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Erratum

Further Studies on SS Bonds in Cereal Glutelins

J. A. D. Ewart

J. Sci. Fd Agric. 1972, **23**, 567–579

On p. 577, line 32, equation (1) should read equation (2).

J. Sci. Fd Agric. 1972, **23**, 919–924

Variability in Fatty Acid Composition in Peanut I. Bunch Group

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(Accepted for publication January 1972)

Oil content and fatty acid composition of the 50 bunch peanut types have been reported. The oil content showed little variation, (49.1 ± 2.5%) and of the major component acids, oleic acid showed little variation (44.5 ± 5%) whilst the palmitic (P) and linoleic (L) contents were reciprocally related (P + L ≃ 48%).

1. Introduction

Fatty acid composition largely determines the quality of oil. Catteneo,[1] French,[2] Jong-Ching and Chinshi[3] and Grishiva and Kuznetsov[4] reported variations in fatty acid composition among peanut varieties. Sekhon, Ahuja and Sandhu[5] described fatty acid composition of the six Punjab peanut varieties. The observed varietal differences in fatty acid composition have been attributed to genetic causes.[6,7] Manipulation of the composition of oil through plant breeding techniques thus has a practical objective.

In the present study 50 bunch peanut types were analysed to study their variation in oil content and fatty acid composition.

2. Experimental

Representative seed samples of 50 bunch peanut types were obtained from the world germplasm collection grown at the Punjab Agricultural University Farm, Ludhiana, in the rainy ("kharif") season of 1970. Oil was extracted by the method of Kartha and Sethi.[8] The methyl esters of fatty acids were prepared according to the method of Luddy *et al.*[9]

The AIMIL Model II A gas chromatograph with flame ionisation detector, having a 6 mm × 2 m column packed with 15% (w/w) diethyleneglycol succinate (DEGS) on celite, was used for the separation of methyl esters of fatty acids. The instrument was operated at 175 °C with a gas flow rate of 35 ml/min. The fatty acids were identified from their retention times and their respective peak areas were directly converted to relative percentages.

3. Results and discussion

The data show a narrow range of variation among varieties, both for oil and fatty acid composition (Table 1). The percentage of oil varied from 47.00 (B 239 and U 1-2-4) to 52.00% (Bogal Kot and Uganda erect); palmitic acid from 9.7 (G 1042) to

TABLE 1. Oil and fatty acid composition of peanut

Sample no.	Variety/ strain	Origin	Oil content (%)	Acid composition (%)						
				Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Higher fatty acids
1	B3	Bihar	48.0	0.4	12.0	0.6	2.1	46.2	35.3	3.5
2	Chandodi	Madhya Pradesh	47.50	0.5	16.9	0.4	2.7	42.1	34.2	3.2
3	NG 51	Madhya Pradesh	51.00	T	15.8	0.7	3.4	44.1	32.3	4.2
4	NG 337	Madhya Pradesh	48.00	0.5	15.8	0.7	4.0	45.2	30.1	3.7
5	R 4	Madhya Pradesh	51.50	0.8	17.3	1.8	2.9	42.7	30.1	4.5
6	AH 32	Tamil Nadu	48.00	0.2	12.4	0.5	2.6	41.8	39.0	3.4
7	HG 1	Tamil Nadu	50.00	T	12.3	1.2	3.3	41.6	36.6	5.1
8	TMV 2	Tamil Nadu	47.50	0.7	11.2	0.9	2.3	42.6	38.1	4.2
9	Akola No. 10	Maharashtra	48.00	T	15.6	0.7	2.0	41.3	35.8	4.2
10	Balloli local	Maharashtra	49.25	0.6	14.8	0.4	3.0	40.0	36.5	4.6
11	Bogal Kot	Maharashtra	52.00	0.6	15.1	0.2	3.0	45.2	33.1	2.9
12	Dohad No. 1	Maharashtra	50.00	0.7	13.2	0.7	1.8	42.5	37.2	2.5
13	Faizpur	Maharashtra	50.00	0.6	17.4	0.9	2.3	41.9	31.7	4.2
14	Faizpur 1–5	Maharashtra	49.00	0.9	14.7	1.2	3.7	45.0	30.4	3.9
15	Improved Small Japan	Maharashtra	49.25	0.7	14.6	0.6	3.4	43.1	34.9	2.8
16	Jhalod No. 6	Maharashtra	48.50	T	16.7	0.7	3.4	47.3	26.9	4.1
17	Khandesh	Maharashtra	49.12	0.2	14.5	0.4	2.7	43.8	34.8	3.6
18	Kopergaon No. 3	Maharashtra	48.50	T	15.3	0.2	4.3	51.9	25.6	2.7
19	Limidi No. 4	Maharashtra	51.00	T	10.3	0.2	2.8	46.5	35.9	4.3
20	No. 1	Maharashtra	50.00	T	18.4	0.8	4.6	46.9	26.9	2.4
21	Shidapur local	Maharashtra	51.40	T	10.2	0.5	2.9	47.3	35.0	4.1
22	Sulebhani Bijapur	Maharashtra	47.75	1.0	18.1	1.1	3.0	47.3	24.5	6.0
23	Taluka Farm Local	Maharashtra	51.00	T	12.3	1.0	2.2	47.6	33.2	3.7

24	G 1042	Mysore	47.50	0.8	9.7	0.5	2.5	44.1	38.7	3.1
25	HG 8	Mysore	48.00	T	10.8	0.9	2.2	42.5	37.1	6.5
26	Var. 28–204	Africa (Senegal)	48.50	T	11.9	0.5	2.4	48.7	32.9	3.7
27	Var. 47–5	Africa (Senegal)	49.00	0.9	12.5	0.1	3.2	44.4	36.3	2.7
28	Bulindi	East Africa	51.00	0.5	14.8	0.6	3.5	46.9	30.3	3.3
29	B 239	East Africa	47.00	T	13.8	0.4	2.5	44.7	35.0	3.7
30	BB 2	East Africa	47.50	T	11.6	0.8	2.6	42.8	38.0	4.3
31	Barbaton	East Africa (Kenya)	48.25	0.7	13.1	0.6	1.9	46.0	33.8	3.9
32	A 9	East Africa (Tanganyika)	51.75	1.8	13.3	1.0	2.8	41.7	37.7	2.9
33	A 13	East Africa (Tanganyika)	47.75	1.5	16.9	2.3	3.0	50.7	20.5	5.1
34	A 14	East Africa (Tanganyika)	48.50	T	13.0	1.4	2.3	45.7	34.8	2.9
35	A 15	East Africa (Tanganyika)	47.50	0.7	14.4	0.9	2.6	40.4	37.6	3.4
36	A 65	East Africa (Tanganyika)	49.00	1.4	17.4	0.7	2.7	42.3	31.9	3.7
37	Brengold	South Africa	49.00	0.7	18.3	0.2	2.2	48.6	26.7	3.3
38	Natal Common	South Africa	50.00	0.1	13.5	0.6	3.7	43.2	35.1	3.9
39	Uganda erect	South Africa	52.00	0.6	12.1	0.6	2.5	45.0	35.4	3.8
40	Valencia	Australia	51.75	1.0	16.9	1.0	2.9	39.0	36.4	2.9
41	Chinese	China	50.00	0.5	13.4	1.0	3.2	43.8	34.3	3.8
42	Chunjia	China	48.25	0.6	11.4	0.8	2.9	45.4	34.0	4.9
43	Israel 123	Israel	51.25	T	23.1	0.9	2.4	41.7	36.6	5.3
44	R 7-47-10	Sudan	48.50	T	14.0	1.1	3.3	44.4	33.9	3.3
45	S 7-1-10	Sudan	47.50	T	11.4	0.2	2.0	47.6	35.9	2.8
46	U 1-2-3	Sudan	47.75	T	11.6	0.6	1.1	44.6	38.3	3.0
47	U 1-2-4	Sudan	47.00	0.5	14.2	1.1	2.9	40.8	34.4	6.1
48	U 2-1-1	Sudan	50.00	T	14.6	0.6	3.9	40.6	36.7	3.5
49	U 1-47-3	Sudan	47.50	T	10.0	T	2.0	47.6	36.7	3.7
50	AK 12-24 (Std)	Maharashtra	49.00	T	13.4	0.7	3.5	45.0	33.5	3.8
		Mean	49.14		13.9		2.9	44.5	33.8	3.8

T = trace.

TABLE 2. Correlation coefficients among oil and fatty acid components

	Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Higher fatty acids
Oil	+0.109	+0.117	+0.007	+0.280*	−0.037	+0.002	−0.275
Myristic acid		+0.240	+0.450**	−0.054	+0.114	−0.065	−0.010
Palmitic acid			+0.256	+0.573**	−0.045	−0.758**	−0.042
Palmitoleic acid				−0.069	−0.686**	−0.654**	+0.285*
Stearic acid					−0.141	−0.640**	−0.097
Oleic acid						−0.629**	+0.142
Linoleic acid							−0.039

*Significant at $P = 0.05$.
**Significant at $P = 0.01$.

18.4% (No. 1); stearic from 1.1 (U 1-2-3) to 4.6% (No. 1); oleic from 39.0 (Valencia) to 51.9% (Kopergaon No. 3); linoleic from 20.5 (A 13) to 39.0% (AH 32) and higher fatty acids from 2.4 (No. 1) to 6.1% (U 1-2-4). Greater variability was thus found in the unsaturated acids (oleic and linoleic) than in the saturated fatty acids, whereas Grishiva and Kuznetsov[4] observed greatest variability in the saturated fatty acids. Oleic acid formed the major fatty acid component in all the entries tested and varieties with low linoleic acid content usually contained more saturated fatty acids. These results corroborate the findings of Jong-Ching and Chinshi.[3] Within the varieties tested the mean values for oil, palmitic, stearic, oleic, linoleic and higher fatty acids were 49.1, 13.9, 2.9, 44.5, 33.8 and 3.8%, respectively.

Varieties A 13, Kopergaon No. 3, Brengold, Sulebhani Bijapur, Jhalod No. 6 and No. 1 had high amounts of oleic and palmitic acids but had low linoleic acid. Varieties with high oleic and low linoleic acid are preferred because their oil has a greater stability (Fore *et al.*[10] and Bratcher *et al.*[11]). Such varieties are also more suitable for hydrogenation purposes where linoleic acid is converted into oleic acid without formation of stearic acid.[12] Furthermore, a high proportion of linoleic acid is not desirable because increased unsaturation in oil increases the cost of hydrogenation due to increased demand for hydrogen.[13] A high oil content was another desirable character and 18 varieties registered more than 50% of oil.

Correlation coefficients among oil and its constituents are presented in Table 2. Oil content showed a significant positive correlation with only stearic acid (0.280). Among the fatty acids, myristic acid had a highly significant positive correlation with palmitoleic acid (0.450). Palmitic acid had a highly significant positive correlation with stearic acid (0.573) and negative with linoleic acid (− 0.758). Palmitoleic acid was strongly negatively correlated with both oleic and linoleic acid (− 0.686 and − 0.654) while the association was moderately positive with the higher fatty acids (0.285). Highly significant, negative correlations were observed between stearic and oleic acids with linoleic acid (− 0.640 and − 0.629).

A high negative correlation of palmitic, stearic and oleic acids with linoleic acid suggests that it is possible to obtain varieties having high amounts of saturated fatty acids and oleic acid with low content of linoleic acid.

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Digestion of the Dry Matter, Nitrogen, Phosphorus, Sulphur, Calcium and Detergent-fibre Fractions of the Seed and Pod of *Stylosanthes humilis* contained in Terylene Bags in the Bovine Rumen

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The rates of digestion of the separated seed and pods of *Stylosanthes humilis* in Terylene bags in the rumen of cattle were studied. The rates of disappearance of dry matter, N, P, S, Ca and detergent fibre fractions and lignin were examined. The magnitude of the errors of determination were assessed statistically for dry matter digestion. Four bag replications were necessary to detect differences of 20% between samples.

