AFID indicated the presence of only one nitrogen-containing compound in the sample viz. the peak of Fraction C. This peak gave the following mass spectrum:

m/e: 124(100), 138(87), 151(55), 137(33), 123(20), 41(14), 105(14), 152(14)

This closely resembles a recently published[14] spectrum for 2-methoxy-3-*sec*-butylpyrazine, viz.

m/e: 124(100), 138(90), 137(50), 151(46), 105(14), 123(12), 152(12)

The subsequent acquisition of an authentic sample of the above pyrazine enabled its mass spectrum and retention properties on both Columns 2 and 3 to be compared directly with those of the peak in Fraction C. All the data were in perfect agreement, thereby confirming the identity of the substance. The actual retention data were checked in the following manner: Fraction A from a 30 μl sample of the essence was trapped in the normal manner and the trap was "spiked" with a sample of the authentic pyrazine by carefully injecting into the centre of the trap 0.1 μl of a 0.1% v/v solution of the pyrazine in pentane. The trap was sealed and analysed on Column 2, to produce a chromatogram identical to that in Figure 2(b), except for a substantially larger peak coincident with the peak of Fraction C. To check the retention time on the polar Column 3, the peak of Fraction C was trapped from Column 2 on two PLOT capillaries in separate runs; one of the traps was "spiked" with the authentic pyrazine as described above, before being sealed. Analysis of both traps on Column 3 produced only one peak in each case having the same retention times, the peak in the "spiked" sample being the larger of the two.

The nature of the mass spectra taken over the carrot-like fraction B in Figure 2(b) indicated the presence of unresolved components. This region was therefore trapped from Column 2 and then

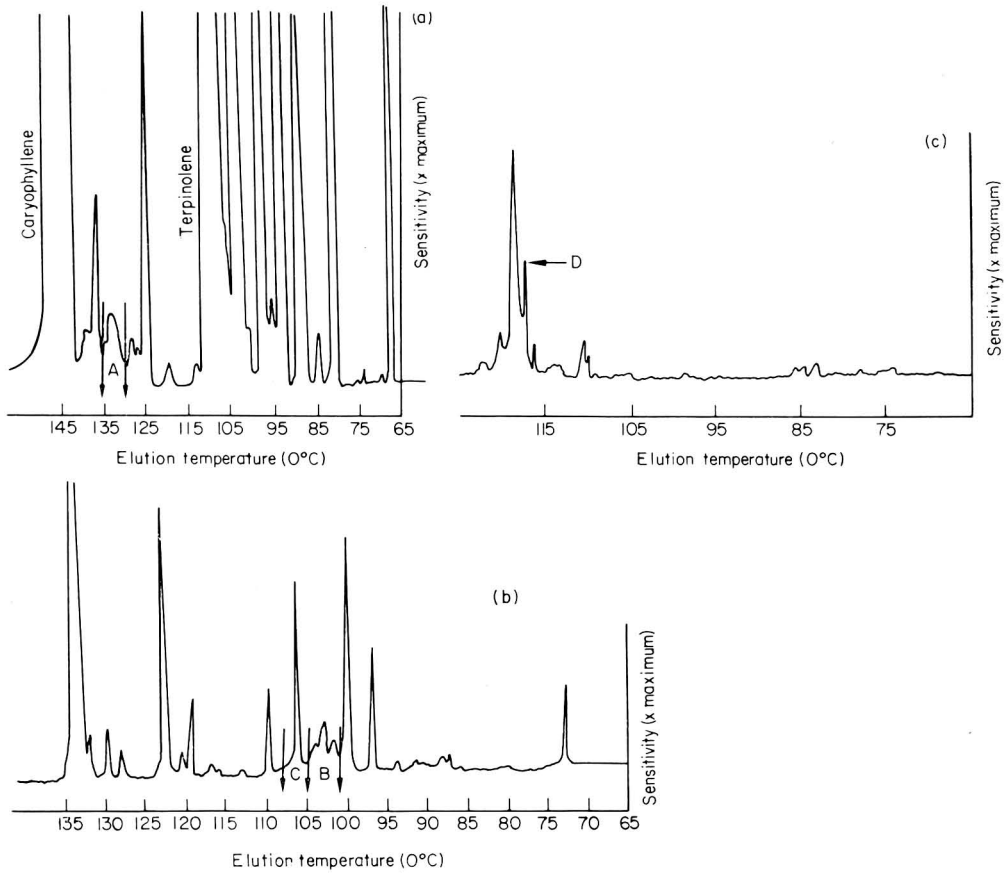

Figure 2. (a) Chromatogram of 30 μl carrot essence on Column 1 for trapping of Fraction A. (b) Chromatogram of Fraction A on Column 2. (c) Chromatogram of Fraction B run on Column 3. Conditions are as given in the text.

rerun on Column 3 to produce the chromatogram shown in Figure 2(c). The carrot-like aroma was shown to be associated entirely with the peak marked D, which gave a rather weak mass spectrum exhibiting the following *m/e* values (in order of decreasing intensity):

m/e: 43, 84, 54, 141, 117, 55, 97, 81, 132

It has not been possible so far to find a published mass spectrum which might indicate the chemical nature of this compound. It is difficult to assess the extent to which this compound, on its own, contributes to the aroma of carrots. It is present only in small amount in Fraction A and when isolated as peak D has a rather weak aroma and would therefore appear not to be too important. However its contribution may well be more significant in the presence of other components such as those of Fraction A.

In recent years a number of 2-methoxy-3-alkylpyrazines have been found to play a key role as character impact compounds in the aromas of some important plant products e.g. 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-isobutylpyrazine and 2-methoxy-3-*sec*-butylpyrazine in peas (Leguminosae)[15] and galbanum oil (from Ferula spp., Umbelliferae).[16] The 2-methoxy-3-isobutylpyrazine has also been found in bell peppers (Solanacae),[17] while 2-methoxy-3-ethylpyrazine[18–20] and 2-methoxy-3-isopropylpyrazine[21] have both been reported to be important odour components

of potato (Solanacae) aroma. The discovery of the 2-methoxy-3-*sec*-butylpyrazine in peas and now also in carrots is yet further evidence that 2-methoxy-3-alkylpyrazines may be widely distributed in the plant kingdom.

Although present in the various products at extremely low concentrations viz parts/10^{11} to 10^{12}, their great potency as odorants is a consequence of their low detection thresholds (estimated by Murray *et al.*[15] as 1 part in 10^{12} of water for 2-methoxy-3-*sec*-butylpyrazine). While a detailed sensory evaluation of the role of the latter compound in carrot aroma remains to be carried out, its ready detection by the nose (but not by instrumental analysis) in the headspace vapour of raw carrots clearly suggests that it makes a significant contribution to the aroma of this vegetable. This view is supported by the actual amount present in the whole carrot which we estimate to be a few parts/10^{10}. The pyrazine is claimed to possess a predominantly "bell-pepper" like aroma.[22] In our opinion, it exerts its effect in carrot by imparting the slightly sharp, raw, earthy, rooty character to the aroma, which complements the sweeter, oily, perfumey contributions of the major terpenoid constituents. Murray *et al.*[15] have suggested that the biosynthetic origins of 2-methoxy-3-alkylpyrazines in plants might well resemble their laboratory method of preparation i.e. amidation of a common amino acid (isoleucine in the case of 2-methoxy-3-*sec*-butylpyrazine), followed by condensation with an α-β-dicarbonyl compound to form a 2-hydroxy-pyrazine which is then methylated. If the observation of Heatherbell and Wrolstad[5] referred to earlier is correct, viz. that the raw carrot-like aroma generated enzymically from odourless carrot substrates is due to the production of very small amounts of an important odorant, it would clearly be of great interest if it were possible to detect the presence of a compound such as 2-methoxy-3-*sec*-butylpyrazine as a constituent of enzyme-induced carrot aroma. Its detection would not only provide further information on its sensory contribution to the aroma of the vegetable, but in addition, would make it worth while to test the validity of the proposed biosynthetic pathway, for example, by looking for the presence of the pyrazine after incubation of various likely synthetic substrates (such as mixtures of isoleucine and glyoxal) with active preparations of flavour-producing enzymes obtained from carrots.

Future work along these lines is envisaged using the type of analytical approach which has led to the discovery of the pyrazine in carrots. The application of such techniques should be generally useful for obtaining information on trace constituents frequently present in very complex mixtures of food aroma volatiles, and which make a contribution to the aroma totally out of proportion to the amounts present.

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Note added in proof

Since this paper was submitted, a report has appeared (Murray, K. E.; Whitfield, F. B. *J. Sci. Fd Agric.* 1975 **26,** 973) confirming the general occurrence of one or more 2-methoxy-alkylpyrazines in the headspace volatiles from all but four of 27 raw vegetables examined, including the specific occurrence of 2-methoxy-3-sec-butylpyrazine in carrots.

J. Sci. Fd Agric. 1976, **27**, 152–158

The Source of the Acyl Moiety in the Biosynthesis of Volatile Banana Esters

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Using headspace analysis of incubated banana slices, it has been shown that addition of acetyl-coenzyme A leads to marginal increases in acetic acid esters, whereas butyryl-coenzyme A gives up to five-fold increases in headspace concentrations of isobutyl and isopentyl butyrate. The increases in esters were much more marked, when the appropriate alcohol was added as well, those with butyryl-coenzyme A being up to 14 times greater. Thus acyl-coenzyme A can act as acylating agent in ester formation and, since in the absence of banana slices little change can be detected, the slices supply some essential factor.

1. Introduction

Although esters typify the substances giving aroma to fruits, still relatively little is known of their biosynthesis.[1] In the earliest work on banana ester synthesis,[2] it was shown that, excepting the very volatile compounds, L-leucine-U-^{14}C is incorporated mainly into isopentyl acetate and isopentyl alcohol, L-isoleucine-U-^{14}C into isopentyl and butyl acetates, and L-valine-U-^{14}C into isobutyl acetate and isobutyl alcohol. L-Isoleucine-1-^{14}C and L-valine-1-^{14}C were not incorporated. More recently, Myers *et al.*[3] obtained 1.3% incorporation of L-leucine-U-^{14}C in banana volatiles, mostly into isopentyl alcohol, but some into isopentyl acetate. In similar experiments with banana slices, Tressl *et al.*[4] obtained 0.5% incorporation of ^{14}C from L-leucine-U-^{14}C, four-fifths of it going into esters. After hydrolysis of the volatiles, the radioactivity was found in the alcohols and acids in roughly equal amounts, that of the former being 76% in isopentanol and 23% in 2-heptanol, whereas 35% of the latter was in 3-methylbutyric acid and 62% in 4-methyl-2-oxovaleric acid. With valine-U-^{14}C, incorporation was only 0.15%, 68% of the activity being found in isobutyric acid and 3-methyl-2-oxobutyric acid. Several esters were labelled, isobutyl acetate the most strongly. After hydrolysis of the aroma concentrate, the ratio of activity alcohols:acids was 1:3. When 8-^{14}C-octanoic acid was supplied, most of the radioactivity was recovered in the volatiles, much of it unchanged, but a considerable proportion appeared in 1-octanol and a range of esters.[5] These were derived from either octanoic acid or 1-octanol, the most radioactive of them being octyl octanoate. Some labelling of 2-pentanone, 2-pentanol, 2-heptanone, *cis*-4-hepten-2-ol, butyric acid, hexanoic acid, and heptanoic acid was detected. Palmitate-U-^{14}C, although 11% incorporated into the aroma concentrate, did not give rise to any radioactivity in esters up to 2-heptyl butyrate.[6] In contrast, 1-^{14}C-butyrate and acetate-U-^{14}C, 8.3 and 4.5% taken up, respectively, appeared almost exclusively in esters of butyric acid and acetic acid, respectively, isopentyl being the principal alkyl component. Only 1% of the activity appeared in the alkyl moieties.

There is thus some direct evidence of the origins of both the alkyl and the acyl components of the volatile esters of the banana, but no evidence has been produced of how the two come together. Speculations about possible reactions is easy. The aim here is to provide some relevant experimental data.

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2. Experimental

2.1. Gas chromatography

A Perkin-Elmer 451 Fractometer was used with FID; 2 m × 6 mm stainless steel column, containing 20% Silicone Oil MS 550 on 60–72 mesh Chromosorb W (average number of theoretical plates 2400) at 125°C; N_2, 0.49, 33; H_2, 1.3, 50; air, 1.4 kgf cm^{-2}, 150 ml min^{-1}.

2.2. Incubations in the absence of banana

Incubations were carried out in flasks (100 ml, r.b.) with Clearfit joints, each fitted with a Teflon stopcock[7] and shaken mechanically in a waterbath at 30 ± 1°C. In each flask was placed phosphate buffer (0.05 M, pH 6.0, 10 ml) and distilled water (1 ml) and either acyl-CoA solution, acid anhydride solution, or acid solution (1 ml).

Acyl-CoA solution was prepared, modifying Ochoa's method[8] somewhat, from coenzyme A (Boehringer, 5 mg), dissolved in water (1 ml), and cooled to 0°C, when aqueous potassium hydrogen carbonate (M, 0.05 ml) was added, followed by acid anhydride (2.0 μl). The cooling retards the decomposition of the coenzyme in solution; the hydrogen carbonate deals with acid produced by the acylation and by the hydrolysis of the anhydride. Nitrogen was bubbled through the solution for 15 min to mix the reagents and to exclude air, which might oxidise thiol groups. Acid anhydride solution was prepared similarly, omitting coenzyme A and potassium hydrogen carbonate. For the acid solution, acid anhydride was replaced by the corresponding acid (2.55 μl acetic acid, 2.25 μl butyric acid). *S*-Acylation of coenzyme A was assessed by the use of Ellman's Reagent[9] (DTNB, 3,3′-dithiobis(6-nitrobenzoic acid), specific for free −SH groups) and always exceeded 70%. To some incubations alcohols were added.

Samples (5.0 ml) of the headspace were withdrawn periodically from each flask by means of a gas-tight syringe (Hamilton, 5 ml) and injected into the gas chromatograph.

2.3. Incubations in presence of banana slices

Banana slices (Giant Cavendish; 15 g) were placed in flasks as above and phosphate buffer (10 ml) and distilled water (1 ml) added. Depending on the experiment, acyl-CoA, acid anhydride, or acid solution or water (1 ml) was added, with or without alcohols (1.0 or 2.0 μl). The banana slices (17.5 mm diam., 2 mm thick) were prepared using a No. 12 cork-borer and a hand microtome and were randomised in tap water. The Ripeness Index (RI)[10] of the bananas was noted and, for each experiment involving comparisons between four flasks, only slices from bananas taken from the same hand and at the same RI were used.

Samples of the headspace were examined from time to time as above. No correction was applied to the results for the repeated withdrawals of 5 ml vapour.

3. Results

3.1. Incubations without banana slices

Neither acetyl-CoA nor acetic anhydride gave rise to any ethyl acetate in the presence of ethanol (10 μl). In the presence of propanol (5 μl), small peaks corresponding to propyl acetate were detected, but only traces of isobutyl and butyl acetate in the presence of corresponding alcohols (5 μl of each). In the presence of 2-pentyl and isopentyl alcohol (5 μl of each), no 2-pentyl acetate was detectable and only traces of its isomer. Overall there appears to be virtually no reaction leading to esters in dilute aqueous solutions containing lower alcohols and either acetyl-CoA or acetic anhydride.

The results with butyryl-CoA and butyric anhydride were even more clear cut. In the presence of ethanol (10 μl) and propanol (5 μl), no ethyl or propyl butyrate was detected. In the presence of isobutanol and butanol (3 μl of each), only traces of the corresponding butyrates were apparent, and no butyrates were formed in the presence of 2-pentanol and isopentanol (2 μl of each).

Neither acetic nor butyric acid produced even a trace of ester in the presence of different alcohols under the conditions used.

3.2. Incubations with banana slices

Banana slices alone led to headspace chromatograms typified by Figure 1. Twelve peaks, B–M, are clearly resolved, another nine minor ones or shoulders being detectable but not invariably. Identities were assigned to the peaks on the basis of previous work on the headspace over bananas[11] and the retentions of the corresponding authentic compounds (see Table 1). Reproducibility between experiments carried out on the same lot of bananas in parallel was qualitatively excellent and quantitatively (peak height) lay within ± 12%. Peak intensity is reduced by the presence of buffer.

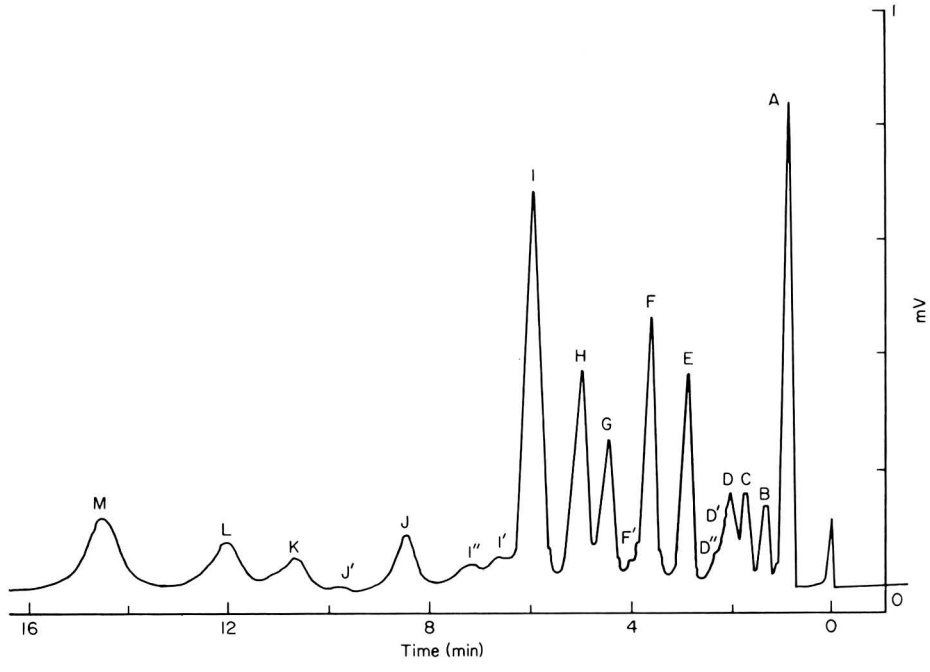

Figure 1. Typical chromatogram of banana headspace. A single sliced banana was placed in a 500 ml round-bottomed flask which was then sealed. After 80 min incubation at 30°C, 5.0 ml of the headspace vapour was withdrawn and injected on to the chromatograph (conditions as in Experimental). Sensitivity × 32.

Peak E (mainly 2-pentanone) increased appreciably (about 25%) over the 5 h incubation, the concentration of ethyl acetate tended to remain constant, and the peaks for isobutyl, 2-pentyl, and isopentyl acetate fell off (up to 70%), the more severely, the riper the banana. Isopentyl acetate was clearly more affected than the 2-pentyl isomer. Butyrates, present in lower concentrations, remained constant.

Banana slices incubated in the presence of acetyl-CoA, acetic anhydride, or acetic acid provided marginal evidence of higher levels of isobutyl and isopentyl acetates in the first case. Experiments with butyryl-CoA, butyric anhydride, and butyric acid gave up to five-fold increases in headspace concentrations of isobutyl and isopentyl butyrates, with some suggestion of concomitant decreases in the corresponding acetates, the implication being that the alcohol radical may be the limiting factor. Banana slices were therefore incubated not only with acyl-CoA, the anhydride, and the acid, but in the presence of added alcohol. The acetates corresponding to the alcohol increased in concentration several-fold for butanol, isobutanol, and isopentanol, acetyl-CoA being more effective than the anhydride or acid (see Table 2, experiments 1 and 2). The increase for 2-pentanol was only marginal. Surprisingly, in some cases there was a concomitant increase in the butyrate ester when acetate radicals were supplied, though the form in which they were provided was immaterial.

Table 1. Retention data for chromatograms on Silicone Oil MS 550 column

Peak in Figure 1	Retention[a] (mm)	Identity	Reference compound	
			Name	Retention[b] (mm)
A	10.5	air		
B	16	acetaldehyde	acetaldehyde	17
C	21	ethanol	ethanol	22
			methyl acetate[c]	20.5
D	25	ethyl acetate	ethyl acetate	25.5
			1-propanol	23.5
D′	27		isobutanol[c]	27
D′′	29.5			
E	35	2-pentanone	2-pentanone	35
			1-butanol	33.5
			2-pentanol	35.5
			1-propyl acetate	36
E′	(42)	isopentanol	isopentanol	42
F	44.5	isobutyl acetate	isobutyl acetate	45
F′	50.5	ethyl butyrate	ethyl butyrate	51
G	55.5	1-butyl acetate	1-butyl acetate	55
			hexanal	56
H	62	2-pentyl acetate	2-pentyl acetate	62
I	74	isopentyl acetate	isopentyl acetate	74
I′	83.5	1-hexanol	1-hexanol	81.5
I′′	90	1-pentyl acetate	1-pentyl acetate	89.5
J	106	isobutyl butyrate	isobutyl butyrate	104.5
J′	121			
K	133	1-butyl butyrate	1-butyl butyrate	132
L	151	1-hexyl acetate	1-hexyl acetate	150
		2-pentyl butyrate	2-pentyl butyrate	150
L′	(167.5)			
M	181.5	isopentyl butyrate	isopentyl butyrate	180
M′	(231.5)			

[a] Retentions in brackets are taken from other chromatograms.
[b] Retentions of standards corrected to isopentyl acetate = 74 mm.
[c] No peak at appropriate retention on Carbowax 1540 column.

When butyryl-CoA was used together with alcohol, the concentration of the corresponding ester increased remarkably, though there were concomitant increases of up to five-fold in other butyrates. Thus in the presence of butyryl-CoA, ethanol gave rise to substantial concentrations of ethyl butyrate, which had been barely detectable in Figure 1. Propanol gave a parallel result. For isobutanol and butanol, the corresponding esters increased up to 70 times and for isopentanol up to 30 times, but considerably less for 2-pentanol (see Table 2, experiments 3 and 4). Modest increases also occurred for butyrates other than that of the alcohol added, as would be expected from the earlier results. In all these experiments, butyric anhydride proved to be effective, though to a less degree than butyryl-CoA. The real increases due to acyl-CoA are more marked in the earlier stages. Thus, at 100 min, the mean increases with butyric anhydride were 23–50% of those with butyryl-CoA. Alcohols stimulated the formation of the corresponding acetates in all cases, irrespective of the presence of butyryl-CoA or butyric anhydride. The effects of replacement of butyric anhydride by butyric acid lay within the experimental error.

4. Discussion

In the absence of banana slices, in buffer at pH 6 neither acetyl- nor butyryl-CoA nor the corresponding acid anhydride or acid was able to acylate significantly low-molecular weight alcohols at

Table 2. Formation of volatile substances on incubation of banana slices (15 g in buffer) with various substances for 300 min[a]

	Banana ripening stage	I	II	III	IV
Experiment 1	RI 5				
Acetyl-CoA solution (ml)	(yellow	1	—	—	—
Acetic anhydride solution (ml)	with green	—	1	—	—
Distilled water (ml)	tips)	1	1	2	2
Isobutanol (μl)		2	2	2	—
1-Butanol (μl)		2	2	2	—
Isobutyl acetate[b]		63	43	41	18.5
1-Butyl acetate[b]		22	14.5	14.5	4
Experiment 2	RI 7				
Acetyl-CoA solution (ml)	(all-yellow	1	—	—	—
Acetic anhydride solution (ml)	flecked	—	1	—	—
Acetic acid solution (ml)	with	—	—	1	—
Distilled water (ml)	brown)	1	1	1	2
2-Pentanol (μl)		2	2	2	—
Isopentanol (μl)		2	2	2	—
2-Pentyl acetate[b]		47.5	42.5	38	28.5
Isopentyl acetate[b]		41	23.5	21.5	8
Experiment 3	RI 5				
Butyryl-CoA solution (ml)	(yellow	1	—	—	—
Butyric anhydride solution (ml)	with green	—	1	—	—
Distilled water (ml)	tips)	1	1	2	2
Isobutanol (μl)		2	2	2	—
1-Butanol (μl)		2	2	2	—
Isobutyl acetate[b]		38.5	33	41	5
1-Butyl acetate[b]		17.5	15	17.5	1.5
Isobutyl butyrate[b]		196	142	14	3
1-Butyl butyrate[b]		96	74	7.5	1.5
Experiment 4	RI 7				
Butyryl-CoA solution (ml)	(all-yellow	1	—	—	—
Butyric anhydride solution (ml)	flecked	—	1	—	—
Butyric acid solution (ml)	with	—	—	1	—
Distilled water (ml)	brown)	1	1	1	2
2-Pentanol (μl)		2	2	2	—
Isopentanol (μl)		2	2	2	—
2-Pentyl acetate[b]		55.5	39.5	46	27
Isopentyl acetate[b]		68.5	56	55	18
2-Pentyl butyrate[b]		33	15	18	3.5
Isopentyl butyrate[b]		153.5	116	93.5	5.5

[a] See Experimental for details concerning preparation of solution.
[b] Figures quoted for volatiles are peak heights in arbitrary units.

the dilutions investigated. In the presence of banana slices, the situation was very different for the C_4-acyl derivatives, up to five-fold increases in the headspace concentrations of isobutyl and isopentyl butyrates being observed, but for the C_2-acyl derivatives the increases in the corresponding esters were only marginal. In the former case, some concomitant decreases in the corresponding acetates were observed, suggesting that the alcohol concentration may be limiting.

Incubation of banana slices with alcohol as well as a source of acyl moieties led to more dramatic increases in headspace ester concentrations, those for isobutyl and butyl butyrates giving factors as high as 70.

When banana slices were incubated with alcohol and a source of acyl moieties, addition of further acyl-CoA after, for example, 3 h led to a renewed, marked increase in the headspace concentrations of the appropriate esters.

When banana slices were incubated not only with alcohols and butyryl-CoA but with either 0.001 M mercuric acetate or silver nitrate, ester formation was very greatly reduced.

Two facts emerge: (a) acyl-CoA can act as acylating agent in ester formation and (b) banana slices supply some essential factor(s). The first does not rule out a role for acyl carrier protein (ACP) derivatives, since these are formed biochemically from the corresponding CoA compounds. It is possible that, in climacteric banana fruit, the availability of ATP as an energy source and/or other cofactors, such as inorganic ions, needed to "activate" acids (i.e., to convert them to their acyl-CoA derivatives; see, for example, references 12 and 13) is in some way limited. Irrespective of whether ester synthesis proceeds via either ACP or CoA derivatives, the natural fruit tissue would require ATP as an energy source for acid "activation" and this need is bypassed by the supply of acyl-CoA in the experiments reported here. The second fact may be interpreted as the supply of enzyme(s) and/or cofactor(s), because ester formation does not result from incubation of acyl-CoA with alcohols in the absence of banana fruit. However, much work remains to be done.

There are some points on which these studies are reinforced by those reported recently from elsewhere.

If the alcohol concentration can be the limiting factor in ester synthesis, as indicated here, then formation of active acetate and butyrate at different points of the diurnal cycle would lead to the out-of-phase cycling of acetate and butyrate esters found by Tressl and Jennings;[14] in fact, it would be expected to provide gearing, the pool of ethyl radicals in the form of alcohol, augmented by ester hydrolysis, being swung first one way and then the other. That L-leucine-U-^{14}C is incorporated into isopentanol and its acetate, even though their headspace concentrations do not change,[3] is proof that the system is indeed a dynamic one.

In interpreting the results of headspace analysis, it must be borne in mind that, compared with the ester, the formation of a given amount of alcohol is much less evident, since a much smaller proportion of it is in the vapour phase. This is clear from comparisons with concentrates of volatiles prepared by vacuum distillation.[3,15] Nevertheless, there appears to be a close relationship between the concentration of the corresponding alcohol and its esters, at least for isopentyl alcohol,[3,14] though it may well be complex.[15,16] The length of post-climacteric storage, i.e., the RI, also greatly affects the concentration of volatiles.[15,17,18]

Acknowledgements

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Since submitting this paper for publication, the work of Ueda *et al.*[19] has become available. These authors also find that added isopentanol is converted into isopentyl acetate by ripe banana slices. The process is inhibited by iodoacetate or azide or by boiling the pulp. In nitrogen, as compared with in air, the extent of reaction was greatly reduced. The presence of acetate or acetylphosphate increased the formation of the ester, but CoA in presence of yeast extract and cofactors did not. Except for this last finding, on which Ueda *et al.* themselves threw doubt because of the presence of KF in the yeast extract, these results are in agreement with those presented here.

Drawert[20] has recently indicated that he too finds that added alkanoates can be vigorously converted into esters by banana discs.

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Fatty Acids of Boal Fish Oil by Urea Fractionation and Gas–Liquid Chromatography

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The fatty acid composition of body and liver fats of boal, Wallago attu (Schn.), a cat fish, belonging to the family Siluridae and commonly known as fresh-water shark has been determined by urea fractionation and gas–liquid chromatography (g.l.c.). The percentages of major component acids were found to be, 16:0, 10.5; 16:1ω9, 7.6; 18:0, 7.2; 18:1ω9, 17.4; 18:2ω9, 8.4; 18:3ω3, 6.1; 20:4ω3, 3.7 and 22:6ω3, 4.4. In addition, a number of minor component acids have been detected and estimated. The liver oil fatty acids have also been determined without fractionation and the percentages of major component acids found were 16:0, 23.5; 18:0, 12.7; 18:1ω9, 7.0; 20:4ω3, 13.7; 22:6ω3, 11.2.

The oil has been studied for the first time for its fatty acid composition.

1. Introduction

The boal fish, a cat fish belonging to the family Siluridae is most abundantly found in the eastern region of India and in Bangladesh. Among the fishes without scales in the eastern region of India, boal is perhaps the most widely available in various sizes and is used extensively as a cheap source of animal protein. In this respect, the fish is of considerable economic importance. It grows wild in fresh and slightly saline water, is carnivorous and is also known as "fresh water shark". The fatty acids of the boal fish oil have been studied in detail by urea fractionation and g.l.c. The liver fatty acids have also been studied without fractionation.

2. Experimental

The experimental techniques used consisted of (a) extraction of the lipid according to Bligh and Dyer;[1] (b) saponification and methylation of free fatty acids according to Metcalfe and Schmitz;[2] (c) urea fractionation of the mixed esters according to the counter current technique of Summerwell;[3] (d) hydrogenation[4] of aliquots of each fraction; (e) g.l.c. of fractions and their hydrogenated products; (f) peak identification; (g) quantitation by triangulation technique; and (h) u.v. and i.r. spectrophotometry. All experimental steps with the samples were carried out using nitrogen gas atmosphere whenever necessary.

2.1. Urea fractionation

The urea fractionation technique used was similar to that of Summerwell[3] based on the counter current distribution theory of Craig.[6] The distribution was carried out in a series of 100 ml erlenmeyers fitted with ground joints holding air condensers. Five crystallised fractions and a final filtrate fraction were obtained. The fractionation technique has been reported elsewhere.[11]

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2.2. Gas chromatography

Gas–liquid chromatography of the samples were performed by an F and M Model 700 gas chromatograph with dual column and dual flame ionisation detector. A stainless tube column, 6 ft × $\frac{1}{8}$ in, was packed with 15% DEGS on 100–200 mesh gaschrom-P obtained from Applied Science Laboratories, USA. Nitrogen carrier gas was used at 40 ml/min at 25 lb/in^2. The column temperature was 165°C and detector and injection port temperatures, 225°C.

2.3. Peak identification

The peaks were identified using (a) cod liver oil fatty acids as the secondary standard according to Ackman;[5] (b) semilogarithmic plots[8] drawn from the relative retentions and carbon chain length of the fatty acids in cod liver oil[10] and fitting the logarithm of relative retention times of the fatty acids under investigation into the prepared plots; and (c) hydrogenation data.

3. Results

A full grown fresh boal fish weighing 6.45 kg yielded 4.50 kg of lean flesh after separation from head, viscera and bones. The flesh was homogenised in a mincer and a 1 kg portion was extracted for the lipids according to Bligh and Dyer.[1] The liver weighing 68 g was extracted in a similar manner. The nonsaponifiables were determined according to the AOCS method. The percentages of oils, iodine values (Wij's) etc. are presented in Table 1. The urea fractionation technique is schematically represented in Figure 1 (C represents crystals and F represents filtrate). After crystallisation at

Table 1. Oil and nonsaponifiable contents and the iodine values of the oil, free fatty acid and mixed methyl esters of the body and liver fats of Wallago attu (Schn.)

	Body	Liver
1. Oil (%)	2.55	7.5
2. Iodine value of oil (Wij's)	118	151.0
3. Iodine value (fatty acids)	127	158.0
4. Iodine value (mixed methyl esters)	112	143.0
5. Nonsaponifiable (%)	3.2	2.5

each stage the filtrates were transferred to the next flask containing crystals and the combined F and C were then allowed to crystallise. This is indicated by the underlining of F and C. The transfers have always started from the highest numbered flasks and ended at the first one. The relative retention times (RRT) of various esters were determined in relation to oleic acid (18:1 ω9 = 1.00). The equivalent chain length (ECL) values are obtained by fitting the logarithm of retention times of esters in a standard curve of logarithm of retention times against carbon chain lengths drawn from the retention times of known saturated esters.[7] The u.v. and i.r. spectrum of the mixed methyl ester did not show the presence of any bands due to conjugated or trans double bonds nor any band due to other classes of fatty acids. The fatty acid compositions of nonfractionated body and liver fats are presented in Table 2. Table 3 shows the fatty acid compositions of the six urea fractions of the body fatty acid methyl esters and their hydrogenated products. It also shows the overall fatty acid composition computed from the composition of the fractions and the RRT and ECL of the various fatty acids.

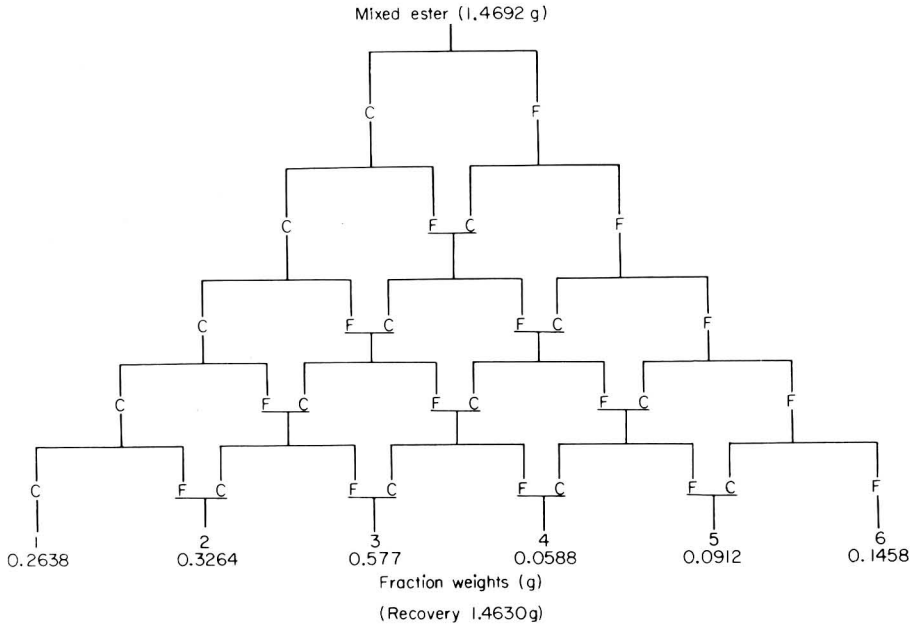

Figure 1. Schematic representation of urea fractionation procedure.

4. Discussion

The polyenoic acids of animal origin particularly of fishes are remarkably homogeneous in the sense that they contain methylene interrupted cis-double bonds. Under the circumstances their identification becomes relatively systematic. In the present study the spectral measurements did not indicate any other type of unsaturation and they were identified through secondary standards and semilogarithmic plots following Ackman's procedures.[5,8] Although a very detailed composition of the fractionated fatty acid esters of the body fat has been obtained, owing to the complexity of marine oil fatty acids it is possible that esters with identical retentions may have been eluted simultaneously from the column and recorded as single peaks. Such critical separations are possible for marine oil fatty acids with open tubular columns.[9] The monoenoic acids in most cases have been represented by the most probable isomers found in fish oils, although it is likely that others may be present[9] and could not be separated by the g.l.c. column used. In some cases, particularly with monoenes, it was not possible to assign any probable structure. Among the acids found, the acids having identical ECL values and forming critical pairs are, 13:1, 14–iso; 16:2ω4, 17:1; 16:4ω3, 19:1; 18:3ω3, 20:1; 18:4ω6, 20:2ω9; 20:3ω6, 22:0; 20:4ω6, 22:1; 21:4ω6, 22:3ω6; and 20:5ω3, 24:0. It is evident from Table 3 that owing to the presence of different degrees of unsaturations among these pairs, they have been mostly crystallised in different fractions and did not produce overlapped peaks. Moreover, as such pairs contained fatty acids of different chain lengths, on hydrogenation they formed saturated acids of different chain lengths which were identified by comparison of retention times with authentic acids and also by cochromatography with the latter acids. Finally, the structure assignments of such acids were confirmed by comparison of the compositions of the nonhydrogenated and hydrogenated samples. The g.l.c. responses of the critical pairs were obviously overlapped in the analyses of the nonfractionated body and liver oil fatty acids and were estimated by comparing the analyses of nonhydrogenated and hydrogenated samples. Considerable errors were found in the quantitation of minor components, whereas in the case of major components, the errors were tolerable. The identifications were also supplemented by the retention data of the cod liver oil fatty acids studied in this laboratory.[10]

Table 2. Fatty acid composition of mixed methyl esters of liver and body fats of Wallago attu (Schn.), expressed as w/w %

Component acids[a,b]	Liver		Body	
	NH	H	NH	H
10:0	—	—	0.2	0.1
11:0	—	—	0.3	0.2
12:0	—	—	0.7	1.0
12:1ω?	—	—	0.4	—
13:0	—	—	0.4	1.1
13:1ω?	—	—	0.5	—
14:0	Tr	—	3.1	4.1
14:1ω?	—	—	1.4	—
15:0	Tr	—	1.9	2.3
15:1ω?	—	—	0.8	—
16:0	23.5	26.8	10.7	18.4
16:1ω9[c]	3.9	—	7.3	—
17:0	—	—	2.9	4.8
17:1ω?	—	—	2.2	—
18:0	12.8	36.5	5.7	45.8
18:1ω9[c]	17.0	—	19.9	—
18:2ω9	3.8	—	10.0	—
18:3ω3	1.5	—	6.7	—
18:4ω3	1.4	—	3.0	—
19:0	—	—	1.5	1.2
20:0	—	14.0	1.0	13.9
20:1ω9[c]	1.0	—	3.2	—
20:2ω9	—	—	1.1	—
20:3ω6	—	—	1.2	—
20:4ω6	—	—	0.8	
20:4ω3	13.7	—	4.4	—
20:5ω3	—	—	1.5	—
22:0	1.1	22.1	—	7.0
22:4ω6	4.3	—	1.2	—
22:5ω6	3.6	—	1.6	—
22:5ω3	1.1	—	1.7	—
22:6ω3	11.2	—	3.1	—

[a] Short hand notation implies chain length:number of double bonds.