The cell contents constituted 80% of the seed dry matter, but only 20% of the pod dry matter. Lignin content of seed was negligible (0.5%), but was high in pods (18%).

The seed was rapidly digested (77% of dry matter after 48 h). This was largely due to almost complete digestion of the cell contents. The pod was only slightly digested (14% at 48 h). Protein and phosphorus appeared to be selectively solubilised, but calcium was only slowly solubilised in both seed and pod.

The cell-wall constituents present were compared to the concentrations of hemicellulose and cellulose found in seed and pod by more definitive chemical methods.

1. Introduction

Intact seed pods of legumes can be an important component of the diet of grazing ruminants. The seed and pod of the tropical legume, *Stylosanthes humilis*, has been shown to be of high nutritional value and a valuable source of nitrogen, phosphorus, sulphur and calcium for the animal,[1] although the extent of its contribution to the diet of grazing cattle is not yet known. In northern Australia, once seed has set in March–April, about half of the nitrogen, phosphorus and sulphur in the plant tops of *S. humilis* is found in the seed.[2,3] Studies on the digestion of the separated seed and pod were carried out because of the possibilities of altering the seed to pod weight ratio (the pod

contains a single seed) and the seed size. D. F. Cameron (1969 personal communication) found seed weight in *S. humilis* genotypes to range from 1.3 to 3.1 mg of dry matter.

The seed and the pod were digested in Terylene bags in order that samples which had been immersed in rumen fluid for different intervals could be fractionated chemically to provide rate of digestion data of each fraction. The nylon, Dacron or Terylene bag technique gives results comparable to those obtained by *in vitro* digestion techniques[4] with similar variation between samples. The bag technique allows the sample to remain discrete and in sufficient amount to permit further component analysis. The main disadvantage is that small solid particles can be lost or gained through the closely woven bag.

In this paper, rate of digestion of seed and of pod of *Stylosanthes humilis* for up to 72 h residence in the rumen is described. The digestion of dry matter, nitrogen, phosphorus, sulphur and calcium and the detergent–fibre fractions of van Soest[5] were examined.

2. Experimental

There were two experiments. Experiment 1 was designed to examine variability of dry matter disappearance from bags. The two feeds used were intact seed pods of *Stylosanthes humilis* and *Stylosanthes humilis* hay. Both feeds were milled through a 1-mm aperture screen. In experiment 2, the seed and pod were separated and the digestion of each was examined. The seed was from the same source as that used in experiment 1. Seed was removed from the pods by using a Christy and Norris 20-cm laboratory mill with a large screen at low speed.

The material was then separated on a Clipper seed cleaner (A. T. Ferrell and Co, Saginaw, Michigan, U.S.A.). Final separation was done by hand under an illuminated magnifying lens. The separated seed and pod were then milled using a 2-mm aperture screen.

2.1. Materials

The rumen digestion studies used two steers fitted with rumen cannulae with 10-cm diameter openings. The steers were each fed once daily 4.1 kg of dry matter, consisting of lucerne hay (*Medicago sativa*) (2 kg), spear grass hay (*Heteropogon contortus*) (1 kg), Townsville stylo hay (*Stylosanthes humilis*) (1 kg) and Townsville stylo intact seed pods (100 g). This ration was fed for at least 4 days before each experiment started and during each experiment.

The bags used were made from closely-woven Terylene cloth (37 picks/cm on 43 ends/cm warp; range of apertures 30 to 93 μm diameter) measuring 15 cm × 6 cm were used and holding 8 to 10 g of ground dried material. The bags were placed in polyethylene 1-l jars measuring 17 cm × 10 cm diameter, perforated on all sides with about 100 1-cm holes. Each jar held up to 8 bags together with a 900 g steel weight. Five jars could be accommodated readily in the ventral sac of the rumen.

At the different sampling times, bags were removed from the jars and were washed under running water for 30 min. They were then centrifuged in a basket centrifuge and dried at 60 °C in a forced-draught oven for 24 h. The contents were then weighed to determine dry matter loss and kept for chemical analysis.

2.2. Design of experiments

In experiment 1, 5 jars were inserted in the ventral rumen of steer 1; in each jar, there were 4 bags of each of the 2 feeds examined, giving a total of 40 bags. There were 4 sampling times after 20, 44, 68 and 91 h digestion. At each sampling time, one bag of each feed was removed from all 5 jars, giving 5 replications.

In experiment 2, two steers were used and 4 jars were inserted in the ventral rumen of each steer. Four bags of seed were placed in 2 jars in each animal. One bag was removed from each of these 4 jars at 3, 6, 12 and 24 h of digestion, giving 4 bag replications at each time.

In the other 2 jars in each animal, 4 bags of seed and 3 bags containing pod were placed in each jar. One bag of seed was removed from each of these 4 jars at 12, 24, 48 and 72 h of digestion; and one bag of pod was removed from each of the 4 jars at 24, 48 and 72 h.

At all sampling times, there were 4 bag replications on both seed and pod, and the 12 and 24 h sampling were over-lapped so that there were 8 replications of bags containing seed. This was done to examine if it was permissible to compare results obtained from bags contained in different jars.

2.3. Chemical analyses

In experiment 1, material from individual bags was analysed for N, P and S. In experiment 2, all chemical analyses representing a particular time of digestion were carried out on samples obtained by bulking the 4 bag replications.

Nitrogen was determined after Kjeldahl digestion by the indophenol blue method using an Autoanalyzer (Technicon Instruments Corp., N.Y.). Phosphorus was determined on the same digest by development of the phosphomolybdate complex using an Autoanalyzer, and 1-amino-2-naphthol-4-sulphonic acid as reducing agent. Sulphur was determined by a barium sulphate turbidimetric method using an Autoanalyzer.[6] Calcium was determined on a hydrochloric acid extract of the ash by atomic absorption spectrophotometry in the presence of 1000 parts/million of strontium.[7]

Neutral detergent fibre (n.d.f.) was determined by the method of van Soest and Wine;[8] acid detergent fibre (a.d.f.) and crude lignin by the method of van Soest.[9]

Neutral detergent solubles (n.d.s.) were calculated from (100 − n.d.f.); "hemicellulose" from (n.d.f. − a.d.f.); and "cellulose" from (a.d.f. − crude lignin).

3. Results and discussion

Rate of dry matter "digestion" or disappearance (d.m.d.) data for both experiments are shown in Table 1. The data on hay digestion are not presented as they are inappropriate to the content of this paper but have been included in the examination of the magnitude of error terms associated with the bag technique for which experiment 1 was primarily designed. The results obtained were used to assess the number of bags necessary to detect "true" differences between mean values. Using the $P = 0.05$ significance level, 10 bag replications were required to ensure a probability of 0.8 of detecting a 10% difference between mean values (e.g. 50 to 55% d.m.d.). Five bags were sufficient to detect differences of 15% and 4 bags, differences of 20%.

TABLE 1. Dry matter digestion or solubilisation of seed and of pod (experiment 2) and of seed + pod (experiment 1) of *Stylosanthes humilis* in Terylene bags in the bovine rumen

Experiment 2					Experiment 1		
	Seed		Pod			Seed + pod	
Time digested (h)	d.m. Digested (%)	Coeff. of variation (%)	d.m. Digested (%)	Coeff. of variation (%)	Time digested (h)	d.m. Digested (%)	Coeff. of variation (%)
3	28.4	3.8	—[a]	—	20	42.4	9.1
6	36.7	9.6	—	—	44	48.0	7.7
12	53.4	7.7	—	—	68	58.5	3.7
24	75.2	8.0	9.9	11.7	91	56.9	3.9
48	76.8	9.1	13.8	15.3			
72	83.2	2.8	13.3	22.7			
Least significant difference (at $P = 0.05$)	7.01		3.13			4.70	

[a] No sample at these times.

In experiment 2, differences in the extent of digestion of bagged seed between the 2 animals were examined. There was no difference between overall d.m.d. mean values of each animal (60.51 and 60.14). At $P = 0.05$, the least difference for significance was 3.48.

Between-jar variation in d.m.d. in both experiments was not significant. Bag variation was confounded with jar variation but since the latter was not significant, it can be presumed that these effects were also not significant.

3.1. Digestion of dry matter

Digestion after 68 h of the seed + pod sample (58.5%) agreed well with that of the separated seed and pod after 72 h when allowance was made for the weight ratio of seed to pod of 62.4 to 37.6 in an intact seed pod. The calculated digestion value of seed + pod from experiment 2 was 56.9%. These values are similar to *in vivo* digestion values of intact seed pods by cattle and sheep.[1]

Cattle consuming intact seed pods as part of their diet excrete unchanged about 5% of the intact seed pods consumed. A further 5% of whole seed consumed is stripped of its pod and is also excreted. The actual amounts excreted depend on level of intake of intact seed pods (Playne, personal communication). Thus, separation of seed and pod and milling of these fractions as in this study should result in a somewhat greater rate of digestion of the seed fraction than would occur in practice.

3.2. Digestion of nitrogen, phosphorus, sulphur and calcium

The solubilisation of these fractions is shown for both experiments in Table 2. After 48 h in the rumen, solubilisation of P was rapid and complete in the seed + pod and in the seed, whereas solubilisation of N and S was slower but exceeded 73%.

Calcium was more slowly leached from the seed which indicated it was associated with the cell wall constituents of the material.

TABLE 2. Digestion or solubilisation of nitrogen, phosphorus, sulphur and calcium in seed and pod of *Stylosanthes humilis* in Terylene bags in the bovine rumen

	Experiment 2								Experiment 1			
	Seed				Pod					Seed + pod		
	N	P	S	Ca	N	P	S	Ca		N	P	S
Initial concentration present (%)[a]	7.38	0.63	0.51	0.70	1.13	0.05	0.07	1.74	Initial concentration present (%)[a]	4.99	0.38	0.35
Percent digested									Percent digested			
after: 3 h	32	35	36	0	—[b]	—	—	—	after: 20 h	69.0	94.1	69.6
6 h	42	55	47	0	—	—	—	—	44 h	79.0	94.2	73.1
12 h	59	84	64	23	—	—	—	—	68 h	80.4	97.2	82.3
24 h	85	98	88	63	58	83	3	10	91 h	79.6	95.9	79.6
48 h	86	99	90	68	63	79	7	13	Least significant difference ($P = 0.05$)	7.9	3.9	4.8
72 h	93	99	94	88	59	65	4	5				

[a] As percentage of dry matter.
[b] No samples taken at these times.

In the pod, concentrations of N, P and S were much lower than the levels in the seed, however, Ca concentration was higher as has been reported previously.[1] Solubilisation of N, P, S and Ca in the pod was slower than that in the seed and was incomplete after 72 h (Table 2). Overall, protein appeared to be selectively digested (compare N and S digestions with d.m. digestions). Some of the calcium was bound, especially in the pod, possibly by some relatively indigestible constituent such as hemicellulose.

3.3. Digestion of cell contents and cell-wall constituents (c.w.c.)

The seed and the pod materials in experiment 2 were fractionated into cell contents (n.d.s.) and the following cell-wall constituents—hemicellulose, cellulose and lignin, before and after residence in the rumen for varying periods. The amounts of digestion and/or solubilisation of each fraction which occurred is shown in Figure 1. The method of fractionation,[8,9] although rapid and convenient for large numbers of samples, is relatively unspecific.[10,11] Thus, the hemicellulose fraction would contain some protein[5] and some pectic substances, but would fail to include some of the xylan constituents which hydrolyse incompletely in the acid detergent.[10] Similarly, the cellulose values would include such incompletely hydrolysed hemicellulose components.

The cell contents of the seed fraction formed a high proportion of the seed dry matter (79.9%) and were rapidly solubilised and digested. The process being largely complete after 24 h in the rumen in agreement with the findings of van Soest.[5] Hemicellulose was some 61% digested and cellulose 34% digested after 72 h. The lignin present (0.5% of dry matter initially) was scarcely digested at all.

The lignin recorded in seed may in fact be cutin, which is commonly present on seed coats.[12] The cellulose of the seed was expected to be highly digested because of the virtual absence of lignin. Only 34% was digested which is suggestive of some factor other than lignin incrustation being responsible for limiting cellulose digestion. This has been discussed earlier[11] and these results substantiate the findings made then using more definitive methods of polysaccharide analysis.

Digestion of all fractions in the pods was small and even after 72 h in the rumen only 38% of the cell contents had been removed. This was surprising as crude protein in the pod was probably a major constituent of the cell contents (Table 2) and itself was digested to the extent of 60% of that originally present.

The c.w.c. were heavily lignified and were the major constituents of the pod. They were scarcely attacked even after 72 h in the rumen.

Values obtained by more definitive methods of analysis for polysaccharides present in the seed and pod of *Stylosanthes humilis*[11,13] were compared with the results obtained here using the analysis scheme of van Soest and co-workers.[5,8,9] Cellulose concentrations in the seed were 12.8, 14.6 and 13.3% of dry matter; and in the pod, 44.3, 27.7 and 34.8% from the results of Pang Way and Richards,[13] Dekker, Richards and Playne[11] and of this study, respectively.

It was not possible to make good estimates of the concentrations of hemicellulose A and B fractions present from the results of Dekker, Richards and Playne[11] because of the analytical approach taken. However, if it is assumed that the neutral detergent soluble fraction does not contain polysaccharide, and this is supported by the virtual

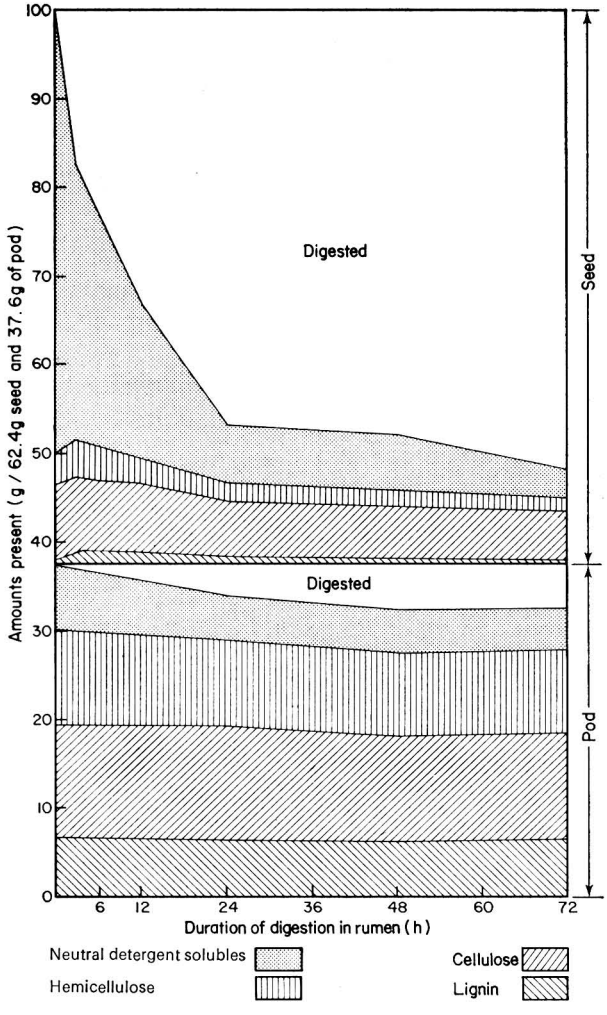

Figure 1. Amounts of cell contents and the cell-wall constituents (hemicellulose, cellulose and lignin) in seed and in pod digested or solubilised with time of residence in the rumen. Amounts represented are g of d.m. of each fraction present in 62.4 g of seed and 37.6 g of pod dry matter.

absence of polysaccharide in hot water extracts of seed,[14] then the hemicellulose A + B fraction can be assessed by difference:

$$\text{hemicellulose A + B} = \text{dry matter} - (\text{n.d.s.} + \text{cellulose} + \text{lignin}).$$

From these estimates, hemicelluloses A + B concentrations were 2.0% for seed and 32.0% for pod. In the present study, hemicellulose values were 6.3 and 27.5%, respectively. Pang Way and Richards[13] found hemicelluloses A + B to be 4.1% for seed and 27.7% for pod.

In general, the results obtained on seed and pod were similar by all three methods and in spite of the crude extraction techniques and the relative lack of specificity of the analytical methods of van Soest,[5,8,9] the results obtained gave values which were nutritionally meaningful.

Acknowledgements

The authors are grateful to Professor G. N. Richards, of the Chemistry Department, James Cook University, Townsville, for helpful discussions; to Mr J. B. F. Field of C.S.I.R.O., Townsville, for statistical analyses and advice; to Mr R. G. Megarrity of C.S.I.R.O., Townsville for N, P and S analyses; and to Mr R. P. Le Feuvre and Mr T. Magner for technical assistance.

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Chemistry of Agaroids, Carrageenans and Furcellarans

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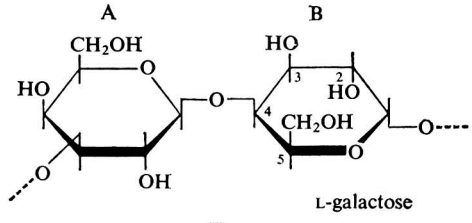
The differences in the fine structure of these algal polysaccharides which are all polydisperse linear galactans are described. The effect of these differences on the conformation of the macromolecules and consequently on the physical properties, in particular the gelling properties, of the individual polymers is discussed. The evidence that gel formation occurs through the association of chain segments into a three dimensional framework which is held together by hydrogen bonds is reviewed.

Agaroids, carrageenans and furcellarans are all galactans, that is polysaccharides made up of galactose units. They are all essentially linear molecules comprising alternately 1,3- and 1,4-linked galactose units (I), i.e. alternate A and B units, in a chain, although



L-galactose

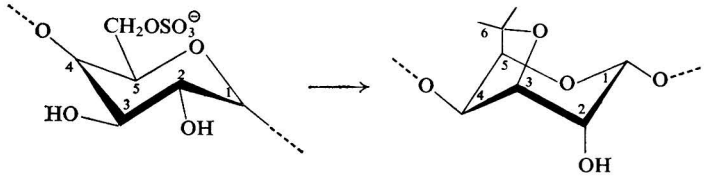
(I)

some of the units may be masked by modification and substitution and evidence of some branching has been obtained in furcellaran. They can be regarded as a family of polysaccharides which differ from one another in the proportions of D- and L-galactose, in the extent to which the galactose is modified into the 3,6-anhydro-derivative, in the amount and position of sulphation and methylation of the individual sugar residues and in the presence of other monosaccharides such as xylose and uronic acid and such substituents as pyruvic acid and glycerol.

It is understandable that the greater part of the work on the extracts from red weeds has been devoted to these three main groups in view of their preparation on an industrial scale, but a number of other genera of red seaweeds have been examined, and the extracts from a high proportion of them show the same general features. Some of these will be described briefly in order to show how they compare with the more important products.

Although most species contain a mixture of galactans which differ in some of the ways mentioned above, galactans with certain distinctive features are found in groups of seaweeds and these similarities are thus of interest in a consideration of the botanical relationships.[1]

Before the differences and similarities between the agaroids, the carrageenans and the furcellarans can be described it is necessary to emphasise the interconvertibility

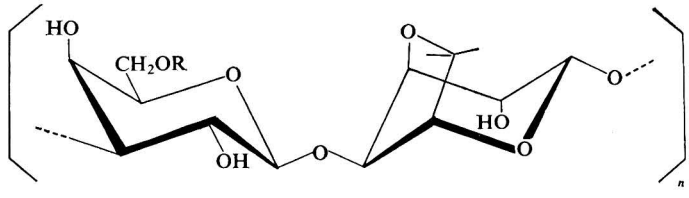


(II) Galactose-6-sulphate (III) 3,6-Anhydrogalactose

of 1,4-linked galactose-6-sulphate (**II**) into 3,6-anhydrogalactose (**III**). Enzymes present in the algae can effect this transformation,[2,3] which can just as readily be brought about in the laboratory[4] and in the factory, by alkaline treatment of the polysaccharides.

Each of the title names covers a whole spectrum of polydisperse polysaccharides and indeed both the agaroids and the carrageenans have been fractionated into several components. While, for example, there is a clear distinction between the agaroids and the other types, the carrageenans include such a wide range of different polysaccharides that it is botanical origin rather than classification on chemical grounds that puts furcellaran as a different type.

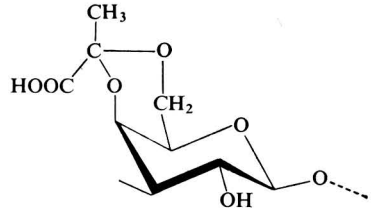
Agar, which is made commercially from a number of species, of which the most important are from the genera *Gelidium* and *Gracilaria*, has been fractionated into agarose and agaropectin,[5,6] the proportions varying with the species used.[7] Agarose is virtually devoid of sulphate and consists of chains of alternate β-1,3-linked-D-



(**IV**), Agarose R = H or OMe

galactose and α-1,4-linked 3,6-anhydro-L-galactose (**IV**).[6] 6-*O*-Methyl-D-galactose may also be present and the amount of this varies from about 1 to 20% depending upon the algal species.[8] More recently 4-*O*-methyl-L-galactose has been found in agar from *Gelidium amansii*.[9] Nevertheless, agarose may be considered to occupy one end of the family spectrum. Agaropectin, on the other hand, is more complex and is probably a mixture of polysaccharides.[5] It contains sulphated residues (3 to 10% sulphate), glucuronic acid and in some species a small proportion of pyruvic acid linked in acetal linkage (isolated as 4,6-*O*(1-carboxylethylidene)agarobiose dimethyl acetal[10] which

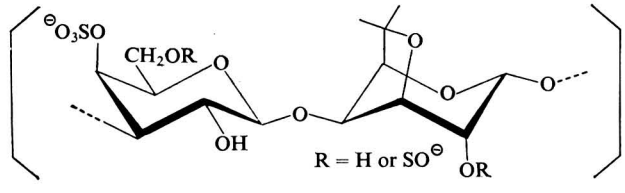
has been assigned the configuration in which the methyl group is equatorial[11] to the 1,3-linked galactose residues (**V**).



(V)

Some seaweeds which are not at present used commercially yield extracts which are similar to agar in containing the L-isomer of 3,6-anhydrogalactose or galactose-6-sulphate. Examples of these are galactans from the *Porphyra* species, but here the repeating pattern is masked, not only by 6-*O*-methyl on the D-galactose units, but as much as 50% of the anhydro-L-units are replaced by L-galactose-6-sulphate.[5] *Laurentia*[12] and a number of genera belonging to the Grateloupiaceae[13,14] have C-2 of the D-galactose units substituted by methoxyl. In the extract from *Aeodes orbitosa*[15] this is the major methyl sugar, comprising one in every six of the galactose residues. 4- and 6-*O*-methyl residues are also present, the former being present on the L-galactose units. It should also be mentioned that the last named extract also contains glycerol. In *Anatheca dentata*,[16] a member of the Solieriaceae, 3-*O*-methylgalactose has been found as a constituent of the galactan.