[b] The ω-values indicate the methyl end chain from the centre of the double bond furthest removed from carboxyl end.

[c] Other isomers may be present.

Tr, Trace quantity present but could not be estimated.

NH, Nonhydrogenated sample.

H, Hydrogenated aliquot of the sample.

The body and liver fats contained fatty acids typical of marine origin. The liver contained higher unsaturated acids in larger amounts. It is interesting to note that 16:1ω9, 18:2ω9 and 18:3ω3 are present in larger amounts in body fat and 16:0 and 18:0 in larger amounts in liver fats.

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Table 3. Weight % composition, RRT and ECL of various fatty acids in the different urea fractions and their hydrogenated products of the body fatty acids of Wallago attu (Schn.)

Component acids[a,b]	(1)[d]		(2)		(3)		(4)		(5)		(6)		% Total	RRT	ECL
	NH	H	NH	H	NH	H	NH	H	NH	H	NH	H			
10:0	0.4	0.3	0.3	0.1	0.1	0.1							0.2	0.08	11.0
11:0	0.5	0.4	0.4	0.2	0.2	0.2							0.3	0.10	11.0
12:0	0.9	1.0	1.2	1.0	1.1	1.0	1.0	1.7		1.0		1.3	0.9	0.14	12.0
12:1ω?					0.1		0.6		1.1		1.5		0.3	0.15	12.3
13-iso					0.1	0.1	0.2	0.1					0.1	0.16	12.8
13:0	1.0	0.8	1.3	1.2	0.4	0.5	0.4	1.1		0.4			0.6	0.19	13.0
13:1ω?			0.2		0.3		0.9		0.6				0.3	0.22	13.6
14-iso	0.8	0.7	0.2	0.1	0.1	0.1							0.2	0.22	13.6
14:0	5.8	5.5	6.2	6.6	3.1	4.5	2.6	4.5	0.9	2.0	2.0	3.0	4.0	0.25	14.0
14:1ω?			1.1		1.7		2.2		1.1		1.2		1.2	0.29	14.5
14:2ω?									0.3				0.1	0.37	15.3
15-iso	1.5	1.3	0.5	0.3	0.3	0.2	0.2	0.1					0.5	0.30	14.6
15:0	5.6	5.5	3.5	4.0	1.2	2.0	1.3	2.3	0.7	0.8		0.7	2.3	0.33	15.0
15:1ω?			1.1		1.0		1.1		0.4		0.8		0.8	0.40	15.6
16-iso			0.2	0.2	0.1	0.1	0.1	0.1					0.1	0.40	15.6
16:0	22.4	26.4	16.0	26.5	7.7	20.8	3.6	14.2		6.8		7.3	10.5	0.46	16.0
16:1ω9[c]	3.1		8.9		11.6		5.6		2.1		1.3		7.6	0.55	16.6
16:2ω7[c]					0.9		2.3		2.6		2.6		0.9	0.60	16.9
16:2ω4									0.7		0.7		0.1	0.70	17.4
16:3ω?							1.4		0.3		0.6		0.1	0.80	17.8
16:4ω3							1.2		1.7		2.6		0.4	1.30	19.4
17-iso	2.2	2.3	0.2	0.1	0.1	0.1	0.1	0.1					0.5	0.55	16.6
17:0	12.7	12.5	4.3	6.1	1.6	3.1	1.4	4.0		1.0			3.9	0.63	17.0
17:1ω?	1.0		2.0		2.2		3.1		1.3				1.7	0.73	17.4
18:0	19.8	32.0	12.3	43.9	2.2	55.1		40.2		26.2		21.9	7.2	0.85	18.0
18:1ω9[c]	9.2		26.4		22.0		14.5		4.4		4.5		17.7	1.00	18.5
18:2ω9	0.8		3.2		16.4		11.5		6.6		1.9		8.4	1.10	18.9
18:3ω6					0.3		0.5		1.9		3.0		0.5	1.50	19.9
18:3ω3					12.1		10.9		8.6		3.5		6.1	1.75	20.4
18:4ω3					0.8		1.9		5.0		8.3		1.5	2.10	21.0
19:0	1.9	2.8	1.0	1.7						0.2		0.7	0.6	1.15	19.0
19:1ω?	0.8		1.0										0.4	1.30	19.4
19:4ω6									0.5		0.7		0.1	3.10	22.3
20:0	2.3	5.6	0.5	6.0		6.9		17.6		25.1		24.8	0.5	1.55	20.0
20:1ω9[c]	4.0		6.1										2.1	1.75	20.4
20:2ω9					1.6		1.1						0.7	2.00	20.85
20:3ω6					1.1		2.0		2.1		1.0		0.8	2.75	21.95
20:4ω6					0.9		2.3		2.6		2.4		0.8	3.65	22.8
20:4ω3					2.9		8.6		13.7		3.7		3.7	4.10	23.5
20:5ω3					0.8		3.0		7.2		8.7		1.8	5.30	24.0
21:0	0.8	0.8	0.4	1.1				0.2				0.2	0.2	2.00	21.0
21:1ω?	0.2		0.9										0.2	2.30	21.3
21:4ω6							0.3				0.4		0.1	4.90	23.8
22:0	0.2	0.7	0.3	0.5		4.8		14.0		35.9		39.9	0.1	2.80	22.0
22:1ω11[c]	0.5		0.3										0.2	3.60	22.7
22:3ω6					0.3		0.5						0.1	5.00	23.8
22:4ω6					1.5		2.6		2.1		0.5		0.9	6.50	24.7
22:5ω6					0.8		2.5		5.9		6.9		1.5	8.20	25.5
22:5ω3					2.0		3.9		4.9		1.7		1.4	9.50	26.0
22:6ω3					0.4		5.2		20.7		29.7		4.4	10.50	26.2
23:0	1.0	0.9											0.2	3.70	23.0
24:0	0.4	0.5											0.1	5.20	24.0
24:1ω9[c]	0.2												0.1	5.80	24.3

[a] Short hand notation implies chain length:number of double bonds.
[b] The ω-values indicate methyl end chain from centre of the double bond furthest removed from carboxyl end.
[c] Other isomers may be present.
[d] Fraction number.
NH, Non-hydrogenated fraction. H, Hydrogenated aliquot of the same fraction.

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Triglyceride Composition of *Sesamum indicum* Seed Oil

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The fatty acid composition of *Sesamum indicum* seed oil was determined by gas–liquid chromatography. The percentages of individual acids were found to be: palmitic, 11; stearic, 6; arachidic, 1; oleic, 43; linoleic, 39. Triglyceride composition was calculated from the fatty acid compositions of the native oil and of the monoglycerides produced from it by pancreatic lipase hydrolysis. The oil is composed of 8, 41 and 51%, GS_2U, GSU_2 and GU_3 respectively. Analysis by special thin-layer chromatography combined with densitometry also indicated similar triglyceride composition for the sesame seed oil. Changes effected on the triglyceride composition by randomisation were also determined by both methods of analysis.

1. Introduction

Sesame oil, obtained from the seeds of *Sesamum indicum* (oil content 44–54%) is of great commercial significance. Nearly a third of the world's crop of sesame is produced in India. In 1971–72, the quantity of sesame oil produced in India was of the order of 143 000 tons. Sesame oil, an edible oil, is readily processed to produce a stable product with good colour and good resistance to the development of rancidity. In the indigenous system of medicine, sesame oil is consumed in large quantities as an ingredient of the oil-based formulation.

The fatty acid composition of sesame oil has been determined earlier by different groups of workers.[1–8] Techniques like low temperature crystallisation, spectrophotometric analysis for polyethenoid acids, urea complexation and gas–liquid chromatography, mostly in combination have been used in all of these earlier investigations. Reported compositions vary within certain ranges. Oleic acid (35–45%) and linoleic acid (37–47%) have been reported to be the unsaturated constituents while palmitic (8–15%) and stearic acids (3–5%) represent the saturated constituents. These variations in composition may be explained by the limitations of the experimental techniques used or by the influence of environmental factors. Though the fatty acid composition of this commercially important oil has been investigated by different groups of workers, studies on its glyceride composition have not been carried out so intensively.[3,6] Chakrabarty and Hilditch,[6] using the technique of segregation by low-temperature crystallisation, found that the triglycerides of sesame oil constituted 39% oleodilinolein, 15% dioleolinolein and 37% saturated oleo linolein. Hilditch observed that sesame oil, because it contains over 40% of glycerides in which two linoleic groups are present, should exhibit *prima facie* some degree of proneness to oxidative rancidity. But sesame oil has a good reputation for being resistant to oxidative rancidity, a feature which has been attributed to the antioxidant properties of the small proportion of sesamol[9] present in the unsaponifiable fraction of the oil. However, limitations of the method based on segregation by low temperature crystallisation in the study of triglyceride composition of linoleic rich oil have been mentioned by Hilditch and Williams.[10] This technique is now considered to be inadequate for the study of the triglyceride composition of seed oils containing a high percentage of unsaturated acids, though Hilditch and Williams[10] found the technique to be quite satisfactory for fats with less than 50% linoleic acid content. Recently developed techniques like selective enzymic hydrolysis and thin-layer chromatography in combination give more definite information about the triglycerides composition.

Barrett *et al.*[11] analysed cotton seed oil (linoleic acid 47% and oleic acid 22%) by a special thin-layer chromatographic technique combined with densitometry and by selective enzymic hydrolysis. Cotton seed oil with a linoleic acid content similar to that of sesame oil, showed on investigation by Barrett and coworkers[11] a considerable deviation from the triglycerides composition determined earlier by Hilditch and Maddison[12] using low temperature crystallisation as the method of segregation. The percentages of triglycerides with four, five or six double bonds in the cotton seed oil were determined by the later workers to be 27, 13 and 15 respectively, while the corresponding percentages reported by Hilditch and Maddison[12] were 18, 28 and 0. It is also of significance that using the method of low temperature crystallisation Hilditch and coworkers[6, 12] reported the contents of dilinoleo glycerides to be 44 and 46% in sesame and cottonseed oils respectively, the percentage content of linoleic acid being identical, (47%) in both samples.

The triglyceride composition of sesame oil has not been investigated by the recently developed techniques. The present communication reports the determination of triglyceride composition of sesame oil of Indian origin by techniques of selective enzymic hydrolysis and by special thin-layer chromatography combined with densitometry preceded by the determination of the mixed fatty acid composition by combined techniques of urea complexation[13] and gas–liquid chromatography. The investigation was also extended to randomised oil to study the changes in the triglyceride composition effected by interesterification.

2. Experimental procedures

2.1. Materials and methods

Sesamum indicum seeds were procured from the local market. Lipolysis was carried out as suggested by Coleman[14] at pH 8.5 and 37.5°C using a purified pork pancreatic lipase preparation with the addition of Ca^{++} ions and bile salts. The partial glycerides were separated on a thin layer (0.3–0.4 mm) of silica by developing with a solvent system of n-hexane–diethyl ether–acetic acid (80:20:0.25). The monoglyceride fraction thus isolated and the original triglyceride were converted into methyl esters by the semimicro method of Luddy *et al.*[15] Gas–liquid chromatography of methyl esters was carried out with an F and M analytical gas chromatograph (Model 700-R-12) equipped with a flame ionisation detector. The column (6 ft × $\frac{1}{4}$ in) packed with 10% polyester of diethylene glycol adipate on 60–80 mesh Gas chrom Z, was operated at 166°C with a carrier gas flow of 40 ml/min. Peak areas were determined as the product of peak height and the width at half height; the weight percentages obtained were converted to mole percentages. For densitometric analysis, the native oil and its randomised product were fractionated on a thin layer of silica gel impregnated with silver nitrate using the solvent system n-hexane–diethyl ether–acetic acid (80:20:0.25). The different fractions thus separated into classes according to their degree of unsaturation, were determined by densitometric analysis as developed by Barrett *et al.*[11] A photo volt Multiplier Photometer Model 520A which is fitted with a stage and a motor for automatic transportation of 20 × 20 cm chromatoplates across the slit and is also coupled with recorder and integrator, was used for the purpose.

Randomisation of the native oil was carried out by the process suggested by Chakrabarty and Bhattacharya.[16] To a solution of 50 g of refined and bleached oil in 75 ml of n-hexane was added drop by drop, the catalyst sodium methoxide (methanolic solution, 0.4% w/w of the oil solution). Rearrangement was continued to the equilibrium state. After 60 min the randomised sample was isolated by the usual procedure after destroying the catalyst with dilute hydrochloric acid.

3. Results

On extraction with petroleum ether (b.p. 40–60°C), *Sesamum indicum* seeds yielded 35% of a light greenish yellow oil. On analysis by standard procedures, the oil obtained from it showed the following characteristics: free acid (10.8% as oleic), refractive index at 30°C (1.4700), specific

gravity at 30°C (0.9122), unsaponifiable (1.9%), saponification equivalent (298.6), Iodine value (110.3 by Wij's 30 min) and acetyl value (14.0).

The mixed fatty acids (100.6 g), free from non-saponifiables, (saponification equivalent—281.0; Iodine value—113.3) were next segregated into four fractions (A–D) by urea complexation techniques, increasing stepwise the proportion of urea to fatty acids. Methyl esters of the mixed fatty acids of *Sesamum indicum* seed oil and also of the fractions (A–D) were analysed by g.l.c. and the results are shown in Table 1.

Table 1. Fatty acid composition (wt %) of *Sesamum indicum* mixed fatty acids and of the fractions A, B, C and D obtained by urea complexation

Sample	% Yield	Iodine value	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{18:1}$	$C_{18:2}$
MFA	—	113.3	11.5	6.6	—	42.2	39.7
A	4.1	21.6	29.9	39.6	8.8	18.0	3.7
B	18.9	48.3	33.6	19.8	2.9	36.1	7.6
C	26.1	96.3	9.7	0.9	—	73.9	15.5
D	50.9	150.8	0.7	0.8	—	32.6	65.9
Computed values(A–D)	—	111.9	10.5	6.0	0.9	43.4	39.2

Computed values for MFA. Iodine values—112.0. Saponification equivalent—278.7.

The fatty acid compositions of the 2-monoglycerides obtained from lipolysis of the *Sesamum indicum* seed oil and its interesterified product were determined by g.l.c. The results along with the fatty acid composition (mol %) of the triglycerides are given in Table 2.

Table 2. Fatty acid composition (mol %)

Sample	Fatty acids			
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Sesamum indicum triglycerides	12.5	6.5	41.6	39.4
2-Monoglycerides from:				
(a) Native seed oil	1.6	—	40.5	57.9
(b) Randomised oil	6.8	3.7	38.6	50.9

The triglyceride compositions of *Sesamum indicum* seed oil and its randomised product were calculated from the fatty acids composition of the original triglycerides and the 2-mono lycerides formed, using the assumption of Vanderwal,[17] and Coleman.[14] The results are given in Table 3.

Table 3. Triglycerides composition[a] (mol %) of *Sesamum indicum* seed oil and its interesterified product

Glycerides[b]	SSS	SSU	SUS	USU	SUU	UUU
Seed oil	0.1	0.6	7.6	0.9	39.6	51.2
Randomised oil	0.5	3.8	4.8	6.2	32.0	52.7

[a] P—$C_{16:0}$ O—$C_{18:1}$ and L—$C_{18:2}$

[b] Major glycerides include (figures in parentheses refer to randomised oil): POO 6.2 (5.1), PLO (8.8) (6.8), PLL 6.2 (5.2), OOO 7.1 (7.1), OOL 10.2 (11.2), OLO 10.3 (9.5), OLL 14.6 (14.8) and LLL 5.3 (5.7).

The native seed oil of *S. indicum* and its randomised product were fractionated on a thin layer of silica gel impregnated with silver nitrate using the solvent system n-hexane–diethyl ether–acetic acid (80:20:0.25). The proportion of different fractions thus separated into classes according to their degree of unsaturation were determined by densitometric analysis as developed by Barrett *et al.*[11] Results are given in Table 4.

Table 4. Percentage triglycerides composition[a] of *Sesamum indicum* seed oil

Sample	Method	S_3	U_1	U_2	U_3	U_4	U_5	U_6
Seed oil	t.l.c.	—	2.9	19.3	28.7	29.0	14.4	5.7
	SEH	0.1	3.5	14.6	27.9	30.3	18.3	5.3
Randomised oil	t.l.c.	—	1.2	10.5	31.1	33.7	21.1	2.4
	SEH	0.5	4.2	14.1	26.4	29.9	19.2	5.7

[a] S_3, U_1, U_2, U_3, U_4, U_5 and U_6: saturated triglycerides and triglycerides with respectively 1, 2, 3, 4, 5 or 6 double bonds.

4. Discussion

The component acid analyses of *S. indicum* are summarised in Table 5. The present results are in general agreement with those already reported and the somewhat different findings of Mehta *et al.*[7] may arise from the experimental procedures used.

Table 5. Fatty acids composition[a] of the seed oil of *S. indicum*

	Saturated acids (% wt)			Unsaturated acids (% wt)	
Habitat	$C_{16:0}$	$C_{18:0}$	$C_{20:0}$	$C_{18:1}$	$C_{18:2}$
China[1]	7.8	4.7	0.4	49.4	37.7
Siberia[2]	← 16.0	→	—	37.0	47.0
India[3]	9.1	4.3	0.8	45.4	40.4
India[4]	8.2	3.6	1.1	45.3	41.2 (a)
Nicaragua[5]	← 14.3	→	—	41.7	44.0
India[6]	9.4	5.7	1.2	35.0	48.4 (b)
India[7]	← 6.7	→	—	22.5	67.2 (c)
India[8]	14.8	5.7	0.4	39.5	37.5 (d)
USA[18]	← 12.9	→	—	41.6	43.8
Source not stated[19]	10.0	5.0	—	40.0	43.0 (e)
Present work	10.5	6.0	0.9	43.4	39.2

[a] The minor component acids recorded were as follows (% wt): (a) $C_{14:0}$, 0.1; $C_{16:1}$, 0.5; (b) $C_{14:0}$, 0.3; (c) $C_{18:3}$, 3.6; (d) $C_{16:1}$, 0.4; $C_{18:3}$, 1.1; (e) $C_{18:3}$, 2.

The triglyceride composition of sesame seed oil has been examined earlier in detail by Chakrabarty and Hilditch[6] using the crystallisation technique. Recently, triglyceride compositions of five samples of sesame oil determined by gas chromatography indicate that the oil is constituted mainly of C_{50}, C_{52} and C_{54} triglycerides.[21] The percentage content of these major components has been reported as 3–4.5, 23–28 and 68–74 respectively.

The triglyceride composition of sesame seed oil determined by Chakrabarty and Hilditch[6] and in this work is compared in Table 6. The investigation shows a wide variation from that determined by Chakrabarty and Hilditch.[6] The present investigation shows a much lower content of oleo-linolein. Similarly the content of glycerides in which two linoleic groups are present has been found to be 28% in the present instance while Hilditch and coworkers[6] reported the content to be more than 40%. This investigation has also indicated that the sesame oil contains 7% triolein and 5% trilinolein. Furthermore, it becomes evident from Table 3, that the content of SSU, SUS, USU and SUU changed from 1, 8, 1 and 40% to 4, 5, 6 and 32% respectively by the process of randomisation carried out for 60 min.

In the present investigation sesame oil has also been analysed by special thin-layer chromatography combined with densitometry.[11] The triglycerides composition of sesame oil and of its randomised product as determined by this technique is presented in Table 4 along with the composi-

Table 6. Triglyceride composition of *Sesamum indicum* seed oil

Glycerides[a]	Mol %: Chakrabarty and Hilditch[6]	Mol %: Present work
SSS	—	Trace
OSS, SOS	3	4
SSL, SLS	1	5
SOO, OSO	—	10
OSL, SOL, SLO	37	21
SLL, LSL	4	10
OOO	—	7
OOL, OLO	15	20
OLL, LOL	39	18
LLL	1	5

[a] S, saturated; O, $C_{18:1}$; L, $C_{18:2}$.

tion determined by the methods of selective enzymatic hydrolysis. There is general agreement between t.l.c. and SEH results.

Sesame seed oil was studied by Mattson and Volpenhein[20] by an enzymatic hydrolysis method for the composition of fatty acids acylating the 2-position of glycerides. The present findings which are similar to those of Mattson and Volpenhein[20] agree well with the hypothesis of Gunstone[22] that the 2-position of the triglyceride is preferentially esterified with C_{18} unsaturated acids.

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Metal Contaminants in Various Food Colours

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Arsenic, cadmium, chromium, cobalt, copper, lead, manganese, nickel and zinc have been estimated in 18 permitted and 18 non-permitted food colours. Arsenic, chromium, copper and lead were found to be within the maximum allowable international limits in all the permitted colours, but they were in excess in a number of non-permitted colours. There is a case for establishing the maximum allowable concentration (MAC) of cadmium, cobalt, manganese, nickel and zinc in food colours.

1. Introduction

At a meeting of the Joint FAO/WHO Expert Committee on Food Additives, the need for establishing international specifications for the identity and purity of food colours was emphasised.[1] Impurities in food colours may arise from the raw materials, the equipment used during their manufacture or the containers used for storage and packing. In respect of metallic impurities, the maximum allowable concentration (MAC) has already been prescribed[2,3] for arsenic (3 parts/10^6), chromium (20 parts/10^6), copper (10 parts/10^6) and lead (10 parts/10^6). However, no such limit has been fixed for cadmium, cobalt, manganese, nickel and zinc, which may find their way into food colours owing to unhygienic manufacturing practice. In the present communication, therefore, an attempt has been made to evaluate the extent of contamination of various dyes with the above metals.

2. Experimental

2.1. Materials

Thirty-eight colours (minimum dye content 85%) were examined for their contents of arsenic, cadmium, chromium, cobalt, copper, lead, manganese, nickel and zinc. The colours included nine single colours (amaranth, carmoisine, erythrosine, fast red E, green S, indigo carmine, ponceau 4R, sunset yellow FCF and tartrazine), and nine blends (apple green, chocolate brown, egg yellow, lemon yellow, lime juice yellow, pineapple yellow, raspberry red, rose pink and vino deep) permitted by the Government of India,[3] and 18 non-permitted colours (acid magenta, auramine, blue VRS, brilliant crocein scarlet, butter yellow, congo red, fluorescein, green crystal, malachite green, metanil yellow, methyl violet, nigrosine, orange II, orange red, red 6B, rhodamine B, sudan II and sudan III). These 18 non-permitted colours were detected by us earlier in as many as 70% of the coloured eatables examined during a ten-year survey of 12 575 foodstuff samples collected from various parts of Uttar Pradesh.[4]

2.2. Apparatus

A Perkin-Elmer double beam atomic absorption Spectrophotometer Model-303, equipped with a Boling air-acetylene burner was used.

2.3. Methods

For the determination of metals other than arsenic, a 1 g sample of the dye was weighed accurately in a silica crucible and heated first at a low temperature and then at 500°C for 5 h in a muffle furnace. The ash was wetted with glass-distilled water and again heated at 500°C for 2 h. The carbon-free residue was dissolved in 0.25 ml warm concentrated AnalaR hydrochloric acid and the volume made up to 25 ml with double glass-distilled water. For arsenic content, a 1 g dye sample was digested in Kjeldahl flasks with a mixture of nitric acid–sulphuric acid (4:1, v/v). The clear solution was finally diluted to 10 ml with 5% (v/v) nitric acid. Standard curves were prepared for each metal, along with the test samples.

3. Results

3.1. Precision of the analytical technique and detection limits

Precision of the technique was assessed by analysing replicates and determining percentage recoveries. The standard deviation for the 10 replicates was between 0.4–1.3% at 10 parts/10^6 and 0.8–4.3% at 1 part/10^6 level. With the exception of arsenic, recoveries of various metals were between 95.6–99.4%. In the case of arsenic, the percentage recovery was between 80.4 and 88.6. The detection limits were 0.1 part/10^6 for cadmium, chromium, cobalt, copper, manganese, nickel and zinc, 0.5 part/10^6 for lead and 1 part/10^6 for arsenic. The concentration of metals in various permitted and non-permitted colours are given in Table 1.

Table 1. Metal concentration (parts/10^6) in permitted and non-permitted food colours

	As	Cd	Cr	Co	Cu	Mn	Ni	Pb	Zn
Permitted single[a]									
Amaranth (CI Food Red 9; 16185)	1.0	3.8	3.1	2.5	4.5	2.1	3.2	6.0	6.1
Carmoisine (CI Food Red 3; 14720)	3.0	5.0	3.8	2.6	4.0	2.2	4.0	7.2	2.6
Erythrosine (CI Food Red 14; 45430)	2.3	7.6	4.4	2.3	4.5	1.4	3.6	7.8	1.8
Fast red E (CI Food Red 4; 16045)	3.0	7.6	1.2	1.7	3.0	2.0	4.0	2.6	4.1
Green S (CI Food Green 4; 44090)	1.0	15.4	3.7	4.9	0.5	1.4	3.4	0.0	4.5
Indigo carmine (CI Food Blue 1; 73015)	2.0	14.7	4.0	2.9	4.5	2.2	6.3	7.8	3.7
Ponceau 4R (CI Food Red 7; 16255)	2.0	2 7	3.8	2.9	2.3	1.9	2.5	1.8	4.3
Sunset yellow FCF (CI Food yellow 3; 15985)	1.0	3.5	3.8	3.8	3.4	1.7	1.8	5.3	1.2
Tartrazine (CI Food yellow 4; 19140)	3.0	3.5	3.4	2.9	6.1	1.5	1.7	4.4	2.2
Permitted blend[b]									
Apple green (tartrazine + indigo carmine)	1.0	22.4	4.4	3.6	2.3	1.9	4.3	7.8	3.7
Chocolate brown (tartrazine + indigo carmine + carmoisine)	2.0	5.4	7.4	4.0	2.3	4.7	3.9	7.8	2.8
Egg yellow (tartrazine + sunset yellow + carmoisine)	2.8	2.3	3.7	1.8	2.3	1.9	3.9	0.0	1.4
Lemon yellow (tartrazine + sunset yellow)	1.0	24.7	6.5	8.3	3.9	1.6	5.7	0.0	1.9
Lime juice yellow (tartrazine + sunset yellow)	1.5	6.8	4.4	3.6	4.5	2.6	3.4	2.4	2.8
Pineapple yellow (tartrazine + indigo carmine	3.0	11.5	2.9	5.5	2.3	3.7	11.7	5.4	1.4

Table 1 *continued*

	As	Cd	Cr	Co	Cu	Mn	Ni	Pb	Zn
Raspberry Red (amaranth + fast red E)	2.5	5.4	3.7	3.6	4.5	3.3	2.9	0.0	1.4
Rose pink (ponceau 4R + amaranth)	2.5	13.8	3.7	3.7	2.3	3.2	4.3	0.0	3.7
Vino deep (tartrazine + indigo carmine + carmoisine)	3.0	2.3	7.4	1.2	4.5	7.0	16.1	7.8	9.7
Non-permitted single[a]									
Acid magenta (CI Acid Violet 19; 42685)	2.5	26.2	2.9	8.0	3.2	1.6	8.1	0.0	2.9
Auramine (CI Basic yellow 2; 41000)	2.0	1.6	4.4	2.2	4.5	0.7	4.3	15.6	2.8
Blue VRS (CI Food Blue 3; 42045)	3.8	5.4	49.2	0.9	2.3	1.4	1.9	18.6	2.8
Brilliant Crocein Scarlet (CI Acid Red 23; 27290)	2.0	3.0	7.4	4.0	7.7	10.0	30.6	13.8	18.0
Butter yellow (CI Solvent yellow 2; 11020)	3.3	2.3	3.7	1.2	2.3	1.9	5.3	7.8	1.8
Congo red (CI Direct Red 28; 22120)	1.8	21.5	5.9	8.3	5.0	3.3	8.1	0.0	28.4
Fluorescein (CI Acid yellow 73; 45350)	5.6	8.4	5.2	0.9	2.3	0.9	7.3	5.4	2.8
Malachite Green (CI Basic Green 4; 42000)	5.0	7.7	8.0	0.6	2.3	0.9	2.9	16.4	7.3
Metanil yellow (CI Acid yellow 36; 13065)	2.0	5.3	4.0	0.6	2.3	1.4	4.6	7.8	1.8
Methyl violet (CI Basic violet 1; 42535)	2.6	2.7	4.4	2.5	21.4	2.1	4.1	9.0	32.2
Nigrosine (CI Acid Black 2; 50420)	4.0	6.1	19.9	3.1	18.2	0.9	5.7	7.8	58.2
Orange II (CI Acid Orange 7; 15510)	3.2	8.4	4.4	0.6	15.9	1.9	4.8	0.0	2.8
Red 6B (CI Food Red 11; 18055)	2.6	9.1	2.9	2.7	2.1	1.9	2.4	0.0	1.7
Rhodamine B (CI Food Red 15; 45170)	7.0	6.1	4.4	4.3	11.4	1.2	4.3	0.0	1.4
Sudan II (CI Solvent Orange 7; 12140)	4.0	2.3	4.4	1.5	2.3	2.3	3.9	7.8	1.8
Sudan III (CI Solvent Red 23; 26100)	6.0	3.9	3.7	1.2	2.3	0.9	3.9	34.2	1.8
Non-permitted blend[b]									
Green Crystal (auramine + blue VRS)	4.0	3.0	5.2	2.7	4.5	0.9	1.4	7.8	7.8
Orange Red (Orange II + carmoisine)	6.0	20.8	5.9	4.3	4.5	3.2	6.3	0.0	1.4

[a] Common and CI name and number (1956).
[b] Blends are the mixture of two or more colours.

3.2. Permitted colours

The concentrations of arsenic, chromium, copper and lead in all the permitted colours (single or blend) were found to be within the prescribed limits. The amounts of cobalt, manganese, zinc and nickel (with the exception of pineapple yellow and vino deep) were within 10 parts/10^6. Cadmium, which ranged between 2.3 and 24.7 parts/10^6, was more than 10 parts/10^6 in green S, indigo carmine, apple green, lemon yellow, pineapple yellow and rose pink.

3.3. Non-permitted colours

Arsenic content in blue VRS, butter yellow, fluorescein, malachite green, nigrosine, orange II, rhodamine B, sudan II, sudan III, green crystal and orange red ranged between 3.2 and 7.0 parts/10^6, the maximum allowable limit being 3 parts/10^6. Copper levels, which ranged from 2.1 to 21.4 parts/10^6, exceeded the prescribed limit of 10 parts/10^6 in methyl violet, nigrosine, orange II and rhodamine B. Lead varied between 0 and 34.2 parts/10^6. Five dyes, namely, auramine, blue VRS, brilliant crocein scarlet, malachite green and sudan III had more than 10 parts/10^6 of lead, the permissible limit. Chromium values, except in the case of blue VRS (49.2 parts/10^6), were within the limit of 20 parts/10^6. Nickel concentration was within 10 parts/10^6 in all the colours, except brilliant crocein scarlet which contained 30.6 parts/10^6. Cadmium content, except in acid magenta (26.2), congo red (21.5) and orange red (20.8) and zinc content except in brilliant crocein scarlet (18.0), congo red (28.4) and nigrosine (58.2), were within 10 parts/10^6. Cobalt and manganese in all the colours ranged from 0 to 10 parts/10^6.

4. Discussion

The results indicate that appreciable amounts of various metals are present in food colours. Excess of even essential metals like chromium, copper, cobalt, manganese and zinc is toxic. Chromium causes ulcerative dermatitis[5,6] and pulmonary carcinoma;[7,8] copper leads to brass chills[9] and cobalt to polycythemia, heart failure[10] and even malignant tumours.[11–13] Excessive manganese results in neurological syndromes,[14] pneumonitis and sexual disturbances[5,15,16] and zinc in metal fume fever.[10,16] Among the non-essential metals, cadmium has an extremely long biological half-life; even low exposures are known to result in considerable accumulation leading to lung and kidney[17–19] damage and carcinomas.[20–22] Similarly, reports of nickel causing dermatitis,[23] respiratory disorders and carcinoma[24,25] are also known.

The main considerations for food colour contaminants should thus be (a) will it jeopardise the safe use or usefulness of the colour, (b) will it be avoided by good manufacturing practice, and (c) is it of sufficient consequence to justify a limitation. According to the report of the National Academy of Sciences, Washington,[26] the average annual per capita consumption of synthetic food colours is about 5.5 g. The maximum and average daily per capita intake were calculated to be 53.5 and 15 mg respectively. Thus although food colours are employed in relatively small quantities and traces of impurities may pose no immediate serious health hazards, prudence dictates that limits be established for impurities such as cadmium, cobalt, manganese, nickel and zinc also. Moreover, restrictions at this stage will encourage the industry to select good quality raw materials, processing equipment and containers.

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Nutritional Value of Soya Protein and Milk Coprecipitates in Sausage Products

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Sausages containing 60% of meat proteins replaced with soybean or milk calcium coprecipitates were prepared. Growth rates and plasma amino acid levels in rats fed diets based on these products, together with vitamin and available amino acid analysis indicated that the nutritional values of the non-meat proteins were similar but were both markedly lower than meat sausages. Nutritional damage to the protein in the sausages during cooking was only apparent in the all-meat sausage.

1. Introduction

In recent years there has been an increase in the use of proteins derived from meat and vegetable sources to replace meat in foods. The texture and flavour of meat are the most difficult properties to simulate and the greatest success has been with comminuted foods such as sausages and small-goods.

Thomas *et al.*[1] replaced up to 60% of the meat in sausage with milk coprecipitates, soya protein preparations and sodium caseinate without affecting the emulsion stability, water retention or organoleptic properties of the sausages.

With the development of improved stabilisers and flavourings it should be possible to replace all of the meat in comminuted foods with mixtures of other proteins such as blood, lupin, sunflower, casein, whey and leaf protein concentrate.

Meat contributes to the average Western diet a large percentage of protein (28%), fat (29%), calcium (2%), iron (29%), vitamin A (25%), thiamin (17%), riboflavin (20%) and nicotinic acid (36%).[2]

Vegetable and milk proteins are deficient in many of the vitamins and minerals present in meat.[3,4] Similarly, the nutritional value of proteins derived from soybean, yeast and casein is lower than meat protein owing to deficiencies in essential amino acids.[5,6]

Owing to the widespread use of milk and soybean proteins in comminuted foods sold to the public,[7] the present study was planned to determine the nutritional value of sausages containing these proteins compared with an all-meat sausage.

2. Experimental

2.1. Test materials

The protein preparations used were a milk coprecipitate (Trade name—Low Cal) and an isolated soya protein product (Trade name—Promine D) containing 92% protein. The milk coprecipitate was prepared by spraying calcium chloride into milk heated to 95°C and spray-drying the resultant precipitate. The milk coprecipitate contained 83% protein, 9.6% moisture, 7.5% ash and 1.5% lactose.

2.2. Preparation of sausages

The control fresh sausages contained 48.2% frozen mutton (18.5% protein, 17.3% fat, 63.2% moisture), 21.7% pork fat, 20.5% ice and water, 7% wheaten binder, 2% salt, 0.2% polyphosphates, 0.4% preservative and seasoning. Sausages having 60% of the meat protein replaced by each of the protein additives were prepared by reducing the amount of mutton in the mix and adding the appropriate quantities of the non-meat protein, pork fat and water to give similar levels of protein and fat to that in the control mix. The ingredients were mixed and chopped for $2\frac{1}{2}$ min at low speed in a silent cutter and then at high speed for 2 min to obtain emulsification.

The sausage meats were then freeze-dried and stored under nitrogen at $-20°C$ until use.

A portion of each of the sausage meats was cooked on a hot plate at 190°C for 20 min.

Protein, moisture and fat analyses were made on all raw ingredients before formulation and on the finished products according to the AOAC methods.[8] The conversion factors for calculating crude protein from nitrogen analysis were 6.25, 6.38 and 5.71 for meat, milk coprecipitate and soya protein products respectively.[17]

2.3. Chemical analysis

The total essential amino acid content of the sausage was determined after acid hydrolysis[9] using a TSM-1 Technicon Amino Acid Analyser. Methionine and cystine were determined in the acid hydrolysates after oxidation of the sausages with performic acid.[10] The residual lysine content of the sausages was analysed by the method of Roach *et al.*[11]

The available essential amino acid content of the sausages was determined after amino acid analysis of the faeces of rats fed the sausage-based diet compared to a nitrogen-free diet.[12] Thiamin, riboflavin and nicotinic acid were determined by the methods described by Pearson.[13]

2.4. Feeding experiment

Diets were prepared from the freeze-dried sausages, starch and a vitamin and mineral mixture,[14] so that the diets contained 10% sausage protein and with an estimated metabolisable energy content of 18.1 kJ/g.