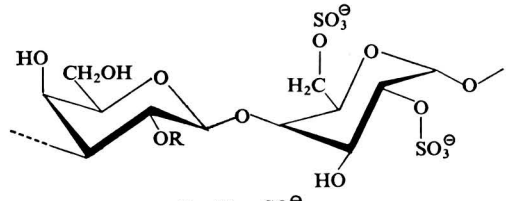
Both the carrageenans and the furcellarans appear to be devoid of methoxyl residues. A further major difference from the agaroids is the absence of L-galactose and the higher sulphate content (approximately 24% in carrageenan). This polysaccharide has been fractionated into κ- and λ-carrageenan; the κ being precipitated as a gel in the presence of potassium ions.[17] κ-Carrageenan (**VI**) differs from agarose in that C-4 of the 1,3-



(VI), κ-carrageenan

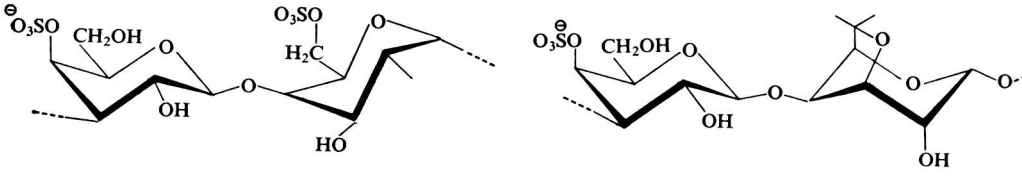
linked galactose residues are sulphated, in addition to the fact that the 3,6-anhydro-residue is the D-sugar, and some of these units are replaced by galactose-6-sulphate and both types of 1,4-linked units are partly sulphated at C-2.[5]

λ-Carrageenan is heterogeneous and its composition[18] and structure[19] vary widely with the source. Rees[20] in fact suggested that the name should be reserved for **VII** alternately 1,3-linked D-galactose sulphated on some units at C-2 and 1,4-linked D-galactose 2,6-disulphate—a polysaccharide that is soluble in potassium chloride.



R = H or $SO_3^{\ominus}$

(VII)



(VIII), μ-carrageenan

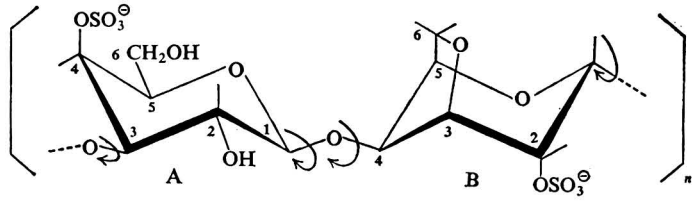
Some seaweed extracts contain also μ-carrageenan (**VIII**), which consists essentially of 1,3-linked D-galactose-4-sulphate and 1,4-linked D-galactose-6-sulphate with variable proportions of the latter present as the 3,6-anhydride. Thus, analysis for the 3,6-anhydride provides evidence of the presence of μ-carrageenan in λ-carrageenan extracts. So far it has only been possible to separate these two polysaccharides after treatment with alkali when the 1,4-linked 6-sulphated galactose units in μ-carrageenan are converted into the anhydride and this polysaccharide, which might be thought of as a κ-carrageenan, is then rendered insoluble in the presence of potassium.[20] A useful distinction between κ- and λ-carrageenans is that the former has a maximum content of 3,6-anhydro derivative and the λ-carrageenan a minimum content of this derivative.

A further example of carrageenan is ι-carrageenan extracted from such weeds as *Eucheuma spinosum* and *Agardhiella tenera* where the majority of the molecule consists of regular alternation of 1,3-linked 6-sulphated galactose and 1,4-linked 3,6-anhydro-galactose 2-sulphate (**IX**), but about 10% of the latter is replaced by 1,4-linked galactose-2,6-disulphate (**X**) and 1% of 1,4-linked galactose-6-sulphate (**XI**).

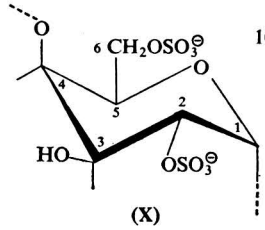
Extracts from other weeds show variable constitutions. That from *Ahnfeltia durvillaei*, for example, is intermediate in constitution between κ- and ι-carrageenans.[21] In fact the proportion of the different fractions in the agaroids and the carrageenans varies not only from weed to weed, but is also dependent upon the geographic position in which the weed grows and on seasonal variations.

Furcellaran has many similarities with the κ-fraction of carrageenan. Enzymic studies have shown that 56% of it is exactly like κ-carrageenan.[22] It has, however, a lower sulphate content, only 2 in 5 of the sugar residues carry sulphate. Nevertheless, this sulphate is dispersed on C-2, C-4 and C-6 as it is in κ-carrageenan.[23] But only about half of the D-galactose units are sulphated at C-4 and the molecule may be branched.

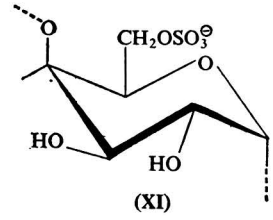
D-Xylose seems to be ubiquitous in all these galactan extracts and in some of the extracts a small proportion of uronic acid is also found. As yet none of the investigators have been able to determine what, if any, part these monosaccharides play in the



(IX)



(X)



(XI)

macromolecular structure of the galactans or whether they are present in contaminating polysaccharides.

Only a few examples of the different galactans have been described but enough has been said to show that it is possible in this complex family of polysaccharides to have every permutation and combination of the different modifications detailed at the beginning of this paper.

It is these modifications and substitutions in the individual galactose units which alter the shape or conformation of the macromolecule and it is this that changes the physical properties of the polysaccharides. There are extracts that give stiff gels in dilute solution, that is give gels which retain a definite shape even though they consist of 99.9% of water, and others that have no gelling properties at all.

Of the agaroids, agarose has the greatest gelling tendency, and any deviation from the agarose structure appears to result in a progressive diminution of the tendency to gel. Porphyran in which a proportion of the 3,6-anhydro units is replaced by galactose-6-sulphate has considerably weaker gelling powers. Similarly the extract from *Gloiopeltis furcata* which has a high proportion of the 6-sulphate does not gel but only forms viscous solutions. κ-Carrageenan gives a less rigid gel than agarose, and it requires a somewhat higher concentration (approximately 1%). The gels from furcellaran are intermediate in their properties, being more rigid and brittle than the κ-carrageenan gels but less so than agarose gels. In keeping with this is the fact that the ι-carrageenan gels, in the presence of potassium have more elastic properties than those of κ-carrageenan, and in the presence of calcium, iota gives useful compliant gels whereas κ-carrageenan gives weak brittle gels. It seems that increasing sulphur in the series agarose→furcellaran →κ-carrageenan→ι-carrageenan coincides with increasing elasticity and decreasing brittleness of the gels.

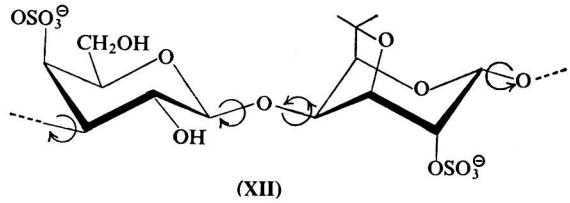
Furcellaran and κ-carrageenan at one end and μ-carrageenan at the other represent the extremes of a spectrum of structural variations. μ-Carrageenan, even in the presence of potassium ions, shows little if any inclination to gel.

Gelation appears to be completely blocked by the presence of D-galactose-2,6-disulphate or D-galactose-6-sulphate in the place of the 3,6-anhydrogalactose, or the presence of 2-sulphate on the 1,3-linked units as in λ-carrageenan which fails to gel even after alkali treatment.

So far the only effect recorded of methoxyl groups on the physical properties of the agaroids appears to be an increase of gelling temperature with increase of methoxyl content, as much as 12 °C for an 8% increase of methoxyl.[24]

Some 10 years ago Rees and his colleagues set out to determine whether the different physical properties could be related to the macromolecular shape or conformation of the polysaccharide chains. Application of X-ray studies to orientated fibres of carrageenans indicated a periodicity in the shape of the stretched out thread. It was found[20] that a single helix could be recognised for the κ- and ι-carrageenan chains from the pattern of diffracted X-rays having a pitch of 26 Å. A second chain is parallel to this helix, but displaced by half this distance, so that alternate layer lines are cancelled to give a crystallographic repeat of 13 Å. It was possible to deduce knowing the structure of the chains, that there are 3 distinct segments (i.e. six hexoses) in each turn of the helix.

A consideration of the polysaccharide chains led to the conclusion that the 4 angles, marked by arrows in **XII**, of the glycosidic links were the only variables. Even so, at



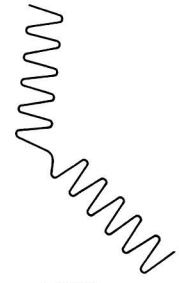
(XII)

10° steps there are 36^4 possibilities and the only possible way of solving this was by feeding all the possible ways into a computer. When this was done it was found that only 47 of these gave helices, and only 17 double helices. On rigorous treatment of these results only one double helical form with hydrogen bonds linking the two strands and with the anions on the outside was reasonably possible. Only the presence of the 3,4-anhydro allows the sugar ring to be so constrained as to have 3-equatorial C—H bonds, an arrangement that increases the flexibility of the chain and allows winding and unwinding of the double helix, and it must be remembered that any mechanism of winding up a double helix requires a free chain end.

The question then arose as to what held the double helices together in stable conformation. Since these gels melt on heating and reform again on cooling the molecules cannot be held together by covalent bonds. Isolated hydrogen bonding would not be strong enough nor would chelation. However, numerous hydrogen bonds could be the explanation. Such bonds would have to be formed between an hydroxyl group on one molecule and an oxygen atom on the second chain. Specific hydrogen bonding in both κ- and

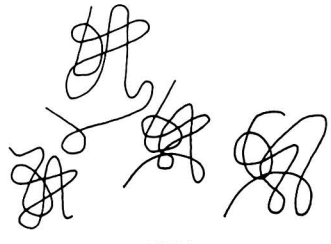
ι-carrageenan has been confirmed by infrared and deuteration–dichroism studies.[19] It can be seen from the formula **IX** that there are hydroxyl groups at C-2 and C-6 and it is considered that hydrogen bonds join O-2 and O-6 of the galactose residues in different strands of the same double helix. If this is so then every unsubstituted hydroxyl in ι-carrageenan would be engaged in hydrogen bonding within the double helix, making the conformation very stable. In natural ι-carrageenan the regular alternation is replaced by a λ-like segment [10% of the 3,6-anhydro units are replaced by 2,6-disulphate units (**X**)] and this causes a displacement of several Angstrom units in the regular alternating pattern, which results in a change in direction relative to the

(XIII)



(XIV)

idealised polysaccharide (**XIII**) shown schematically to (**XIV**) which in the native polysaccharide has a "kinked" helix. Thus a single molecule may have a number of kinks in it and each bit of the helix between the kinks can form double helices with helices from different molecules. In this way a complete three dimensional network can be built up. The solution that exists before gelation is a typical solution of polymer mole-



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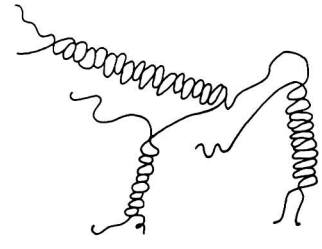

(XVI)

cules in random coil formation (**XV**) and gel formation involves the association of chain segments (**XVI**) resulting in a three-dimensional framework with junction zones between the molecules and with water held in the interstices of the framework. Junction formation and destruction is a phase change resembling protein denaturation or the melting of crystals.

On standing it is thought that there is a tendency for the framework to change spontaneously to a more stable state. This causes the phenomenon known as syneresis or the spontaneous release of some water and contraction of the gel volume. This may not occur for several days.