Weanling male rats of the Sprague-Dawley strain were housed individually and fed the diets *ad libitum* for a three-week period. The weight gains and feed intakes were measured and the protein efficiency ratios were calculated.[8] Four replicates of four rats were fed each diet.

2.5. Plasma amino acids

Plasma acids were determined on the protein free plasma[12] of starved rats and rats fed the experimental diets by using a Technicon TSM-1 Amino Acid Analyser. Plasma amino acid ratios were calculated by the method of Whitaker and Patrick.[15]

2.6. Statistical analysis

The analysis of variance of weight gains and protein efficiency ratios was carried out by the methods described by Snedecor and Cochran.[16]

3. Results

3.1. Proximate and chemical analysis

Table 1 shows the crude protein, fat and moisture content of the freeze-dried sausage preparations. The total and available essential amino acid and available lysine content of the products compared to the human requirements are shown in Table 2.

There was a wide variation in the essential amino acid content of the three sausage preparations. The meat-based sausage had a low isoleucine, tryptophan and sulphur amino acid content compared

Table 1. Composition of sausages (%)

Type of protein	Crude protein	Fat	Moisture
Meat	21.5	64.5	2.8
Milk coprecipitate	19.2	61.4	2.5
Soya protein	19.3	63.2	2.5

Table 2. Essential amino acid content of sausages made from different types of protein (g/kg of protein). Available amino acid values are shown in parenthesis

Amino acid	Meat	Milk coprecipitate	Soya protein	Human requirement[17]
Lysine	76 (66)	79 (72)	63 (57)	46
Chemically available lysine	64	72	55	
Histidine	42 (39)	36 (34)	34 (30)	37
Threonine	45 (36)	46 (39)	41 (35)	29
Valine	70 (58)	72 (61)	72 (58)	52
Methionine	25 (18)	20 (16)	18 (12)	16
Isoleucine	47 (39)	43 (36)	45 (37)	48
Leucine	86 (73)	78 (72)	86 (78)	63
Phenylalanine	44 (41)	43 (38)	50 (45)	42
Tryptophan	91 (75)	44 (37)	86 (68)	12
Cystine	11 (8)	7.5 (6)	9 (7)	15
Total sulphur amino acids	36 (26)	28 (22)	27 (19)	31

with the human requirement, while the milk coprecipitate and soya protein sausages had a low histidine, sulphur amino acid, isoleucine and phenylalanine content. The soya protein sausage also had a low tryptophan content.

The vitamin content of the sausage is shown in Table 3. The thiamin, riboflavin and nicotinic acid content of the meat sausage was markedly higher than that of the soya protein or milk coprecipitate sausage.

Table 3. Thiamin, riboflavin and nicotinic acid content of sausages (mg/100 g)

Type of protein	Meat	Milk coprecipitate	Soya protein
Thiamin	0.33	0.15	0.21
Riboflavin	0.41	0.18	0.14
Nicotinic acid	2.8	1.8	1.6

3.2. Feeding experiment

The growth rates and protein efficiency ratios (PER) of rats fed the three diets are shown in Table 4. The average weight gain and PER values for the milk coprecipitates and soya protein sausages indicated that there was no nutritional damage during the cooking process. On the other hand, the average weight gain and PER values for uncooked meat sausage was significantly greater than the milk coprecipitate or soybean products.

Table 4. Mean performance of rats fed sausage-based diets

	Mean weight gain (g) in 21 days		Protein efficiency ratio	
Type of protein	Cooked	Uncooked	Cooked	Uncooked
Meat	102 a[a]	123 b	2.0 c	2.5 f
Milk coprecipitate	85 c	87 c	1.4 g	1.4 g
Soya protein	92 c	96 c	1.7 h	1.7 h
s.e.m.[b]	3.7		0.11	

[a] Values followed by the same letter were not significantly different ($P > 0.05$).
[b] s.e.m., standard error of the mean.[16]

The PER value for the soya protein product was greater than that obtained for the milk coprecipitate but the values for the weight gains were similar.

3.3. Plasma amino acids

The plasma amino acid levels and ratios of rats fed the cooked sausages are shown in Table 5. The sulphur amino acid, methionine appeared to be the first limiting amino acid in each of the diets

Table 5. Plasma amino acid levels and ratios (in parenthesis) of rats fed sausage-based diets (μg/ml)

	Type of protein			
Amino acid	Meat	Milk coprecipitate	Soya protein	Starved
Lysine	86	88	53	57
	(51)	(53)	(−8)	
Histidine	27	32	19	12
	(125)	(172)	(59)	
Arginine	23	30	23	18
	(30)	(65)	(27)	
Threonine	67	102	72	61
	(10)	(65)	(18)	
Methionine	8.6	6.3	7.2	8.7
	(−1)	(−28)	(−16)	
Isoleucine	19	9	19	11
	(73)	(−18)	(73)	
Leucine	23	17	29	19
	(22)	(−7)	(56)	
Phenylaline	18	14	12	10
	(82)	(39)	(18)	
Valine	20	22	32	19
	(6)	(17)	(71)	
Tryptophan	13	12	13	11
	(18)	(11)	(18)	

since the concentration of this amino acid in the plasma was low compared with the starved rat. The ratio values obtained for isoleucine and leucine for the milk coprecipitate and lysine for the soya protein diet were also negative.

4. Discussion

The nutritional damage found after cooking the meat sausage can be seen in animal studies as well as the low values of the available amino acids compared with the total amino acids. This may be due to the Maillard reaction.[19]

Presumably, no nutritional damage could be detected on cooking the milk coprecipitate or soya-protein-based sausages since the reducing sugar content of the purified soya protein would have been low and the lactose present in the milk coprecipitate would have reacted with the protein during the drying process.[20]

The limiting amino acids in all of the sausage preparations appeared to be the sulphur amino acids so that mutual supplementation of the meat proteins and milk coprecipitates or soybean proteins would not be expected.

Similar results were obtained by Kies and Fox[5] who fed diets with different ratios of beef and textured soya protein to human subjects. From nitrogen balance measurements the soya protein was nutritionally inferior to the beef protein and mixtures of the two proteins showed no mutual supplementation effect since both proteins were limiting in sulphur amino acids.

Supplementation of these proteins with cereals would not be expected to increase their nutritional value since cereals are deficient in sulphur amino acids.[12]

The essential amino acids content of the sausages shown in Table 2 indicated that the lower nutritional value of the soya protein and milk coprecipitate sausages was due to a marked deficiency in sulphur amino acids, isoleucine and possibly tryptophan. The nutritional values would be expected to be further lowered if the diets were not supplemented with thiamin, riboflavin and nicotinic acid since these vitamins are deficient in the soya protein and milk coprecipitate preparations (Table 3). If human diets were based predominantly on these non-meat proteins, vitamin deficiencies would be expected. Vegetarian diets are usually rich in folic acid which masks the anaemia of vitamin B_{12} deficiency so that irreparable nerve damage may be the first sign of vitamin B_{12} deficiency.[3,4]

The preparation of coprecipitates of casein and whey proteins provides an effective means of conserving milk proteins in a product that also contains all of the calcium and phosphorus of the casein micelle. Whey proteins, although rich in sulphur amino acids are present in much smaller amounts than casein and increase the PER of casein from 2.5 to 2.7 (Lohry, personal communication).

Supplementation of diets with amino acids which are deficient in the diet, increases the nutritional value of animals fed *ad libitum*, but it has been shown that crystalline amino acids are absorbed more rapidly than dietary amino acids from intact protein resulting in an amino acid imbalance and a reduction by the body to utilise protein efficiently.[18]

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Distribution of Some Volatile Nitrosamines in Cooked Bacon

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Bacon was fried in the normal UK domestic manner, and the rashers, the cooked-out fat and the vapour were analysed for *N*-nitrosopyrrolidine and *N*-nitrosodimethylamine. Quantitative results were based on high resolution mass spectral measurements, and it was found that for both of the nitrosamines by far the greatest proportion occurred in the vapour.

1. Introduction

It is now well established that cured meat products such as bacon contain trace amounts of *N*-nitrosodimethylamine[1] and that on cooking, *N*-nitrosopyrrolidine is formed.[2–4] The origin of the *N*-nitrosopyrrolidine is not conclusively established, but may be formed from proline by nitrosation and subsequent decarboxylation.[4] The present study is restricted to the determination of these two nitrosamines, both of which are known to show carcinogenic activity.[5,6] The distribution of these nitrosamines in cooked bacon, in the resulting cooked-out fat, and in the vapour produced during cooking has been measured.

2. Experimental

2.1. Sample preparation

Pork for the experiments was mild block cured using multi-needle injection. Nine batches each of two cuts were used, middle (back) of pH range 5.5–6.5 and collars of pH range 5.8–6.9. All blocks were given identical treatment in respect of brine composition (21% w/w NaCl), the amount of brine pumped into each block, the ratio of meat weight to cover brine, and immersion time. The injection brine contained 2% w/w sodium tripolyphosphate, but no ascorbate. Target NaCl content was 4% in the water contained in the bacon, and phosphate content 0.3% in the lean meat. The final heat process (smoking) was carried out under fixed conditions of temperature and humidity. The only variables were the levels of sodium nitrite and potassium nitrate. Nitrite levels in the brine were such as to provide 100 or 200 mg/kg of added nitrite in the bacon. Nitrate levels were zero, 250 or 500 mg/kg. The legal limits in the United Kingdom for nitrite and nitrate in bacon as purchased by the consumer are 200 and 500 mg/kg respectively. Bacon of the type used in this work typically contains 40–60 mg/kg of nitrite (as purchased). After smoking the blocks were matured for 6 days. They were then sliced, vacuum packed and stored at 5°C until cooked. Bacon slices for cooking were selected from the packs such that a sample representative of all the bacon in each cure was analysed. Bacon was fried following current domestic UK practice as closely as possible, to produce a crisp well-cooked commodity. Known weights of the representative slices from each cure were fried in preheated thermostatically controlled electric frying pans set at 171°C (340°F). The bacon rashers were turned after 4 min, giving a total cooking time of 8 min. The pans were fitted with ventilated lids over which was placed an inverted wide mouth funnel connected to a water

vacuum pump and collection vessel. In order to preserve a realistic domestic situation, some sacrifice of vapour trapping efficiency was necessary, and there were obviously losses of vapour during turning. However, since trapping was carried out throughout the cooking process, the concentration of nitrosamines in the condensed vapours was assumed to be representative of the concentration which would result from complete trapping of all vapours in an ideal situation. On completion of frying excess fat was drained from the rashers. The rashers and the cooked-out fat were weighed and stored at -18°C pending analysis. The condensate was weighed and stored at $+4^{\circ}$C.

2.2. Analytical procedure

A total of 36 bacon samples were analysed. Each sample of cooked bacon was frozen in liquid nitrogen, ground in a mortar and minced. 250 ml of water and 1 ml of a 2.5 mg/l aqueous solution of *N*-nitrosodipropylamine to check recoveries were added to 250 g of the minced bacon, and the mixture steam distilled at atmospheric pressure; 400 ml of distillate was collected, and 80 g of sodium chloride and 4 ml of 10 N sulphuric acid added to it. This was extracted with 4×40 ml of redistilled dichloromethane, and the combined extracts washed with 70 ml of 1.5 N sodium hydroxide. The dichloromethane extract was dried over sodium sulphate, transferred to a Kuderna-Danish flask and evaporated to 2.5 ml at 45°C on a water bath. The flask was cooled, 800 μl of hexane added and evaporated to about 250 μl. The volume was measured with a 500 μl capacity syringe used to transfer the extract to a septum-fitted vial, which was stored at 10°C prior to analysis. The cooked-out fat was treated in a similar manner, but it was not found necessary to freeze or mince this prior to distillation. Nitrosamines were extracted directly from the vapour condensates and thereafter treated as above. All extracts were examined by combined gas chromatography and mass spectrometry. A Pye 104 chromatograph was fitted with a short polar packed column connected to a high efficiency support-coated open-tubular column. A venting valve was placed between the two columns to prevent overloading of the high efficiency column by solvent and contamination of the mass spectrometer by extraneous material. A parallel reference flow system was incorporated to maintain a constant flow rate of carrier gas through the columns, irrespective of the switching valve mode. A detailed description of this apparatus has been published.[7] The gas chromatograph was interfaced to an MS902 mass spectrometer via a silicone rubber separator.[8] The mass spectrometer was operated under high resolution, and the nitrosamines were detected by monitoring their respective parent ions at the appropriate retention time, with reference to a suitable fragment ion of a fluorinated hydrocarbon. Calibration of the mass spectrometer for a quantitative assessment of the level of *N*-nitrosodimethylamine, *N*-nitrosodipropylamine and *N*-nitrosopyrrolidine, was carried out with standard nitrosamine solutions, four times each day. Precision of the mass spectral results was $\pm 20\%$. The concentrations of nitrosamines in the extracts, as determined by mass spectrometry, were corrected for the losses incurred during the clean-up procedure.[9] For *N*-nitrosodimethylamine, losses occurred mainly in the final stages of evaporation of the extract, whereas *N*-nitrosopyrrolidine was lost predominantly during the initial steam distillation of the foodstuff.

3. Results

The concentrations of nitrosamines in the cooked bacon, cooked-out fat and vapour, expressed in μg/kg, were obtained from the corrected mass spectral results. The concentration ranges of *N*-nitrosodimethylamine in the cooked-out fat (0–30 μg/kg) and vapour (10–160 μg/kg) are shown in Figure 1. Levels of *N*-nitrosodimethylamine in the cooked bacon were all in the region of the detection limit, the highest value being 6 μg/kg. The range of concentration (2–180 μg/kg) of *N*-nitrosopyrrolidine is shown in Figure 2. For both nitrosamines the levels in the cooked bacon are typical of those reported by other workers.[4,10,11] No attempt was made to correlate the concentrations with the nitrite or nitrate levels, as this forms part of a larger programme, the results of which will be published later.

The weights of uncooked bacon, cooked bacon and cooked-out fat were known, and hence the

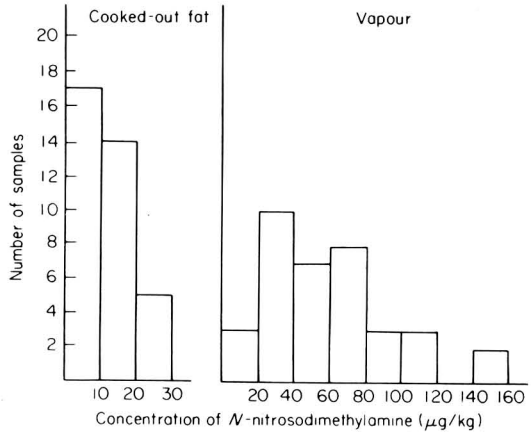

Figure 1. Concentration range of *N*-nitrosodi-methylamine in bacon after frying.

weight lost as vapour during cooking was calculated. From this the absolute quantities of nitrosamines present and their distribution between the cooked bacon, cooked-out fat and vapour were estimated. Results are shown in the form of histograms, for *N*-nitrosodimethylamine in Figure 3 and for *N*-nitrosopyrrolidine in Figure 4.

The distribution by weight of the cooked bacon itself, the cooked-out fat and the vapour for the collars and backs is shown in Figure 5. The backs gave rise to somewhat more cooked-out fat, but less vapour than the collars. No difference in the distribution of the nitrosamines in the backs and collars was detectable, although it is recognised that small variations would be masked by the limitations of the experimental procedure.

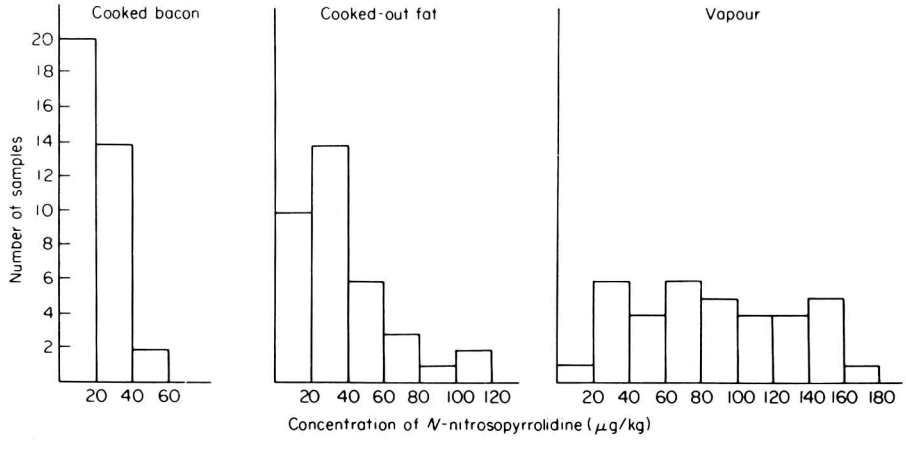

Figure 2. Concentration range of *N*-nitrosopyrrolidine in bacon after frying.

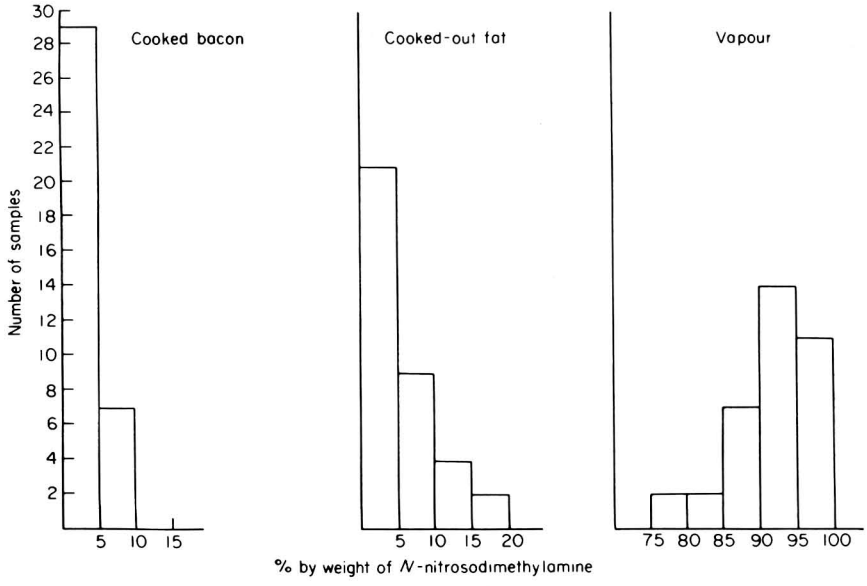

Figure 3. Distribution of *N*-nitrosodimethylamine in bacon after frying.

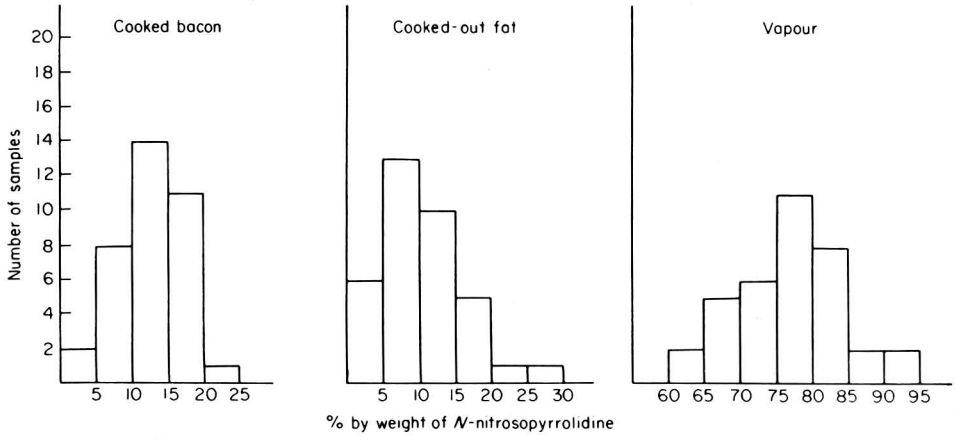

Figure 4. Distribution of *N*-nitrosopyrrolidine in bacon after frying.

4. Conclusions

In bacon fried in a normal UK domestic manner, by far the greatest proportion of *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine is lost in the vapours produced during cooking. Up to 10% of *N*-nitrosodimethylamine is found in the rasher itself and up to 20% in the cooked-out fat. For *N*-nitrosopyrrolidine the corresponding maximum figures are 25 and 30%, respectively.

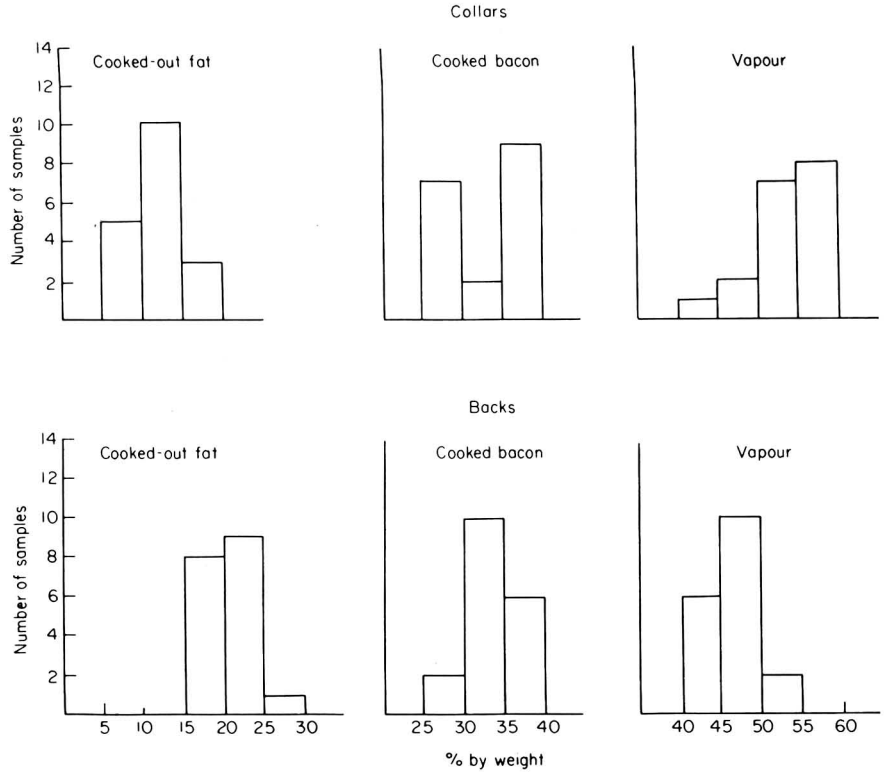

Figure 5. Proportion by weight of cooked bacon, cooked-out fat and vapour in collars and backs.

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Transverse Anisotropy in Beef Muscle

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In beef *sternomandibularis* muscles, a variety of treatments—tetanic contraction, cold shortening, cooking shortening and swelling in alkali—caused dimensional changes which differed markedly with the direction chosen within a plane perpendicular to the fibre direction. In all cases a marked increase (up to twice) occurred in the narrow dimension of the muscle, with little change in the wide dimension. In the cooked meat, shear force across the fibre was independent of the transverse direction chosen whether the muscle was relaxed or cold shortened. The effects were also seen in *rectus abdominis* and *psoas*, but not in *longissimus dorsi* muscles. It is suggested that anisotropy is a result of differing organisation of connective tissue in the two directions, although there was little histological evidence. The force to shear parallel to the fibres varied with direction in a manner consistent with this view.

1. Introduction

The highly organised fibrillar structure of muscle confers its anisotropic properties, that is, its differing behaviour along and across the fibres. It is generally assumed, however, that within a plane at right angles to the fibre, the choice of direction for measurement will not affect any particular property (excluding muscles which contain well defined sheets of connective tissue). We have recently become aware that this is far from the truth for the muscle most used in this laboratory—*sternomandibularis* of the ox. It was first noticed that strips of this muscle immersed in alkali swell much more in a direction parallel to the thin dimension than in a perpendicular direction. We have since studied other stimuli giving rise to dimensional changes, and all of these changes showed the same transverse anisotropy. These included electrical stimulation of fresh muscle, cold shortening and cooking shortening. The material has also been studied histologically in search of an explanation. Other muscles also show the effect.

2. Experimental

Ox *sternomandibularis* muscles were collected soon after slaughter and trimmed. Black ink marks were applied, 10 cm apart, for length measurement. A central ink mark was also applied, at which thickness and width were measured, using a dial gauge and a slide caliper respectively.

In the experiments of Table 1, one muscle of each pair was allowed to go into rigor at near rest length, by placing on a stainless steel tray, covered with plastic and leaving for 24 h, at 15°C. The other was cold shortened by placing on thin film (Stretchwrap) lying on talc-dusted paper in a closed container at 2°C for 48 h. Dimensions were then recorded. Lengths of 16 cm were cut from the 15°C sample and 11 cm from the 2°C sample and all were cooked in plastic pouches in a water-bath at 80°C for 40 min. Dimensions were measured again.

In the tetanus experiment, intact fresh muscles (both of each pair) were measured in the same way. Electrodes were clipped to the ends and electrical DC pulses of 4 ms at 10/s and 110 V were applied. When the tetanus reached a steady state at about 40 s the muscles were measured again quickly. The current was switched off and recovery of length measured after 30 min.

In the alkali experiments, muscles were allowed to go into rigor at 15°C between glass plates

12 mm apart. Four accurately parallel strips about 1 cm wide were cut from each muscle and were trimmed to exactly 17 cm long. (Subsequent length measurements were on overall length.) Since ink marks disappear in alkali, plastic labels were sewn on to the strips, and the centre marked with a thread. After measurement with the dial gauge, the strips were agitated on a rocker in 0.1 N NaOH for 24 h at 2°C, measured, and the fluid changed to 0.1 M phosphate pH 7.0 overnight. After another 24 h in 0.1 M KCl + 0.02 M succinate pH 5.6, the strips were measured again. One strip was cooked at 50°C for 40 min, and one at 70°C and both were measured. The data in Tables 3 and 5 for changes in dimensional changes in alkali and succinate were derived from four strips for each animal, but only one in the case of the cooked strips.

In the experiments on other muscles, where cooking shortening and alkali swelling were measured, samples were taken from carcasses in the chiller, one day *post mortem*. Strips about 1 cm square in cross section were cut with the knife laid parallel to the fibres. In the case of the *psoas* and *rectus abdominis* muscles, one face was parallel to the ventral plane, and in *longissimus* as close as possible to the dorsal plane.

In the *rectus* muscles the central portion was used and the natural faces were left intact. In the case of *longissimus* and *psoas* the anterior ends of the muscles were used and all natural faces were removed. The dorsal or ventral plane was taken as "width" and the perpendicular plane as "thickness". Cooking and alkali immersion occurred at 30 h *post mortem*. In cooking, strips were packed carefully side by side to minimise distortion.

Tetanus and cold shortening experiments on *rectus* were carried out as for *sternomandibularis*, using strips 4–5 cm wide and about 14 cm long.

Shear force measurements were made on the MIRINZ tenderometer[5] using strips 15 mm wide and 10 mm thick. The mean of four bites was taken for each sample, shearing across the fibre and of eight bites, shearing along the fibre.

In statistical comparisons the paired *t*-test was used.

3. Results

3.1. Dimensional changes during rigor and cooking

The *sternomandibularis* muscle of the ox has a cross section resembling a flattened ellipse, with the smallest dimension (thickness) about 1.3–1.5 cm and the largest (width) usually 5–7 cm. Changes in thickness and width have been followed in muscles, in which one was allowed to go into rigor at 15°C, and the other of the pair at 2°C. Both sets of muscles were free of any restraint or wrapping which might deform them. Further changes on cooking were also followed (Table 1).

Table 1. Changes in dimensions of unrestrained muscle samples during rigor onset and cooking, expressed as a ratio of the pre-rigor dimension (12 animals)

Temperature	Final state		Thickness	Width	Length
15°C	Rigor	Mean	1.05	0.98	0.93
		S.D.	0.11	0.08	0.07
	Cooked	Mean	1.55	0.78	0.75
		S.D.	0.17	0.04	0.04
2°C	Rigor	Mean	1.51	1.01	0.61
		S.D.	0.15	0.04	0.05
	Cooked	Mean	1.94	0.83	0.54
		S.D.	0.18	0.06	0.04

At 15°C there was little change during rigor onset (shortening 7%). On cooking the thickness increased by more than half, while the width was reduced to near three quarters of the rigor value.

At 2°C a cold shortening of 39% increased the thickness by half, without affecting width. Cooking increased the thickness but decreased the width in about the same proportion as before.

Thus cold shortening increases thickness but leaves width unchanged, while cooking shortening

increases thickness and decreases width, irrespective of the state of contraction of the muscle. The two treatments thus reinforce each other in producing transverse anisotropy.

3.2. Shear force in relation to plane of shearing

This lack of symmetry in cold shortening and cooking behaviour raised the important question of whether shear force would vary if shearing across the fibre occurred in different directions along a transverse plane. Collected results (Table 2A) show clearly that there is no effect whatever on tenderness in unshortened or cold shortened meat.

Table 2. Shear force values of cooked muscle in relation to the plane of cutting of the 10×15 mm test strip

A. Longitudinal strip, wedge across the fibres (22 animals)

Rigor temperature		15 mm face of strip parallel to thin dimension of muscle	15 mm face of strip perpendicular to thin dimension of muscle
15°C	Mean	51.0	50.7
	S.D.	6.2	6.4
2°C	Mean	106.2	108.1
	S.D.	14.3	16.2

B. Transverse strip, wedge along the fibres (10 animals)

	Axis of strip parallel to thin dimension	Axis of strip perpendicular to thin dimension
Mean	13.9	17.2
S.D.	3.3	3.8

While shearing across the fibre largely reflects the strength of the myofibrils, shearing with the wedge parallel to the fibre appears to measure connective tissue.[1] Further measurements were made in the latter mode on transverse strips cut with the axis along the narrow and wide dimensions of the muscle (Table 2B). There was a small but highly significant difference ($P < 0.001$) in shear force. It should be noted that connective tissue was not cut, but fibres were simply pushed aside. The sign of the difference, the same in all samples, is consistent with a less firm binding together of fibres by connective tissue in the narrow dimension, in line with the anisotropic swelling.

3.3. Swelling in alkali

These experiments were undertaken in a study on the role of collagen in the elastic properties of meat. The dimensional changes observed are included here because they offer the most extreme case of transverse anisotropy. They also demonstrate that this effect is seen in strips of muscle originally of approximately square cross section. (The experiments on this muscle previously described, used lengths with intact natural surfaces.)

In Table 3 it can be seen that strips swell in alkali along the thin dimension by almost two times, whereas the width is unchanged. The deformation is permanent, being hardly altered by neutralisation.

Data are also presented on these strips, cooked under conditions which shrink collagen (70°C) and conditions which do not (50°C). In both cases the proportional shrinkage is about the same for width and thickness. Another sample cooked at 80°C showed dimensional changes similar to the 70°C sample. No anisotropy is evident therefore during cooking.

Table 3. Changes in dimensions of muscle strips after soaking in alkali, and after neutralisation and cooking, expressed as a ratio of the dimension in rigor (10 animals)

Treatment		Thickness	Width	Length
0.1 N NaOH, 24 h, 2°C	Mean	1.94	0.98	1.27
	S.D.	0.14	0.06	0.06
Neutralisation in 0.1 M KCl	Mean	1.98	0.94	1.19
0.02 M succinate, pH 5.6	S.D.	0.18	0.08	0.06
Cooked 40 min, 50°C	Mean	1.67	0.88	1.04
	S.D.	0.14	0.13	0.06
Cooked 40 min, 70°C	Mean	1.56	0.77	0.76
	S.D.	0.19	0.12	0.05

3.4. Tetanic contraction

It seemed desirable to study changes in a situation nearer to the physiological. Measurements were made on electrically stimulated muscles which had reached a steady state in tetanus. The results are shown in Table 4. The anisotropy is still strongly evident, although in contrast to the other treatments, a 20% increase in width was also seen. The contraction of 48% is beyond the physiological range, and was not completely reversed. However, the anisotropy was seen at earlier stages of the contraction.

Table 4. Changes in dimensions during tetanus induced by electrical stimulation, as a ratio of the dimensions before stimulation (20 muscles, 10 animals)

	Thickness	Width	Length (tetanus)	Length (relaxation)
Mean	1.78	0.48	0.48	0.85
S.D.	0.14	0.05	0.03	0.06

3.5. Histological observations

Frozen sections were cut from glutaraldehyde-fixed specimens of the cold shortened *sternomandibularis* muscles, both raw and cooked, and examined under phase contrast. Cutting was longitudinal, in planes parallel to the thick and thin dimensions. It was thought that crimping might occur in one direction only, but this did not prove to be the case. Crimping was about equally pronounced in both planes.

In these sections, or in transverse sections, no preferred orientation of the perimysium could be detected. A sample of the alkali-swollen muscle, neutralised and fixed, gave the same result.

3.6. Other muscles

More limited studies were carried out on a few other muscles, mainly in terms of cooking and alkali swelling of strips cut from the rigor meat. In the case of *rectus abdominis* muscle, electrical stimulation and cold shortening were also included.

The results are shown in Table 5. It is more difficult to measure strips with accuracy due to distortion in processing, but the results suffice to show that anisotropy is not confined to *sternomandibularis*.

Both *rectus abdominis* and *psoas major* muscles show significant anisotropy in dimensional changes during cooking and immersion in alkali, while *longissimus dorsi* did not. *Sternomandibularis* and the central portions of *rectus* and *psoas* have fibres parallel to each other and to natural surfaces, and it is easy to cut strips with fibres parallel to the axis and with faces of known orientation. The skewed fibres of *longissimus* make the cutting of such strips much more difficult. The scatter in values

was generally greater for this muscle and a "not significant" result is to be taken as a lack of evidence for anisotropy, rather than a proof it does not exist.

Electrical stimulation and cold shortening of *rectus* also showed transverse anisotropy, as in the case of *sternomandibularis*, the effect being somewhat more pronounced in cold shortening.

As before, the anisotropy seen in *rectus* and *psoas* was parallel in all treatments although less pronounced than in the neck muscle. It is reasonable to suppose that it would also be observed on electrical stimulation or cold shortening of *psoas*, but not of *longissimus*.

The swelling of *psoas* in one dimension was the highest observed, but it also swelled in the other direction, whereas *sternomandibularis* did not, and the ratio of the swelling in the two directions was less than in *sternomandibularis*. This suggests that the strength of the connective tissue influences the degree of swelling in alkali.

Table 5. Anisotropic effects in strips of other muscles under a variety of treatments, expressed as a ratio of dimensions before and after treatment (8 animals)

Muscle	Treatment		Thickness[a]	Width[b]	Significance[c] ($P<$)	Length
Rectus abdominis	Electrical tetanus[d]	Mean	1.41	1.13	0.001	0.61
		S.D.	0.11	0.04		0.07
	Cold shortening[d]	Mean	1.33	1.07	0.001	0.68
		S.D.	0.12	0.02		0.05
	Cooking	Mean	1.20	0.89	0.001	0.71
		S.D.	0.07	0.09		0.06
	Alkali	Mean	1.61	1.27	0.005	
		S.D.	0.25	0.11		
Longissimus dorsi	Cooking	Mean	1.08	1.03	NS	0.77
		S.D.	0.10	0.20		0.05
	Alkali	Mean	1.32	1.38	NS	
		S.D.	0.31	0.26		
Psoas major	Cooking	Mean	1.20	0.98	0.025	0.69
		S.D.	0.10	0.13		0.02
	Alkali	Mean	2.38	1.40	0.001	
		S.D.	0.44	0.09		

[a] Measured in the dorsal or ventral plane.
[b] Measured perpendicular to the dorsal or ventral plane.
[c] Significance of difference between thickness and width ratios in a paired *t*-test.
[d] Ratio based on pre-rigor dimensions. In all other cases on rigor dimensions.

Samples of all four muscles were studied after prolonged immersion in the alkali and after cooking long enough to weaken the connective tissue (2 h at 100°C). Attempts were made to pull the fibre bundles apart in the two planes previously studied, but with the exception of *psoas*, no clear differences in ease of separation were seen.

Psoas, however, has a distinctly laminar structure in the ventral plane. This is evident in the raw muscle where the laminae readily slide over each other. The laminae separate more easily in cooked or alkali-treated muscle. Sectioning of the cooked *psoas* in two planes showed the collagen to be laminated between the fibre layers. Heat shortening of the collagen had thrown the fibres into a wave with a period of about 1 mm at right angles to the laminae, while in the plane of the laminae, fibres remained fairly straight.

It may be noted that cooking shortening is significantly higher in *psoas* (31%, $P<0.001$) or *rectus* (29%, $P<0.005$) than in *longissimus* (23%). *Psoas* also differs significantly from *sternomandibularis* (25%, $P<0.005$) but the other muscles do not. This is likely to be due to the longer initial sarcomere

lengths in *psoas* and *rectus*, which are stretched by the hanging of the carcass. Carse[2] has found that if *sternomandibularis* is set in rigor at various degrees of stretch and then cooked without restraint, cooking shortening increases with stretch in an almost linear manner from 25 to 55% at twice rest length. It is also known that cooking shortening is about halved in samples of this muscle which have cold shortened.[3, 4]

4. Discussion

The anisotropy is not reflected at all in shear force values of unshortened or cold shortened meat. A great deal of work has been done on the MIRINZ tenderometer, without consideration of this possibility, although normally *sternomandibularis* is cut with the wide dimension of the strip parallel to the thin dimension of the muscle. Davey and Gilbert[5] made a careful study of the effect on shear force of the strip dimensions using this muscle. They concluded that it was the number of fibres to be cleaved that was important, and that it was immaterial whether the thick or thin edge of the strip was presented to the wedge. They did not consider orientation in the transverse plane.