The gelling tendency of a particular polysaccharide is extremely sensitive to its fine

structure confirming that a steric fit is involved. The presence of one D-galactose-6-sulphate in 200 residues has a dramatic effect on gel strength—that is, a single kink which indicates that contacts between a number of consecutive residues on each chain are necessary for gel formation. Irregular 4-sulphate on the 3-linked galactose or irregular 2-sulphate on the 3,6-anhydrogalactose do not interfere sterically. In contrast gelation appears to be completely blocked by the presence of D-galactose-2,6-di-sulphate or D-galactose-6-sulphate in place of the 3,6-anhydride or a 2-sulphate on the 1,3-linked unit.

In this discussion the biological aspect has been neglected. Nevertheless, it is fascinating to pause for a moment and consider how the seaweeds have devised a method to alter radically the properties of their polysaccharides as circumstances demand by these simple modifications of the polymer chains.

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Relevance of Animal Feeding Trials to Human Dietary Requirements

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The late Professor H. H. Mitchell, to whom the science of nutrition owes so much and with whom I had the privilege to work, when discussing the general thesis of his book *Comparative Nutrition*,[1] stated "Certain broad principles may be formulated that describe the metabolic and nutritional behaviour of many species of animal .... However, superimposed upon the apparent fixity of nutritional behaviour among species of animals there are secondary and specific modifications that reflect adaptations to different environmental conditions. These are the modifications that distinguish one species from another."

This view suggests that experiments with animals have relevance to human dietary requirements in a very broad way, but that there are aspects of the nutrition of each species—whether of man or animal—which are peculiar to that species. How one distinguishes between those experiments which are concerned with principle and those concerned with specifics is not easy. The distinction is similar to that raised by Francis Bacon between experiments of light and experiments of use—experiments which increase our understanding of Nature and those which increase our power over Nature. Certainly, many experiments carried out with principle in mind prove to have limited inter-species validity and some which were designed with entirely practical ends in view have proved to have classic generality. What is beyond question is that in the development of the framework of principle in nutrition, the final test of the generality can only come through experiments and observations with a wide variety of living things. This has indeed been the pattern of the past; principles have emerged as a result of a complex interplay of observation and test with a variety of species. Furthermore, though the history of nutrition is far from tidy one can discern a dichotomous pattern in the variety of approach. Light has illuminated, and use has proved useful, but starting points in our understanding have often been experiments of use; equally, starting points for the application of nutritional science to human need have often been experiments of light.

The elucidation of the cause of scurvy provides an excellent illustration both of the approach from the practical problem and the interplay of clinical observation, experiment and therapeutic test with both man and animals. Lind's treatise of 1753[2] is of supreme interest not only as the record of the first therapeutic test in nutrition, but also because it records opinion based on observation. Lind relates the story given by Hakluyt of the Cartier Expedition of 1536 when 110 men disabled by scurvy were cured by an Indian remedy—an infusion of leaves from an evergreen. He records also

the siege of Torun when Bachstrom concluded that the scurvy epidemic was due to an absence in the diet of fresh vegetables. Lind's own experiments on HMS Salisbury confirmed the older beliefs based on observation. That "fresh vegetable chyle" prevented human scurvy was known long before any animal tests were made. The identification of ascorbic acid as the antiscorbutic principle, however, had to wait until a suitable animal test was available. Attempts to guess the causal agent were fruitless. Indeed conjecture following the potato famine led to incrimination of lack of potassium in the diet.[3] The disease was first produced in a guinea pig in 1895; but it was not until 1907 that it was actively studied. As a result of these animal studies, the vitamin was isolated in the 1920's and its structure determined.[4] Once biochemical methods were developed for its determination, work to determine man's requirement could follow and exploration of the needs of species other than primates and guinea pigs begun. The Sheffield experiments to determine human ascorbic acid requirements by direct experiment[5,6] are part of this whole pattern and these have provided the basis of present recommendations.

A similar progression from observations of affected people through therapeutic test and experiment with man to animal studies and back again is also evident with pellagra. Pellagra had been known in the maize growing areas of Italy for centuries and indeed had been attributed to the feeding of mouldy maize. Direct therapeutic tests by Voegtlin in 1914 showed that pellagrins could be cured by diets containing meat, milk and eggs.[7] This was confirmed a year later by Goldberger with the pellagrins of the Mississippi,[8] and it was Goldberger who undertook in 1915 what was probably the first controlled experiment with man designed to produce a dietary deficiency—the Rankine Farm Experiment.[9] Eleven convicts on the promise of a free pardon consumed a pellagrogenic diet, developed the disease, were cured by diet therapy and indeed were pardoned. As with ascorbic acid it was work with animals that finally led Elvehjem[10] to identify the pellagra preventative factor as nicotinic acid. As with ascorbic acid, choice of species of animal was important for the animal analogue of pellagra is black tongue in the dog; rats do not develop a classical pellagra under most conditions. Even so, despite the apparent anomaly posed by the rat it was work by Dame Harriet Chick[11] and by Krehl[12] on rat pellagra that led to recognition of the interrelation between tryptophan and nicotinic acid and their involvement in the aetiology of maize pellagra. Starting from another anomaly—the observation of the absence of pellagra in peoples preparing maize as tortillas—led through animal experiment to the realisation that a proportion of nicotinic acid in foods can be bound and to the isolation by Kodicek of nyacytin,[13] the unavailable form of nicotinic acid. More recently the study of pellagra in the jowar eaters of the Deccan has raised new problems.[14] Millet—the staple item of diet—is adequate in nicotinic acid and tryptophan content, the nicotinic acid appears to be available, yet pellagra occurs. Dog experiments suggest that leucine antagonism is involved.[15]

These two examples illustrate how, starting from the real human conditions—scurvy and pellagra—animal experiment has been a component in elucidation of the causal agent essential before further advance can be made. The generality of conclusions arises from the ubiquity of ascorbic acid and the pyridine nucleotides rather than from the wide species prevalence of dietary requirement for or of deficiency of the vitamins.

With ascorbic acid man, other primates, the guinea pig, the fruit bat and the red-vented bulbul, because of genetic deficiency in the enzyme L-gulonolactone oxidase, cannot synthesise ascorbic acid. That ascorbic acid is an essential to the normal function of cellular systems is the principle that emerges.

Not all sequences of experimentation in nutrition start with the human condition. It is more usual to find that experiments with one species of animal lead to thoughts of possibility which are then explored by therapeutic and experimental means to test the hypothesis of generality. Thus the essentiality of magnesium was first unequivocally demonstrated by McCollum in the rat in 1931,[16] to be followed not only by similar work with other animals but by recognition, first, of abnormalities of magnesium metabolism in man[17] and second, by experimental production of the deficiency in human subjects.[18] The essentiality of cobalt was first shown by work of Underwood[19] and of Marston[20] with sheep to be followed by the discovery of vitamin B-12, and elucidation of its biochemical role.[21] Work by Schwartz and Mertz in the late 1950's[22] demonstrated the essential nature of chromium in the rat. Currently work with man is still at the therapeutic stage through assessment of its possible role in explaining differences in glucose tolerance in diabetics and other patients. Again zinc is now known by direct experiment to be essential for a wide variety of species and to be a naturally occurring deficiency in animals.[23] Work in Iran and Egypt is concerned with exploration of the possibility that a similar natural deficiency exists in certain dwarfs,[24] while much work is in progress in relation to the effects of zinc on wound healing, some but not all of which is of pharmacological rather than nutritional concern.

What emerges from these considerations is that there is a curious interdependence between animal experimentation and nutritional studies with man. In some instances animal studies are essential to provide an understanding of human disease. As important, animal experiments can and do provide an inductive base for the formulation of investigations with man and the strength of this inductive base resides in the fact that we are slowly building up a fabric of principle in nutrition, in which we can begin to distinguish an underlying unity of nutritional response in biological systems. On occasion the animal test provides little more than a tenuous guide or no guide at all because the responses of one species to dietary insult are not the same as those of another. This we have already seen; looking back it is more than probable that, in the early days of the study of scurvy, studies with rats hindered rather than helped advance. Difficulty in species choice still occurs. To our chagrin we have failed to find a laboratory species which responds to the haemolytic toxin in the brassicas.[25] Thus experiments to find its nature have to be made with cattle or infant goats. Similarly favism, which occurs when men and women with an hereditary low glucose-6-phosphate dehydrogenase activity in their erythrocytes eat broad beans, has no analogue to my knowledge in an animal.[26]

We reach the conclusion that some animals are more equal than others when it comes to their nutritional needs and to their responses to dietary deficiency or to natural dietary toxins. Nor is the inequality of response simply an "all or none" phenomenon. Vitamin-E deficiency signs, for example, vary widely with the species.[27] Precisely the same deficient diet given to ducks produces necrosis of the voluntary neck muscles, to turkeys a necrosis of the smooth muscle of the gizzard, to the chicken ischaemic

necrosis of the cerebellum. In man Vitamin-E deficiency increases the sensitivity of his erythrocytes to haemolysis by dialuric acid while in mink the clinical syndrome is an intense steatitis. The reasons for such diversity—the search for a guiding principle to explain it—is of more abiding interest than the mere cataloguing of the curiosities themselves.

It seems to me that in the absence of general principle very great care has to be taken in the interpretation of the results of experiments with one species in terms of another. An extreme view is that relevance can only be established by direct appeal to the second species. This means quite categorically that the proper nutritional study of mankind is the nutritional study of man and that conclusions reached with animals are but a basis for conjecture until direct experimental proof is obtained with man. This extreme view, we know, must be tempered by past experience. We can make some positive statements or reach less rigorously qualified conclusions about man from the results of animal tests. For example, since past experience has shown the ubiquity of mineral element requirements we can conclude that it is very highly probable that vanadium is a dietary essential for man and indeed for all species. The single experiment to demonstrate that it is needed in the diet of the rat, when confirmed, has generality.

It is, however, only right to point out that in the past those concerned with establishing general conclusions in nutrition have been less logically exacting in the criteria they have used. The proof that gaseous nitrogen was not involved in protein metabolism in the animal organism came from a completely inadequate experiment by Boussingault with cattle.[28] We would not accept his proof today, but happily studies using ^{15}N have shown without doubt that Boussingault's conclusion was correct.[29] Also, after years of polemic the proof that carbohydrate was the major source of bodily fat came from the experiments of Lawes and Gilbert with pigs.[30] With our present knowledge we would not accept the generality of their conclusion from the experimental data which they submitted: indeed, we now know that what was then the excluding alternative hypothesis, namely that dietary fat and protein are sources of body fat, is also true under some circumstances.

A further dimension to this problem of the relevance of animal experiments has already been touched upon, namely the existence of hereditary differences within a species. It is now very evident that populations can be selected within species which have very different nutritional needs. This is true of the amino-acid needs of Drosophila,[31] the copper metabolism of sheep and the riboflavin requirements of chicken.[32] Such genetic variation is somewhat distinct from that in individuals with inborn metabolic errors for the latter do not constitute populations in any but a statistical sense. It would seem that the inbreeding of our animal colonies not only reduces the variance of their responses but may also well entail cognisance being taken of between-colony responses. This is certainly true when the disease status of a colony is controlled. Our barrier-maintained rat colony, when subjected to zinc deficiency, no longer shows the cutaneous lesions which for long had been regarded throughout the world as the classic signs of a lack of dietary zinc. Thus when considering animal experiments themselves we may well have to qualify our statements and recognise that we cannot readily draw conclusions about "the rat" or "the pig", but state that our results apply to a particular sub-population. Equally we can ask what precisely do we mean by "man"? Should we

consider not only recognised ethnic differences but also the specific social, cultural and disease environments of the sector of population with which we are concerned? If we conclude that, we should then we are in very considerable difficulty in deciding not only which animal experiments might be relevant to man, but in designing experiments to illumine man's nutritional predicament. Is it, for example, possible to design an animal experiment to provide hypotheses concerned with the effects of hire purchase agreements on choice of diet? Or to explore the influence of cultural mores on the distribution of food within African families or the effect of sedentary existence on energy requirement? Certainly not, yet these are real problems which confront anyone who is interested in the nutritional needs of man.