The structural basis of the anisotropic behaviour in *sternomandibularis* is not yet clear and must be the subject of further study. Since most of the experiments used lengths of intact muscle, it could be argued that the effects are due to the tendency of a tube of elliptical cross section to assume a more circular shape under pressure generated by contraction. However, the anisotropy was also seen in strips, devoid of natural faces, subjected to cold shortening and cooking shortening. The alkali swelling also used strips, and the natural faces, present on two sides, showed no sign of affecting the dimensions. The results from strips of other muscles are in line with these findings.

It appears that the reason for the anisotropic dimensional change, lies not in the external geometry, but in the internal structure of the muscle.

It is surprising that the effects are seen with such a variety of unrelated treatments varying from tetanic contraction to the grossly unnatural swelling in alkali. This suggested that the answer might be found in the organisation of the perimysial connective tissue, rather than within the fibres.

There is some support for this in differing shear force values with the wedge parallel to the fibre, although the anisotropy was much less than that observed in swelling behaviour.

Observation at microscopic level supported the involvement of perimysium only in the case of *psoas*. This muscle is reinforced by layers of connective tissue in the ventral plane, but not at right angles, hence expansion is easier in the latter direction.

The ease of separation of the layers of fibres may well be a major reason, together with long sarcomeres, for the status of *psoas* as the tenderest of all muscles. The connective tissue content is no explanation, since this is almost the same as for a number of other muscles,[6] ranging from tender (e.g. *longissimus dorsi*) to tough (e.g. *triceps brachii*). There may of course be considerable differences in the degree of cross-linking of the collagen.

It seems unlikely that elastin could be of much significance with respect to the anisotropy since *sternomandibularis*, *longissimus* and *psoas* contain elastin only to the extent of 2.5, 2.9 and 3.8% respectively, of the total connective tissue protein, which can be accounted for by the sheaths of the arterioles.[6] *Rectus* contains somewhat more (9.1%).

The anisotropic effect observed is undoubtedly an adaption to function. In *psoas* it could be related to the need for fibre bundles to slide over each other in one plane rather than another. The effect may also avoid a large dimensional change in a muscle which is much wider than it is thick, particularly in the sheet muscle, *rectus*, and to a lesser degree in *sternomandibularis*. The same percentage change would produce a much greater actual change in the width than in thickness. This would be difficult to accommodate in the body, and could cause an undesirable divergence from parallelism between the fibre direction and the line of tension in the muscle.

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Tenderness in Relation to the Temperature of Rigor Onset in Cold Shortened Beef

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Beef *sternomandibularis* muscle was cold shortened at 2°C for 24 h and then transferred to 37°C until *rigor mortis* was complete. In spite of a final shortening of 33%, the mean shear value after cooking was identical with that of unshortened meat which had gone into rigor at 15°C. Meat sent into rigor at 2°C with the same degree of shortening had twice the shear value. Thus raising the temperature to 37°C in the final stages of rigor completely nullifies the toughness seen in cold shortened meat, without affecting the shortening. Small changes in cooking loss run parallel to tenderness. The effects are not due to ageing, and may arise from modification of actin–myosin bonding.

It is concluded that conditions during the last stages of rigor onset are more important to tenderness than the rest of the post-mortem history of the muscle.

1. Introduction

We showed recently[1] that shortening of beef muscle during rigor at 37°C did not produce the toughening to be expected from the well known shortening–toughness relationship.[2]

Here we wish to report briefly on two further experiments in which beef *sternomandibularis* muscle was allowed to cold shorten for 24 h. One part was then held at 37°C for the last stages of rigor to occur, while another was left at 2°C. Although the final shortening was the same, striking differences in tenderness were obtained between the two rigor temperatures.

2. Experimental

Freshly collected ox *sternomandibularis* muscles were trimmed, and to each, two sets of ink marks, 6 cm apart, were applied. The muscles were then halved transversely between the mark sets.

In the first experiment one piece from each animal was wrapped firmly in polythene and was held at 15°C for 24 h, then transferred to 2°C. The three other pieces were placed on thin film (Stretch-wrap), lying on talc-dusted paper in a closed container, and left at 2°C to cold shorten. At 24 h two pieces were removed to 37°C in a metal container (to speed heat transfer) and left for 3 h and 7 h respectively in still air, before returning to 2°C. At 48 h *post mortem* all pieces were cut to 6 cm and cooked as previously[1] in plastic bags in a water-bath, at 80°C for 40 min. They were then assessed on the MIRINZ tenderometer.

The second experiment was carried out in a similar fashion.

Length measurements were made between the marks at various stages during rigor, and overall on the pieces after cooking. Cooking loss was determined by weighing before and after cooking.

All statistical comparisons were made by paired *t*-tests.

3. Results

3.1. Transfer of cold shortened muscle, near rigor, to 37°C

In this first experiment (Table 1) two controls were used, one allowed to go into rigor at 15°C under restraint, and the other at 2°C without restraint. Two other samples of the cold shortened meat were transferred to 37°C at 24 h, when the pH was 0.3 units above ultimate and shortening was only two-thirds complete. Rigor was too advanced at this stage for the cold shortening to be reversible.[3] One piece was held at 37°C for 3 h, in which time rigor was almost complete (subsequent shortening was only 2.5%). It was returned to the cold till 48 h *post mortem*. The other piece was held for 7 h at 37°C, a time used in our previous experiments,[1] and sufficient for completion of rigor from the pre-rigor state.

Table 1. Effect on shear force of warming cold shortened muscle to 37°C in the last stages of *rigor mortis* (12 animals)

		Time–temperature treatment			
		24 h 15°C +24 h 2°C	48 h 2°C	24 h 2°C +3 h 37°C +21 h 2°C	24 h 2°C +7 h 37°C +17 h 2°C
pH	Mean[a]	5.49 (48 h)		5.76 (24 h)	
	S.D.	0.03		0.06	
Shortening (%)					
(a) after 24 h	Mean			23.3	24.6
	S.D.			5.0	5.2
(b) after 1 h more at 37°C	Mean			23.5	25.5
	S.D.			7.1	8.3
(c) after 3 h at 37°C	Mean			29.5	30.6
	S.D.			5.4	6.3
(d) after 48 h total	Mean	1.1	34.5	32.0	33.5
	S.D.	4.5	5.2	5.3	5.5
Shear force	Mean	53.7	114.0	60.9	54.5
	S.D.	5.1	10.7	15.2	15.4
Cooking shortening (%)	Mean	23.8	13.8	13.7	9.8
	S.D.	2.9	3.2	3.6	3.2
Cooking loss (%)	Mean	23.7	19.4	23.4	23.0
	S.D.	3.2	1.5	2.6	2.7

[a] Mean of 8 animals.

In the cold shortened meat, the completion of rigor at 37°C led to a two-fold reduction in shear force in the cooked meat, to the same level as that from rigor at 15°C. The sample in which rigor had not quite been completed at 37°C (3 h) was only a little tougher than that in which rigor had been completed (7 h). In all three samples subjected to cold shortening, the final shortening was almost identical. Thus a two-fold difference in tenderness has been obtained at the same degree of shortening, purely as a result of varying the temperature in the last stages of rigor mortis.

It seems unlikely that ageing at 37°C could account for the drop, since the 3 h/37°C sample was not quite in rigor, and its shear force was not greatly different from the 7 h/37°C sample. This point has been further verified in the next experiment.

Cooking loss was significantly lower in the muscles simply cold shortened by 35% than in the unshortened muscle ($P<0.001$) or in the 3 h/37°C sample ($P<0.001$) or the 7 h/37°C sample ($P<0.005$).

As previously observed, cooking shortening was much less in the cold shortened meat.[1,4] It was also significantly lower in 7 h/37°C samples than in the 3 h/37°C sample ($P<0.01$) or the 48 h/2°C sample ($P<0.001$).

3.2. Transfer of cold shortened muscle to 37°C earlier in rigor, and post-rigor

The second experiment (Table 2) tested the possibility of ageing by holding muscle for 3 h at 37°C, after rigor had been completed at 2°C. The effect of transfer of muscle at an earlier stage in rigor was also investigated.

Table 2. Effect of warming cold shortened muscle to 37°C before rigor and at the completion of rigor (10 animals)

		Time–temperature treatment			
		24 h 15°C +24 h 2°C	48 h 2°C	12 h 2°C +1 h 37°C +35 h 2°C	48 h 2°C +3 h 37°C
Shortening (%)	Mean	1.7	35.0	24.4 (12 h)	
	S.D.	5.0	4.1	4.3	
				18.1 (1 h, 37°C)	
				7.5	
				29.9 (48 h)	35.3
				8.3	6.7
Shear force	Mean	48.5[a]	121.7	92.4	108.6
	S.D.	7.0	12.6	16.1	15.3
Cooking loss (%)	Mean	22.7	19.9	19.4	17.1
	S.D.	3.0	2.6	1.5	5.1

[a] Mean of 5 animals.

When samples were allowed to go fully into rigor at 2°C (48 h) the very high shear force was only slightly reduced by transfer to 37°C for 3 h. It was still twice as high as the value obtained for the same 3 h exposure to 37°C just before rigor (Table 1). This shows unequivocally that the latter result is not an ageing effect.

The experiment also included an attempt to reverse cold shortening by earlier removal from 2°C when the mean pH was 6.3. At this stage cold shortening was 25%, but after an hour at 37°C partial relaxation to 18% shortening occurred. Return to the cold induced a return to 30% shortening, whereas the undisturbed 2°C sample reached 34%. The shear force was intermediate in value between 15°C and 2°C rigor, a result in line with the relative degrees of contraction. It appears that the effect of warming to 37°C is reversed if the return to the cold occurs at an early stage of rigor onset, and cold shortening then reasserts its effect on tenderness.

In this experiment three sets of samples all went into rigor at 2°C, and all showed significantly lower cooking loss than the control which had gone into rigor at 15°C ($P<0.025$ for 48 h/2°C; $P<0.001$ for the other two sets).

4. Discussion

In the previous work, comparisons were made between the tenderness of meat in which rigor had occurred at temperatures ranging from 15 to 37°C. Above 34°C a marked increase in tenderness resulted. The present work comparing rigor at 37°C with rigor at 2°C shows even sharper contrasts.

It appears that the history of the muscle during most of the pre-rigor period is less important than conditions in the final stages of rigor onset. In cold shortened meat in which the point of reversibility has been passed, a transfer to 37°C for the final stages of rigor completely nullifies the effect of an ultimate shortening, the greater part of which has been due to cold. A tenderness level identical with that achieved in unshortened muscle set at 15°C is obtained at a degree of shortening which, if completed at 2°C, leads to a doubling of shear force.

Ageing appears to have been eliminated as a cause. In support of this we have observed in electron micrographs of this muscle after rigor during 7 h at 37°C, that the Z-lines are perfectly intact.

It is possible that the explanation for the dramatic reduction in shear force may lie in the influence of temperature on the bonds formed in rigor between myosin heads and the actin–tropomyosin–troponin complex.

It is interesting that cooking loss appears to behave in parallel to tenderness. Cold shortening causes a decrease in cooking loss if rigor is completed at 2°C, but has no effect if rigor is completed at 37°C (Table 1). In Table 2 the last three treatments all involved completion of rigor at 2°C and all showed a reduction in cooking loss over the unshortened control. These differences are too small to be of practical importance but are probably significant as a reflection of differing myosin–actin interactions.

A reduction in cooking loss due to cold shortening agrees with other recent results of ours[5] (see Table 3)[5] but differs from our earlier report,[6] which found that 29% shortening had no effect on 4 cm pieces or mince. The majority of fibres in cold shortened meat are crimped, and the effective fibre length is therefore greater for the same length of piece than in unshortened meat. Since loss is related to length along the fibre,[6] a slightly lower loss on cooking shortened meat might be expected. Davey and Gilbert[4] however found cooking loss increased with degree of cold shortening. Their result was probably due to the thin strips they used. Longitudinal cutting of crimped muscle cannot avoid sectioning the crimp, leaving leaking fibres across the faces, in numbers increasing with the severity of the crimp. Our muscle pieces had the natural faces intact.

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A Differential Scanning Calorimetric Study of Conversion of Ovalbumin to *S*-Ovalbumin in Eggs

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The presence of *S*-ovalbumin, a more heat-stable form of ovalbumin formed on storage of eggs, can be determined by differential scanning calorimetry of egg white. At a heating rate of 10°C/min, at pH 9, the characteristic denaturation temperature of ovalbumin is 84.5°C, that of *S*-ovalbumin, 92.5°C. The formation of *S*-ovalbumin proceeds through a previously unrecognised intermediate species having a denaturation temperature of 88.5°C. The kinetics of the conversion on storage of eggs at 4, 22 and 37°C have been determined. Differential scanning calorimetry is a rapid and convenient method of determining the quality of eggs held in storage. Freeze-dried preparations of ovalbumin stored in the cold for 20 years showed partial conversion to the intermediate, but not to *S*-ovalbumin.

1. Introduction

Changes in the thermolability of the protein components of egg white are conveniently detected by differential scanning calorimetry.[1] Upon storage of the egg, ovalbumin is converted into a heat-stable form called "*S*-ovalbumin".[2] We have studied the changes in the differential scanning calorimeter (DSC) thermograms of egg white as a function of time of storage. The endotherm characteristic of denaturation of ovalbumin slowly changes into an endotherm characteristic of *S*-ovalbumin. At intermediate storage times, an endotherm corresponding to an intermediate between ovalbumin and *S*-ovalbumin is observed. Since the relative amounts of ovalbumin, *S*-ovalbumin and intermediate can be calculated from the thermogram, DSC appears to be a quick and convenient method for evaluating the quality of stored eggs and of processed egg white.

2. Experimental[a]

2.1. Materials

2.1.1. Eggs

Unwashed, unoiled, nest-run White Leghorn eggs were obtained from a local producer on the day of lay. They were washed in 37°C water containing 1% trisodium phosphate and 1% Phisohex (Winthrop Laboratories), air-dried, dipped in 95% ethanol, and air-dried again. Each egg was wrapped in plastic food wrap to prevent further bacterial contamination. Eggs were randomly divided into three groups stored at 37°C, at room temperature (22°C), and 4°C. Upon removal from storage, each egg was inspected for Pseudomonas contamination with ultraviolet light. Individual egg whites were separated, and blended in a tissue homogeniser. For each egg white examined after storage, the absence of gross bacterial contamination was shown by plating on trypticase agar at 37°C.

[a] Reference to a company or product name does not imply approval or recommendation of the product by the US Department of Agriculture to the exclusion of others which may also be suitable.

2.1.2. Egg white proteins

Our standard ovalbumin was recently prepared from fresh eggs as previously described.[1] Other ovalbumin preparations had also been made from fresh eggs in a similar manner, but had been stored for approximately 12 years at −12 to −18°C, then for approximately 8 years at 2°C. *S*-ovalbumin was prepared by holding an unbuffered pH 10.0 solution of purified ovalbumin at 56°C for 38 h.[3] The *S*-ovalbumin was recovered in dry form by dialysis against distilled water and freeze-drying. Concentration of ovalbumin and *S*-ovalbumin was determined by ultraviolet absorption, using the optical factor $E^{1\%}_{1\,cm} = 7.12$ at 280 nm.[4]

2.2. Methods

DSC thermograms (plots of heat flow as a function of temperature) were recorded on a DuPont model 990 thermal analyser with DSC cell, described previously.[1] Heating rate was 10°C/min. Preparation of samples and interpretation of thermograms have been described.[1,5,6] The fitting of complex thermograms by computer has also been reported.[7] Baselines under the thermograms were constructed as straight lines, between 75 and 98°C for fresh egg white, and between 80 and 105°C for egg white with complete conversion to *S*-ovalbumin, with similar straight line baselines for samples at intermediate storage times.

3. Results

3.1. Thermograms of ovalbumin and *S*-ovalbumin

In agreement with Smith's observations[3] made by other experimental methods, thermograms of ovalbumin and *S*-ovalbumin show the increased stability of *S*-ovalbumin to heat denaturation. When these two proteins are present together in solution, they are heat-denatured independently, at their characteristic denaturation temperatures, 84.5°C for ovalbumin and 92.5°C for *S*-ovalbumin, at pH 8.8 (Figure 1). Within experimental error, their enthalpies of denaturation are the same,

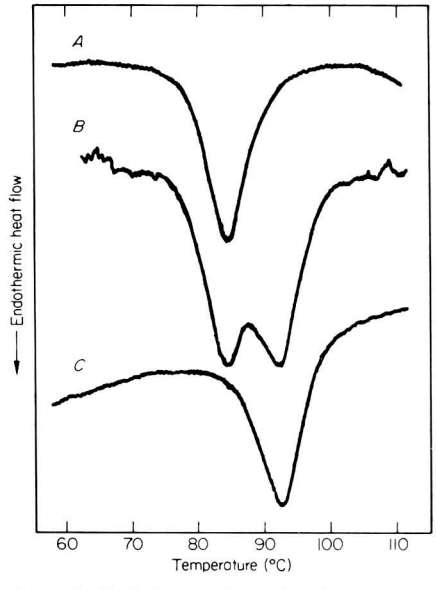

Figure 1. Endotherms of ovalbumin and *S*-ovalbumin. *A*, Ovalbumin; *B*, equimolar mixture of ovalbumin and *S*-ovalbumin; *C*, *S*-ovalbumin. Buffer, 0.05 M Tris, pH 8.8; heating rate 10°C/min.

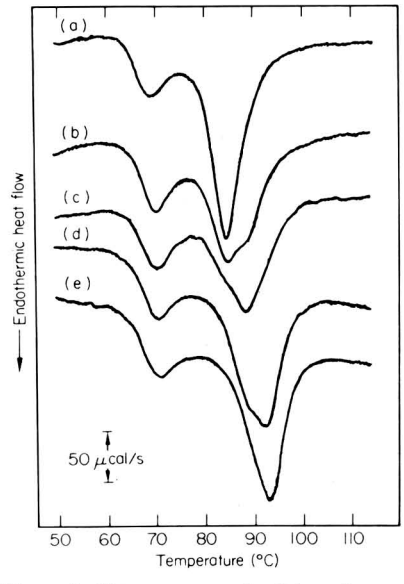

Figure 2. Thermograms of whites of eggs held various times at 22°C. Heating rate, 10°C/min. Samples: (a) fresh egg, pH 8.26, 17.2 mg; (b) 166 h, pH 8.94, 16.7 mg; (c) 331 h, pH 8.99, 15.6 mg; (d) 691 h, pH 9.24, 16.2 mg; (e) 908 h, pH 9.37, 16.4 mg.

3.6 cal/g, as previously reported for ovalbumin.[1] When mixtures of ovalbumin and *S*-ovalbumin containing 10–90% ovalbumin were prepared, the amount of each component could be determined within 5% by graphical resolution of the DSC thermogram of the mixture into the two endotherms characteristic of the two components.