One can argue that problems of this sort are the sole domain of the anthropologist and sociologist and that they have nothing whatever to do with nutrition as a science. This is to take a very restricted view of nutrition as a subject. Nutrition as a subject for inquiry has always had a clear social and cultural relevance, and it would be wrong both to pretend otherwise, and to attempt to make it a simple subdivision of the biological sciences. We can, in fact, go even further to state that many scientific problems in the nutrition of man, some of which have arisen from consideration of animal experiment, may well have to be solved not by the rigorous methods employed in the physical and biological sciences, but through approaches which are more closely related to those employed in social science and demography. The example of diet in relation to longevity will perhaps illustrate this point more fully.

Once more, it was our good friend Francis Bacon who said "A pythagorical or monastical diet to strict rules and always exactly equal seemeth to be very effectual for a long life".[33] A prescient statement of observation which antedates McCay's classic experiments with rats by a mere 400 years. In these experiments in the 1930's McCay showed that "a monastical diet", a reduction in food intake below what was normally consumed to meet appetite, significantly increased the span of life.[34] McCay's experiments have been repeated and refined by many. A longer life obviously implies that the time pattern of terminal disease must change. It has been shown in rats that tumour prevalence increases with the protein and total energy consumption and that the incidence of a number of degenerative diseases of the rat is increased by a full diet.[35] Changes in the age-specific mortality in animals subject to different dietary treatment certainly suggest that diet has unique effects on the initiation or development of specific diseases. We thus obtain from animal experiments not only a general conclusion concerning longevity but a series of more specific ones relating diet to disease. In thinking about the relevance of animal experiments to man we must equally consider these more specific aspects. Indeed we do distinguish in this way. The problems of human nutrition in this wide field of diet and longevity we do subdivide. We thus consider the effects of early undernutrition on subsequent mental development,[36] the effect of early overnutrition on later development of obesity,[37] the effect of meal-eating patterns on obesity,[38] effects of sucrose and of triglycerides on ischaemic heart disease[39] and effects of diet on the later incidence of diabetes, gout and diverticulosis.

It seems probable that the general conclusion drawn from animal experiments that diet, through its effect on disease incidence, affects longevity, is of the nature of a principle and thus applicable to all species including man. With the more specific

conclusions from animal experiments, however, we cannot make such a probability statement; we must be more wary. This implies that we should take what I earlier called the extreme view, namely that relevance of animal studies can only be established by direct appeal to the species concerned. In turn we conclude that we would have to commit ourselves to experiments with man to confirm or refute the possibility. We thus find that, taking this extreme view, we are committed to experiments that last for at least three score years and ten.

Professor Brock,[40] at the end of a symposium on diet in relation to human health, asked the obvious question "How do we set up an experiment the answer to which cannot be expected in less than 50 years?" The answer, very simply, is that we cannot. We cannot set up an experiment with man in the sense that we use this term in animal studies to imply precise genetic, dietary and environmental control. When the time span of the experiment is life itself it is not reasonable to consider volunteers and it is not ethical to consider populations in institutions as sources of potential subjects.

This means that we have to abandon the rigour, the precision and the unequivocality of conclusion of the properly designed experiment and seek other methods of enquiry. Conversely, we have to admit that our accumulated knowledge about the design of animal experiments and about methodical ways of isolating cause may have nothing more than general relevance to the conduct of investigations of problems of human nutrition. Animal experiments provide illumination but, except in the very short term such as in the Sheffield experiment on ascorbic acid, are not models which we can slavishly copy in work with man.

We thus come to what Francis Bacon called "contrived experiences", by which I think he meant the observation of the relevant, or the methods of social science and demography. Here there are enormous pitfalls for the unwary. For centuries comparisons have been made between peoples in different parts of the world with respect to their vigour and disease incidence and incidence has been correlated with attributes of diet or with social habit. David McCay's studies in India[41] are an excellent example. He attributed differences in stature, vigour and indeed personality of different peoples to dietary differences. Such correlations between races are now in disrepute for nature and nurture are known to be difficult to disentangle.

The retrospective study, which was advocated by Francis Bacon when he wrote "Inquire touching the length and shortness of life in men according to their Studies, their Several Courses of life, the Affections of the mind and divers accidents befalling them" is equally open to criticism, for correlation is not causation. There are many anecdotes to illustrate this point. The one I like is the irrefutable evidence that schizophrenia is associated with intense urbanisation, evidence that has led to intense social comment. The evidence was a highly significant correlation between incidence of schizophrenia and distance from the city centres, often confirmed by well conducted survey. The association proved to be one of age and household structure with distance from the centre and not due to an effect of a canyon-like existence on mental disease.

In all this type of survey study, the problem is not to measure the incidence of a disease but to isolate its cause. We have, for example, the paradox pointed out by Yudkin[42] that though consumption of fat has no effect on sugar tolerance the incidence of human diabetes is correlated with the amount of fat consumed. Yudkin believes that

because intakes of fat and sugar are themselves correlated the association of dietary fat with diabetes and with myocardial infarction is only coincidental.There could, however, be a more complex correlation chain. Nor does the prospective study necessarily resolve the situation. Trials undertaken by a Working Party of the Clinical Research Board of the MRC[43] in part suggest that the slightly higher sugar intake of patients who develop myocardial infarction was likely to have been due to an association between the consumption of sugar and the smoking of cigarettes.

Without the powerful tool of direct experiment, investigation of the new nutritional problems of man will be incredibly difficult. It is here perhaps that animal experiments can again prove useful, for we need to explore new ways of isolating cause in population studies. The elucidation of nutritional, environmental and genetic interactions with animals and secular analysis of continuous variation of nutritional inputs in animal experiments could well provide a theoretical baseline for the development of what we can call quantitative nutritional epidemiology.

Finally, I have dealt with the relevance of animal experiment to the nutrition of man, interpreting the subject in terms of man's physical condition, his health, his longevity and his freedom from disease. In the current climate of our time, when science must, it seems, be seen to produce tangible dividends such an analysis is no doubt acceptable. Man, however, does not live by bread alone, nor does he live by dietary essential. There is a further and far more important relevance of animal studies to the human condition, namely its enrichment of our understanding of Nature. No justification, no counterpoint of cost and benefit, no equivocation, no apologium is needed for this aspect.

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Rapid Determination of the Oil Content of Animal Feedstuffs

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The modifications to a beam balance which are described make possible the differential measurement of the specific gravity of two liquids. In this method oil is extracted from animal feedstuffs with tetrachloroethylene and the change in specific gravity is used as a measure of the oil content of the sample. Results suitable for quality control which are obtained within 20 min are comparable to those obtained in 6 h by the official petroleum extraction method.

1. Introduction

In any industrial processing of foodstuffs involving the addition or removal of fat or oil a rapid analytical determination of oil content is desirable. Conventional methods of oil determination such as that used in the animal feedstuffs industry involve solvent extraction steps followed by the gravimetric determination of the oil after the removal of the solvent. These steps are lengthy, and rapid methods which have been described shorten either or both of them.

The amount of lipid material removed from a sample by straight solvent extraction depends on the solvent employed and almost complete extraction of lipid material can only be achieved by systems using both polar and non-polar solvents either mixed or in succession. Alternatively, hydrolysis procedures will release a maximum quantity of lipid although some degradation of compounds such as phosphatides will occur. In order to obtain results comparable to those obtained by petroleum extraction, which is the official method of analysis for animal feedstuffs in Great Britain,[1] it may not be necessary to carry out an exhaustive extraction with another solvent. We have found that shaking a sample of feedstuff with tetrachloroethylene quickly yields an equilibrium extract about 0.5% lower than that obtained by prolonged extraction with petroleum spirit which can be used for the determination of oil for quality control purposes.

Having made the extraction, the determination of oil content can be achieved by the use of various physical measurements and a great saving in time is made if these can be carried out without the removal of the solvent. A review of these techniques has been made by the British Food Manufacturing Industries Research Association.[2]

Many of these methods require calibration against the particular type of fat being examined. In the case of animal feedstuffs this presents problems since the oil may be derived from a large variety of ingredients, such as cereal, vegetable protein, meat and bone meal and fish meal as well as added fat which is itself variable. It is necessary

therefore to choose a physical measurement which is not influenced by the type of oil or fat present. Specific gravity measurement goes some way towards satisfying this condition particularly if the solvent used has a specific gravity much higher than that of oil. Tetrachloroethylene with a specific gravity of about 1.62 is such a solvent and the variations of the specific gravity of oils and fats, 0.91 to 0.95, are small compared to the difference between their specific gravity and that of tetrachloroethylene. Tetrachloroethylene is suitable in other ways, being a good fat solvent with a high boiling point, non-inflammable and having a low toxicity.

Bittenbender[3] has published a method for the determination of the fat content of meat using heptane as the solvent and a sensitive hydrometer for the measurement of specific gravity. This requires very careful control of the temperature to within 0.01 deg. C. Perl, Ince and Wiggall[4] have overcome this disadvantage by the measurement of the specific gravity difference between solvent and extract. Their differential instrument modifies an analytical two pan balance by replacing the pans with identical plummets which hang through the base of the balance into cells, one containing the solvent and one the extract. The cells immersed in a thermostatically controlled water bath are at the same temperature at the time of measurement but absolute control of temperature need be no better than ± 0.1 deg. C according to the authors.

We appreciate the very real advantages that this type of apparatus offers and we have modified their design to make a more compact, robust and cheaper instrument which is as easy to set up or move as an ordinary balance.

2. Differential specific gravity balance

The differential specific gravity balance has the advantage that errors due to changes of temperature and of solvent specific gravity are minimised. It is useful to be able to determine the tolerances that can be allowed in the plummet volumes and the temperature control before these advantages are lost.

The zero of the balance is set when the two plummets are immersed in the solvent and if the plummets are identical the zero will not shift, no matter what the specific gravity of the solvent is. In practice over a range of temperature of 10 deg. C the specific gravity of the tetrachloroethylene will change about 0.016 units. If the plummets have different volumes, V_1 and V_2, the deflection from zero will be given by

$$D(V_1 - V_2) = \text{deflection (g)}$$

where D is the specific gravity difference. For this deflection to be less than 0.5 mg over a 10 deg. C temperature change ($D = 0.016$) the difference in volumes of the plummets must be less than 0.03 ml. A zero shift will also be caused by a temperature difference between the two cells in which the plummets are hung. When the plummets are equal in volume at 20.000 ml then the specific gravity difference between the solvent in the two cells which will cause a zero shift of 0.5 mg is 0.000 025 units. For tetrachloroethylene this is equivalent to a temperature difference of less than 0.02 deg. C.

The effect of temperature on the calibration of the apparatus against solutions of oil in tetrachloroethylene will be dealt with later but if two instruments are to give the same calibration at a single temperature then their plummets should have similar volumes.

A 0.1 % difference in volume will give a 0.1 % difference in deflection for a given fat concentration. For a 20 ml plummet this is a volume difference of 0.02 ml.

This gives the standards to which the balance has to be made, plummets differing in volume by less than 0.02 ml and, probably more important, the temperature of the two cells differing by less than 0.02 deg. C although the absolute control of the temperature is less critical.

2.1. Description of the apparatus

The apparatus consists of a two-pan beam balance from which the pans have been removed to be replaced by glass plummets suspended in water-jacketed cells housed within the balance case. The cells are filled and emptied through a tube at the base of the cell. Water is circulated through the water jackets of both cells by a small centrifugal pump. A diagram of the apparatus is given in Figure 1.

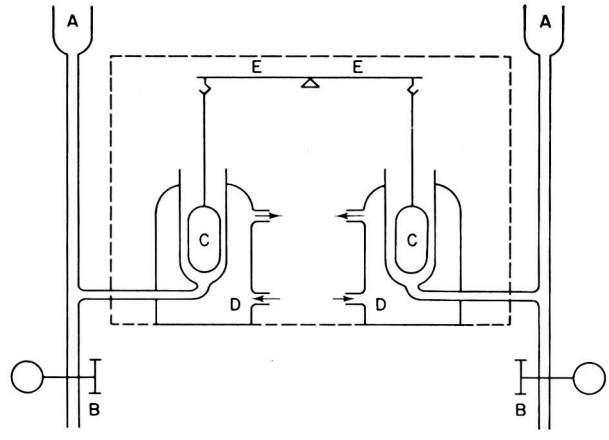

Figure 1. Schematic diagram of the differential specific gravity balance. ———, Balance case; A, filling funnel; B, outlet with pinch clip; C, plummet in cell; D, water jacket; E, balance arm.

2.1.1. The balance

Ideally a balance which displays a milligram difference between the ends of the balance beam on an illuminated scale should be used but other types of balance have been adapted. Air damping is not essential since the plummets moving in the solvent produce a damping effect. The modifications described below were made to an Oertling 52GB model and may require slight alterations when other models are being adapted.

2.1.2. The plummets [Figure 2(b)]

After experimenting with brass cylinders and considering hollow glass, the plummets were finally made in solid glass. This has the advantage that the weight can be used to control the volume and it is quite easy to adjust a pair to better than 0.01 g difference (0.004 ml) by rubbing the larger down with carborundum.

The dimensions of the plummet dictate the size of the cell which can be used and thence the volume of solvent required to immerse the plummet completely. It can be calculated that this volume is at a minimum for a 20-ml plummet when the diameter of the plummet is 18 to 25 mm.

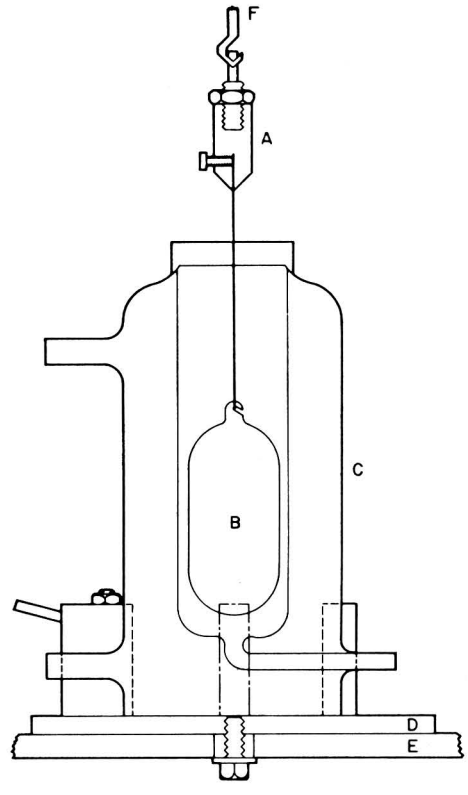

Figure 2. Detailed diagram of a cell unit. A, The suspension; B, plummet; C, water-jacketed cell; D, base plate; E, vitrolite base of balance; F, suspension hook on balance beam.

2.1.3. The water jacketed cell [Figure 2(c)]

The cell is designed to give a minimum clearance between the plummet and cell walls allowing for variations in the bore of the glass tubing used for making the cell. The present design permits the cell to be filled and emptied through the same tube at the base of the cell. The water jacket is produced with a flat base so that the cell is vertical and this simplifies the positioning of this piece of glassware.

2.1.4. The suspension [Figure 2(a)]

The brass hanger allows the height of the plummet to be adjusted so that it just clears the bottom of the cell. Nylon is used to suspend the plummet.

2.1.5. The base plate [Figure 2(d)]

The base plate is triangular with three plastic covered pins, one of which is eccentrically pivoted to allow the water jacketed cell to be clamped in position. The position of the cell and base plate in the horizontal plane can be adjusted until the plummet hangs centrally clear of the sides of the cell. Then the base plate is fixed by tightening the bolt through the base of the balance case against the washer.

2.2. Method of analysis of animal feedstuffs for oil content

The reagent used was tetrachloroethylene (technical grade). The pump was switched on to circulate water at room temperature round the cells and round the bottle of solvent which was placed in the water reservoir. With solvent in both cells the balance was raised and the tare of the balance was adjusted to bring the scale reading to zero.

A 10 g sample of animal feedstuff which had been prepared for analysis was measured into a 150-ml conical flask. 75 ml of solvent was added and the flask stoppered and shaken on a wrist action shaker for 5 min. The extract was filtered through a 15 cm No. 1 Whatman or similar grade filter paper and about 50 ml of clear filtrate was collected. The sample cell was emptied and then refilled with about 20 ml of the extract and then run to waste. Sufficient extract (usually about 25 ml) to cover the plummet completely was used to fill the cell. The balance was raised. A steady reading is usually reached within 30 s. The mg deflection was noted and the oil content of the sample was read from a calibration graph prepared by carrying out this determination on animal feedstuffs of known oil content determined by the petroleum extraction technique. Once this calibration has been made for one instrument it will apply to all instruments having plummets with identical volumes or a correction for different plummet volumes can be made.

3. Experiments and results

In the initial experiments with this instrument the solvent was measured by weight thus making sure that the amount of solvent used was independent of the temperature and a calibration of the instrument using solutions of tripalmitin in tetrachloroethylene was made. (Tripalmitin is a fat available in powdered form and therefore convenient to handle.) The results in Table 1 indicate that over the range of concentrations examined a linear relationship is obtained.

TABLE 1. Calibration of the specific gravity balance using tripalmitin solutions in tetrachloroethylene at 22 °C

Weight of tripalmitin in 120 g of solvent (g)	Balance deflection (mg)	Balance response per g tripalmitin (mg)
0.1	24	240
0.2	48	240
0.4	94	236
0.6	143	238
0.8	188	235
1.0	235	235

These results were obtained with water, thermostatically controlled at 22 °C, circulating round the cells. To check the effect of temperature, calibrations were carried out with solutions of tripalmitin in tetrachloroethylene over a range of temperatures 15 to 32 °C. These results are shown in Table 2, together with the specific gravity of tetrachloroethylene at these temperatures. There is a small reduction in the response of

TABLE 2. Specific gravity balance response at different temperatures to 1 g tripalmitin in 120 g solvent or in 75 ml solvent

Temperature deg. C	Balance response (mg) 1 g of TP/120 g of solvent	Specific gravity of tetrachloro-ethylene	Weight of 75 ml tetrachloro-ethylene (g)	Corrected balance response (mg) 1 g of TP/75 ml of solvent
15.0	234	1.631	122.3	230
23.0	232	1.618	121.3	230
32.5	226	1.603	120.2	226

the instrument at higher temperatures but over a limited range the change becomes negligible. If, however, instead of using a fixed weight of solvent a fixed volume, measured at the temperature of the circulating water, is taken the reduced weight of solvent at higher temperatures compensates for the reduced response and a virtually constant calibration is achieved over the whole temperature range. There is thus no need for temperature control as long as the cells are kept at the same temperature by rapidly circulating water. By the use of a small powered pump which has no heating effect we are able to keep the cells at laboratory temperature which has the added advantage that little time is required to bring the extract to the temperature of the cell and a stable balance reading can be reached more rapidly. In practice, laboratory temperatures vary throughout the day and the temperature of the circulating water is liable to lag behind, but the difference should never be great and a stable reading is always reached in less than a minute.

The method outlined was used to examine extracts of feed ingredients and compound animal feedstuffs and the response of the balance was plotted against the oil content of the samples determined by petroleum extraction. The results lay close to a straight line and a summary is given in Table 3 together with a few typical results obtained on ingredients and compound feeds using the graph as a calibration of the instrument.

It would appear that the simple method of extraction adopted fails to remove a portion of oil from the samples since upon extrapolation the graph cuts the oil axis at 0.5% oil. The slope of the graph is however similar to that obtained using solutions of fat of known concentration indicating that fat and oil in excess of 0.5% is being fully extracted. Increasing the time that the sample is shaken with the solvent from 5 to 30 min does not increase the amount of oil extracted but the effect of temperature has not been tested. Refluxing the sample with tetrachloroethylene is the method successfully adopted by Perl, Ince and Wiggall,[4] but for our purpose, for a rapid method, obtaining a more complete extraction of oil would not increase the sensitivity of the method, i.e. the slope of the calibration graph, nor is it likely to affect the precision. So we feel

TABLE 3. Results obtained on animal feed ingredients and compound feeds

(a) Calibration against petroleum extraction method
Number of samples examined: 30
Range of oil contents: 2.2 to 7.1%
Standard deviation of the results from those obtained by petroleum extraction: 0.15% oil
Equation of the calibration graph

$$\% \text{ oil} = \frac{\text{mg deflection}}{\text{f}} + 0.5$$

the factor f being approximately the volume of the plummet in ml

(b) Typical results obtained using this calibration

	Specific gravity method		Petroleum extraction
	Deflection (mg)	% Oil	% Oil
Feed ingredients			
Wheat meal	16	1.20	1.18
Wheat feed	63	3.30	3.10
Yellow maize	48	2.60	2.63
Expeller groundnut meal	154	7.35	7.16
Sun flower meal	49	2.65	2.51
Meat and bone meal	214	10.00	9.77
Compound feeds			
Chick layer	48	2.60	2.56
Chick broiler	93	4.65	4.60
Turkey feed	48	2.60	2.64
Pig feeds	39	2.25	2.36
	62	3.25	3.12
	100	4.95	4.68
	149	7.10	7.14

justified in adopting a simple and rapid extraction, while acknowledging that it is empirical and only has value by calibration with a recognised method.

To obtain information on the effect of the type of fat on the balance reading, solutions of oils and fats, triglycerides and other lipid fractions in tetrachloroethylene were examined. Table 4 shows the response to 1 g of fat in 75 ml of solvent for triglycerides

TABLE 4. Specific gravity balance response to solutions of different fats at the same concentration 1 g/75 ml of solvent

Fat	Balance response to 1 g/75 ml of solvent
Vegetable oil	216
Vegetable oil	217
Butter oil	221
Commercial cake fat	222
Margarine oils	223
Margarine oils	224
Frying fat	224
Tripalmitin	230
Stearic acid	257
Crude lecithin	172

ranging from oils to completely hard fats and it can be seen that there is an increased response with increasing hardness. This appears to be contrary to expectations since it might be thought that the less dense solute, oil, would give the greater response.

A bigger variation in response is noted between different lipid classes. Fatty acids give an increased response while phosphatides and some emulsifiers which have been examined give a decreased response. Both free fatty acids and phosphatides are likely to be present in animal feed extracts. Phosphatides are likely to be a fairly constant minor constituent of the extracts of animal feedstuffs, but the fatty acid content may vary both with the type of fat added as an ingredient and with the age of the product. However, for a feedstuff with an oil content of 5% a change of free fatty acidity from 10 to 50% would produce an error in the determination of about 0.25% if the response to stearic acid is taken as typical of fatty acids. This error is slightly less than the maximum deviation expected in the method and the variations in free fatty acidity may in part be a cause of this discrepancy. The highly unsaturated oils present in fish meal are liable to oxidation and in the oxidised state become unextractable by petroleum spirit. Application of the rapid extraction with tetrachloroethylene to fish meals yields results similar to those obtained by petroleum extraction while a mixed solvent extraction using chloroform, methanol and water yields a higher quantity by including oxidised oils.[5] These results are given in Table 5. Redissolving the petroleum extractives in tetrachloroethylene and measuring the specific gravity balance response gave deflections slightly higher than direct tetrachloroethylene extraction showing again that about 0.5% oil is unextracted by this rapid technique. Agreement between the rapid and petroleum extraction is generally good and apparently independent of the state of oxidation of the oil.