3.2. Conversion of purified ovalbumin into *S*-ovalbumin

Samples taken from a solution of purified ovalbumin at different times of heating under the same conditions used to prepare *S*-ovalbumin (see section 2.1.2), gave thermograms unlike thermogram *B* of Figure 1. Instead, the thermograms obtained at intermediate times of heating showed a progressive change from a thermogram characteristic of ovalbumin to a final thermogram characteristic of *S*-ovalbumin, and could best be interpreted as made up of three endotherms, for ovalbumin, an intermediate, and *S*-ovalbumin. Since these thermograms are like those for egg white, which will be treated in detail below, representative thermograms are not shown here.

3.3. Thermograms of the white of stored eggs

Samples of egg white obtained after different times of storage of intact eggs, when subjected to DSC without adjustment in pH of the white, gave the thermograms shown in Figure 2. There is a progressive change in the ovalbumin portion of the thermogram, with the peak temperature shifting from 84.5–88.5°C, and then to 92.5°C. Changes in the lysozyme–conalbumin region of the thermogram, at 70°C, are nearly absent. Note that mixtures of ovalbumin and *S*-ovalbumin (Figure 1) do not give a thermogram with a peak at 88.5°C.

Changes produced in the thermograms of egg white by storage are accelerated at elevated temperatures (Figure 3). Changes occur only slowly at 4°C. Storage for 1675 h at 4°C resulted in a

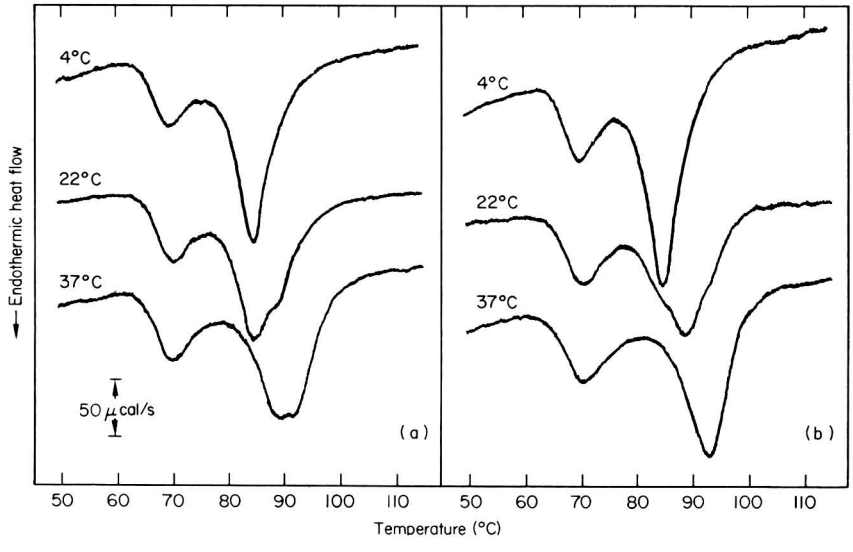

Figure 3. Thermograms of egg white of eggs stored for 1 (a) and 2 (b) weeks. Heating rate, 10°C/min. (a), 190 h at 4°C, pH 8.68, 16.1 mg; 166 h at 22°C, pH 8.89, 14.4 mg; 186 h at 37°C, pH 9.15, 15.6 mg. (b), 335 h at 4°C, pH 8.75, 17.5 mg; 331 h at 22°C, pH 8.99, 15.6 mg; 330 h at 37°C, pH 9.20, 16.5 mg.

thermogram approximately equivalent to that for storage at 22°C for 166 h. Figure 3 provides a convenient reference set of thermograms showing various stages of conversion of ovalbumin into *S*-ovalbumin. The changes in pH for these samples are shown in Figure 4.

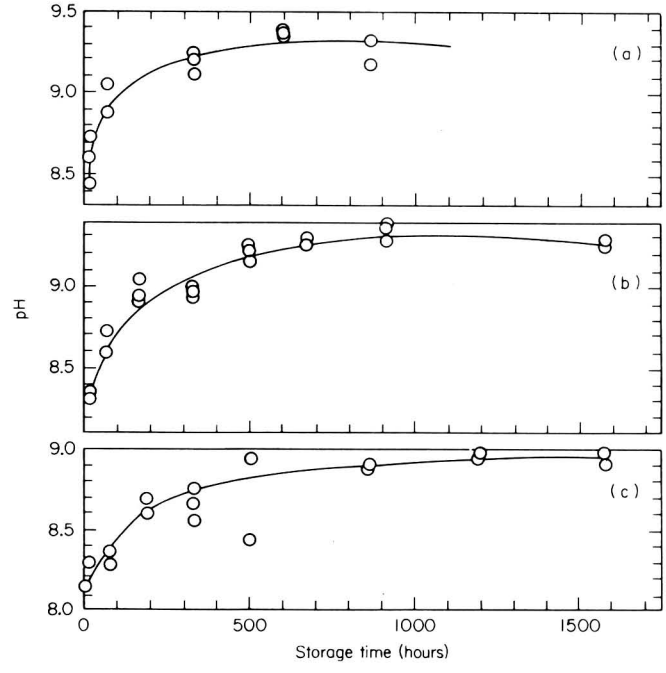

Figure 4. Changes in pH during storage at (a) 37°C, (b) 22°C and (c) 4°C. The pH determined for the homogenised white of individual eggs is plotted as a function of storage time for the three storage temperatures.

Since combinations of the two endotherms of ovalbumin and *S*-ovalbumin will not give a peak at 88.5°C, the presence of additional endotherms must be assumed in order to fit the thermograms of Figures 2 and 3. Addition of a single intermediate endotherm to the endotherms of ovalbumin and *S*-ovalbumin gives an acceptable fit to the observed thermograms. Although the presence of a number of intermediate endotherms is also conceivable, more than two intermediates seems unlikely. The sharpness of the intermediate peak (see Figures 2 and 3) is comparable to that of a single component. Multiple intermediate components would produce a broader, more shallow endotherm, probably not recognisable as a separate peak in the presence of both ovalbumin and *S*-ovalbumin.

3.4. Calculation of the extent of conversion of ovalbumin in egg white

The most plausible pathway for conversion of ovalbumin to *S*-ovalbumin, as stated in Section 3.3, is through a single intermediate species. The conversion has the kinetic behaviour:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where A represents native ovalbumin, B, the intermediate, and C, *S*-ovalbumin, and k_1 and k_2 are first order rate constants. The relative amounts of the three species present were determined from the thermograms obtained for storage at 22 and 37°C by computer fit with Gaussian endotherms centred at 84.5, 88.5, and 92.5°C. It was thought desirable to obtain also the relative amounts of the three species in a simpler way, directly from the heights of the endotherms in the thermogram. Since the sum of the concentration of the three species is a constant, the height of the thermogram at only two temperatures was required, and 84.5 and 92.5°C were chosen since the largest changes in the thermogram occur at these temperatures. An *a priori* fit, based on the endotherms of a pure A and C, assuming that the endotherm for B was similar but shifted in temperature, did not give good

agreement with the results obtained by computer fit of the thermograms. Accordingly, the constants for equations of the type

$$H_t = \alpha H_t^{\alpha} + \beta H_t^{\beta} + (1 - \alpha - \beta) H_t^{\gamma}$$

were determined empirically, by trial-and-error. Here, H_t is the heat flow per unit time (thermogram height) at temperature t, H_t^i is the thermogram height for pure component i at temperature t, and α, β and γ are the fractions of the three components present, $\alpha = A/A_0$, $\beta = B/A_0$, $\gamma = C/A_0$, $\alpha + \beta + \gamma = 1$. When the observed values of H_t were in units of (mcal/s)/(mg of egg white), an adequate fit to the 22°C data was obtained with the coefficients $H_{84.5}{}^{\alpha} = 0.99 \times 10^{-2}$, $H_{84.5}{}^{\beta} = 0.40 \times 10^{-2}$, $H_{84.5}{}^{\gamma} = 0.14 \times 10^{-2}$, $H_{92.5}{}^{\alpha} = 0.12 \times 10^{-2}$, $H_{92.5}{}^{\beta} = 0.22 \times 10^{-2}$, $H_{92.5}{}^{\gamma} = 1.02 \times 10^{-2}$. When the 37°C data were fitted by use of these coefficients, the calculated values of α, β and γ were in reasonable accord with those obtained by computer fit of the thermograms.

An error is introduced into interpretation of the DSC thermograms of egg white by the assumption that the endotherms in the 85–93°C temperature range result entirely from denaturation of ovalbumin and *S*-ovalbumin. Other minor proteins are also denatured in this temperature range. The greatest contribution comes from ovomucoid, which makes up about 10% of the egg white protein. Its denaturation produces a very broad endotherm, with a maximum at 79°C, but extending to 110°C.[6] Almost all of this endotherm was eliminated by constructing baselines as described in Section 2.2.

3.5. Kinetics of the conversion of ovalbumin to *S*-ovalbumin

The amount of conversion of ovalbumin first to an intermediate, then to *S*-ovalbumin, was determined as described in Section 2.2. The amounts of the three components present as a function of time are presented in Figure 5. The curves drawn in this figure are calculated on the assumption

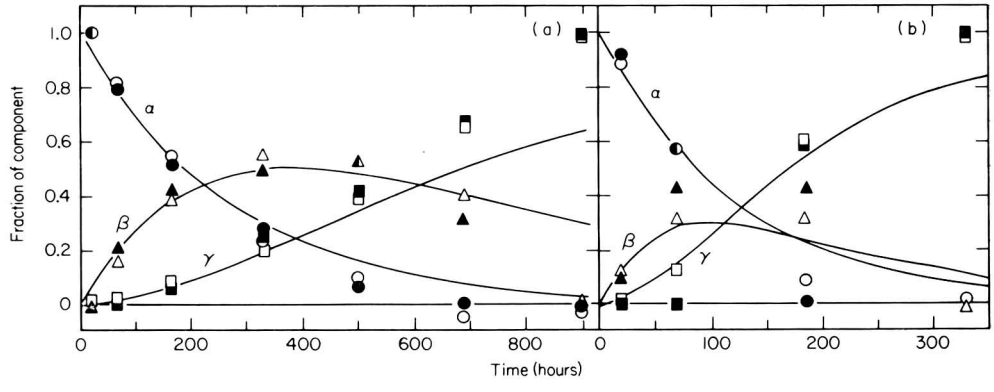

Figure 5. Kinetics of the conversion of ovalbumin to *S*-ovalbumin in stored eggs at (a) 22 and (b) 37°C. Filled symbols were obtained by computer fit of thermograms with bands for ovalbumin (α), intermediate (β) and *S*-ovalbumin (γ); open symbols, by calculation from height of thermogram at two temperatures, see text. Solid lines are calculated for successive first order reactions with k_1 and k_2, respectively, 0.0036 h^{-1} and 0.0018 h^{-1} at 22°C, 0.0080 h^{-1} and 0.0120 h^{-1} at 37°C.

that the reactions are irreversible, and sequential first-order. The rate constants were selected on a trial-and-error basis to fit the early data. The conversion obviously proceeds faster than the calculated rate at later times. It seems very likely that this increase in rate results from the increase in pH of the egg white, shown in Figure 4. Accordingly, the rate constants obtained are only approximate. Over the periods for which the rate constants were determined, taking into account those experimental points which have greatest effect on the calculated rate constants, the pH of the 22°C

and 37°C samples were nearly the same, and averaged 9.0. The difference in rate can thus be attributed to temperature, not pH. The best fit gave the rate constants (in units of h^{-1}) $k_1 = 0.0036$, $k_2 = 0.0018$, at 22°C; $k_1 = 0.0080$, $k_2 = 0.0120$, at 37°C. Estimated error in these rate constants is 25%. Activation energies calculated from only these two data points are: step 1, 10 kcal/mol; step 2, 23 kcal/mol. Since the rate constants k_1 and k_2 are of similar magnitude, neither step is rate-limiting, and an activation energy for the overall process would approximate 17 kcal/mol. An activation energy of 18 kcal/mol was obtained from the initial rate of disappearance of native ovalbumin at the three temperatures.

3.6. Stability of ovalbumin preparations on storage

A number of samples of ovalbumin prepared 20 years ago at this laboratory had been stored as freeze-dried material in the cold (12 years at −12 to −18°C, followed by approximately 8 years at 2°C). As far as known, all were prepared by the same standard method,[8] which was the method used to prepare a fresh sample of ovalbumin. All preparations were recrystallised by addition of ammonium sulphate near the isoelectric pH of ovalbumin, dialysed against distilled water until free of salt, and freeze-dried. To check that these preparations were stored under equivalent conditions, the pH of a 5% solution in water was measured. The recently prepared sample of ovalbumin (JGD) had a pH of 4.9, the stored samples had pHs of 4.4 (EDD) and 4.5 (TFS). These values of pH give, approximately, the pH of the last ammonium sulphate precipitation before solution and dialysis. The DSC thermograms of some of these samples are shown in Figure 6. The 20-year-old samples have apparently been partially converted to the intermediate form upon storage.

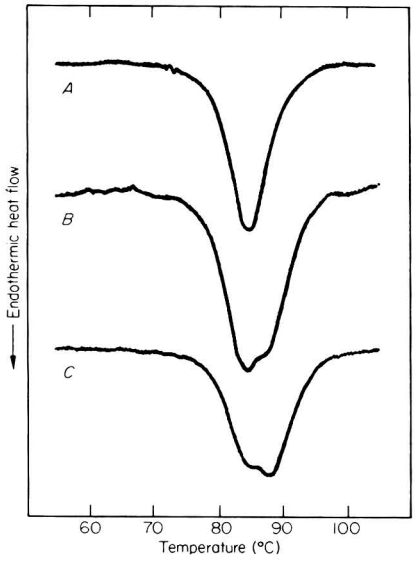

Figure 6. Effect of storage on freeze-dried ovalbumin. *A*, recent preparation of ovalbumin; *B*, sample TFS-17 (4/12/54); *C*, sample EDD (4/8/54). Samples *B* and *C* had been stored 12 years at −12 to −18°C, then approximately 8 years at 2°C. Thermograms of water solutions, 80–90 mg protein/ml, adjusted to pH 9.0 with KOH. Heating rate, 10°C/min.

4. Discussion

4.1. DSC as a measure of the quality of egg white

The egg white from eggs stored at elevated temperatures gives angel cakes with reduced volume.[9] The damaged egg white has a higher coagulation temperature, and an altered ovalbumin. The functional properties of the egg white can be restored by addition of native ovalbumin.[9] The altered ovalbumin has been characterised, shown to be heat-denatured at a slower rate than ovalbumin, and designated *S*-ovalbumin, or stable ovalbumin.[2,3]

The present work shows that differential scanning calorimetry readily distinguishes the denaturation behaviour of ovalbumin from that of *S*-ovalbumin, and that DSC can be used to monitor the conversion of ovalbumin into *S*-ovalbumin. Accordingly, the quality of egg white of eggs held in storage can be readily determined from DSC thermograms. Incubator rejects should be clearly recognisable when tested by DSC. Calorimetric measurements of egg white should also reveal quality changes resulting from processing, particularly when egg white has been exposed to alkaline pH, which accelerates formation of *S*-ovalbumin. Although measurement of pH will generally also indicate the conditions of storage and the quality of the egg white, DSC measurements are still valid when the pH of the egg white has been adjusted after breaking and separating, or during processing.

4.2. The ovalbumin–*S*-ovalbumin transformation

Smith[3] has shown that the ovalbumin–*S*-ovalbumin transformation is not reversible. Completely denatured *S*-ovalbumin appears to refold at a much slower rate than completely denatured ovalbumin.[10] Together, these observations indicate that the source of the increase in heat stability is a change in covalent structure of ovalbumin. This change does not appear to be an alteration of the disulphide linking in the molecule.[11] The transformation is considerably accelerated by increasing pH. In fact, the reaction appears to be first order in hydroxide ion concentration.[3]

It has not previously been recognised that the conversion of ovalbumin to *S*-ovalbumin occurs by way of an intermediate. Smith[3] has stated that different preparations of *S*-ovalbumin from stored eggs gave material with variable properties. It is possible that these preparations of Smith contained significant amounts of the intermediate.

We find it surprising that this transformation also takes place in the freeze-dried state, particularly since the freeze-dried ovalbumin was originally prepared by recrystallisation from acid solution. However, for all old samples examined, conversion appears to have occurred. The samples in which the transformation has proceeded to the greatest extent appear to be composed of about one-half ovalbumin and one-half intermediate. Little or no *S*-ovalbumin appears to be present. Although it may be questioned whether the original material already contained the intermediate before it was freeze-dried, this appears unlikely. However, when these samples were prepared, the existence of *S*-ovalbumin and the intermediate was not known, so the freshly prepared ovalbumin was not tested for the presence of these materials.

The rate of transformation, as observed in stored eggs by DSC, appears to be comparable to the rate of transformation of purified ovalbumin reported by Smith. At 37°C, pH 9.0, we have determined that k_1 is approximately 2×10^{-6} s^{-1} from our storage experiments. At 40°C, pH 9.0, Smith reports a rate of constant of 7×10^{-6} s^{-1}.[3] Our estimate of the activation energy for transformation of ovalbumin also appears to be in rough agreement with that reported by Smith,[3] although both measurements are relatively imprecise. It is clear that the activation energy (15–25 kcal/mol, or about 0.5 cal/g) is not characteristic of complete denaturation of a protein. Such an activation energy would be of the order of 5 cal/g.

4.3. Properties of *S*-ovalbumin

Most of the properties of ovalbumin and *S*-ovalbumin are identical.[3] Differences between these proteins are evident only under denaturing conditions. The coagulation temperatures of fresh and storage-altered egg white differ by 10°C.[9] The maxima in the DSC thermograms of ovalbumin and *S*-ovalbumin differ by 8°C (Figure 1). The enthalpy of denaturation of *S*-ovalbumin is, within experimental error, the same as that of ovalbumin. This is in agreement with Smith's contention that these proteins are very similar in conformation.[3]

4.4. Properties of the intermediate

The presence of a single intermediate between ovalbumin and *S*-ovalbumin appears to agree best with the experimental observations. Since the denaturation temperature of the intermediate (88.5°C) is halfway between that of ovalbumin and *S*-ovalbumin, it is likely that the other properties of the

intermediate closely resemble those of ovalbumin and *S*-ovalbumin. Since the properties of these ovalbumins are so similar,[3] and either ovalbumin or *S*-ovalbumin, or both, are present with the intermediate at different times of heating or of storage, separation of pure intermediate would appear to be a formidable task. We have not attempted it.

The thermal characteristics of the intermediate appear to be those of a specific molecule, rather than of a range of species with properties between those of ovalbumin and *S*-ovalbumin. A progressive change from $A \rightarrow C$ so that no specifically identifiable species B intervened could result if, for example, a gradual increase in cross-linking of the species A occurred. This would result in a gradual stabilisation of the protein as the degree of cross-linking increased. However, this kind of process should not give rise to a new endotherm with a specific denaturation temperature, as we have observed, but instead to many species with a range of denaturation temperatures.

Acknowledgements

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