TABLE 5. Comparison of the oil content of fish meals determined by the rapid specific gravity method, by petroleum extraction and by a mixed solvent extraction

Fish meal sample	Rapid specific gravity method %	Petroleum extraction %	Mixed solvent extraction %
S. African April 1971	8.2	8.2	10.1
S. African Sept. 1971	8.9	9.0	11.0
S. African Nov. 1971	9.5	9.6	11.5
Norwegian Herring July 1971	6.0	6.9	9.2
Norwegian Herring Nov. 1971	9.6	10.4	13.3
S. W. African fish	2.3	2.1	6.5
S. African fish	2.9	2.9	7.4
Home-produced herring	6.0	6.1	9.1
Herring meal	7.1	7.0	10.3
Herring meal	12.6	12.4	14.7
Herring meal	11.3	11.3	13.9
Scotch fish	8.4	8.4	10.7
Scotch fish	7.0	7.0	9.7
Norwegian herring	6.8	7.2	10.2
Iceland herring	7.1	6.8	10.8
Fish pellets	8.0	7.7	10.1

Standard deviation between rapid and petroleum extraction methods, S.D. = 0.35 (0.19 omitting samples 4 and 5 in the Table).

The two stages of the method can be considered separately in the light of the results of the experiments described. The extraction procedure, although incomplete, is adequate for the quality control of compound animal feedstuffs and the results obtained correspond closely to those obtained by petroleum extraction. The measurement of specific gravity change does not preclude the use of other extraction techniques and these would need to be investigated for more fundamental work.

The determination of the oil concentration in the solvent by specific gravity measurement is not independent of the source of oil or fat and some of the factors affecting the response have been noted, but the variations are not great and the use of an average calibration for compound animal feedstuffs is believed to be justified. When the method is to be applied to a range of samples of a single ingredient such as fish meals or meat and bone meals, more accurate results are likely to be obtained by a separate calibration against the particular material.

The necessary adaptations to a beam balance are inexpensive, costing less than £60.00, and allow the oil content of an animal feedstuff to be determined within 20 min of the receipt of the ground sample. If analyses are carried out in batches, sequential analyses can be completed at intervals of 2 or 3 min.

Acknowledgements

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Leaf Analysis as a Guide to the Nutrition of Fruit Crops

IX. Effects of Initial and Supplementary Levels of N and P on Black Currants (*Ribes nigrum* L.) Grown in Sand Culture[a]

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A study was made, by means of a factorial sand culture experiment, of the main effects and interactions of varying initial levels of N and P and of supplementary levels of N and P applied in July, on shoot growth, blossom number, fruit set, berry weight and crop yield of black currants grown as biennials and of their relationships with leaf-lamina nutrient concentrations. Nitrogen influenced crop yield largely through its effect on blossom number, whereas phosphorus influenced crop yield through increased shoot growth and percentage fruit set. Crop response to N was approximately linear (at adequate P levels) over the leaf-N range 2.1 to 2.5% of N in dry matter, whereas crop response to P (at adequate N levels) was curvilinear over the leaf-P range 0.1 to 0.3% of P in the dry matter of samples taken in August of the previous season. Additional-N applied in July increased yield but was strongly dependent on level of initial-P. Additional-P increased yields at the two lower levels of initial-P only.

1. Introduction

The previous paper[1] in this series dealt with the main effects and interactions of N, P, K and Mg on black currants (grown as biennials in sand culture) and enabled the optimum levels of these nutrients (for leaf samples taken in July of the first season) to be defined. The data, however, did not enable one to predict the optimum level of nutrition at other specific growth stages, for instance at the period of flower initiation. The present experiment was designed so that the main NP treatment plots (6 pots) could be split, in early July, into three sub-plots (each of 2 pots), each sub-plot thereafter receiving, respectively, initial treatment only, initial treatment plus additional-N or initial treatment plus additional-P.

It was hoped that the information so obtained would enable the "critical" levels of leaf-N and -P associated with flower initiation and other components of yield to be defined.

2. Experimental

2.1. Pot experiments

2.1.1. Materials

A non-calcareous coarse pit sand was used as the root medium. It was not acid washed (normally carried out only for trace element studies) but was thoroughly leached before

[a] Part VIII in this series is *J. Sci. Fd Agric.* 1969, **20**, 172.

use. Rainwater, for nutrient solutions and irrigation, was collected from the roof of a large glasshouse and filtered before use. Further purifications of water, nutrient salt and sand was not necessary in order to achieve the moderate deficiency levels required for this experiment.

2.1.2. Containers

Industrial 3 gal (13.5 l) grey plastic buckets, provided with drainage holes in the base (covered with discs of coarse-mesh Tygan), were used as containers.

2.1.3. Nutrient solutions

The nutrient compositions were based on the standard Long Ashton solution (as described by Hewitt)[2] which contains the following concentrations of nutrients in terms of mg equiv./l: SO_4, 3; NO_3, 10; PO_4, 4; K, 2; Mg, 3; Ca, 8; Na, 1.33; and Fe (as Chel 138), 5; Mn, 0.55; B, 0.33; Cu, 0.064; Zn, 0.065 and Mo, 0.019 as parts/million.

Nitrate and phosphate, when reduced, were replaced by equivalent amounts of sulphate.

2.1.4. Plants

Hardwood cuttings, cv. Baldwin, were inserted in sand during early September and watered-in. They were allowed to over-winter in a cold glasshouse to encourage callousing and root formation and in mid-March four well-rooted cuttings were transplanted into each container. In mid-April these potted cuttings were transferred from the glasshouse to covered concrete pits in outdoor cages where they remained for two seasons. In early June, when new growths were 6 to 9 in (16 to 23 cm) long, superfluous shoots were removed so as to leave two per cutting, giving a total of eight shoots per pot. Any new shoots formed subsequently were removed. A co-variance correction was made for any shoots lost during the course of the experiment.

2.1.5. Treatments and layout

The initial 24 nutrient treatments (N × P factorial) consisted of six levels of nitrogen in combination with four of phosphorus:

nitrogen 10:12:14:16:18:20 mequiv. NO_3/l
phosphorus $\frac{1}{2}$:1:2:4 mequiv. PO_4/l
potassium 4 mequiv. K/l
magnesium 3 mequiv. Mg/l.

Three replicates, each of 24 treatments, were used, with six pots in each unit whole-plot, giving a total of 432 pots. From the beginning of July (first season) each unit whole-plot was sub-divided into three unit sub-plots of two pots each; one sub-plot continued to receive the initial treatment only, whilst the remaining two sub-plots received either supplementary nitrogen (4 mequiv. NO_3/l) or supplementary phosphorus (1 mequiv. PO_4/l), respectively, in addition to the initial nutrient solutions. The supplementary treatments continued for the first season only.

2.1.6. Management

Nutrient solutions were applied (to the surface of the sand) in sufficient volume to completely saturate the sand, once weekly at first and then with increasing frequency rising to four or five times weekly as the season progressed. (During the callusing and root formation period a basic complete nutrient solution was applied to all cuttings.)

Differential initial N × P treatments were started in mid-April, and supplementary N and P treatments in the first week of July (by adding the requisite amount of calcium nitrate or sodium dihydrogen orthophosphate solutions to the initial nutrient solution).

When required, additional water was applied by means of an overhead irrigation system (Bould).[1] Routine sprays were applied for pest and disease control, using metasystox for aphis, thiodan for big-bud mite, DDT for midge, morestan for mildew and zineb for leaf spot.

2.1.7. Shoot growth

Total primary shoot length, in cm, was recorded for each pot after leaf-fall in the first season (i.e. non-cropping shoots).

2.1.8. Flower number

In May of the second season a single shoot from each pot was chosen (by reference to a book of random numbers) and tagged at a point approximately 15 cm from the tip. The 1st, 3rd, 5th, 7th and 9th trusses down from this tag were subsequently counted for number of flowers; single, double or multiple sprigs were also recorded. The results are given on a two-pot basis as the means of 10 trusses.

2.1.9. Fruit set

At fruit-ripening the tagged shoots were removed and the number of berries on the five selected trusses per shoot were counted, to determine the percentage fruit set.

2.1.10. Fruit weight

The total weight of fruit produced and the weight of two randomly-selected samples of 50 fully-ripe berries, were recorded per sub-plot.

2.1.11. Leaf sampling

Two leaf-lamina samples per sub-plot were taken in the first season, the first on July 3 and the second on August 6. One complete leaf-lamina was taken from the mid-third region of each shoot, giving a composite sample of 16 laminae per unit sub-plot.

2.1.12. Chemical analysis

Leaf samples were analysed for total N, P and K by the methods described by Bould, Bradfield and Clarke.[3]

3. Results

Main effects and interactions have all been examined statistically but for the sake of brevity only the main effects are given in the Tables. Significant interactions are given in the text.

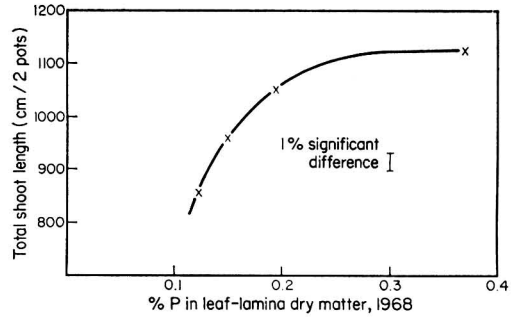

Figure 1. Relationship between leaf-lamina P (August 6) and total primary shoot length in the same season.

3.1. Shoot growth (first season)

3.1.1. Nitrogen

There was no detectable effect of initial level of nitrogen, or of additional nitrogen applied in July, on shoot growth (Table 1) although leaf analyses (Table 4) indicated that leaf N was in the "poverty adjustment" region (Figure 2). Average shoot length was similar to that in previous sand culture pot experiments (Bould).[1] Water, oxygen

TABLE 1. Treatment effects on total shoot length (first season) of black currant (means of 2-pot totals (16 shoots) in cm)

Initial N-means		Initial P-means		Additional N- and P-means	
N10	1017	P½	858	O	993
N12	997	P1	962	+N	1003
N14	1025	P2	1055	+P	1007
N16	992	P4	1130		
N18	986				
N20	989				
Level of significance	n.s.		**		n.s.
S.E.	11.4		9.3		5.2

N.B. In this and subsequent Tables * = 5%; ** = 1%; *** = 0.1% level of significance, n.s. is not significant. Standard errors (S.E.) for N and P means are based on 46 d.f. and O, +N and +P on 96 d.f.

supply and/or denitrification of nitrates may have acted as partial limiting factors on growth. Average growth (>60 cm/shoot) was fairly normal for the cultivar Baldwin.

3.1.2. Phosphate

There was a steady increase (**)[a] in growth with increasing initial P level until a leaf-P value of approximately 0.3% in dry matter (at N20) was reached (Figure 3): this is in line with previous results for black currant (Bould).[1] There was no detectable growth response to additional P applied in July.

[a] On this and subsequent pages: (*) = 5% level; (**) = 1% level of significance.

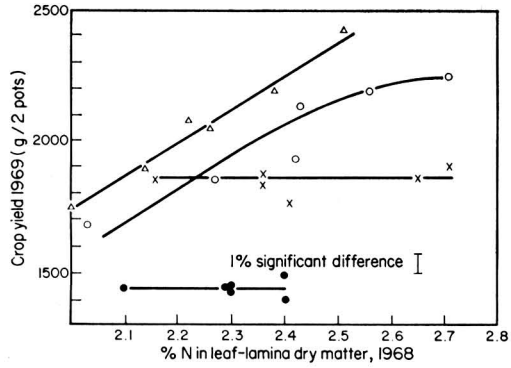

Figure 2. Relationship between leaf-lamina N (August 6), at different levels of nutrient-P supply, and crop yield in the following season. (●) = ½; (×) = 1; (○) = 2; (△) = 4 mequiv. of P/l.

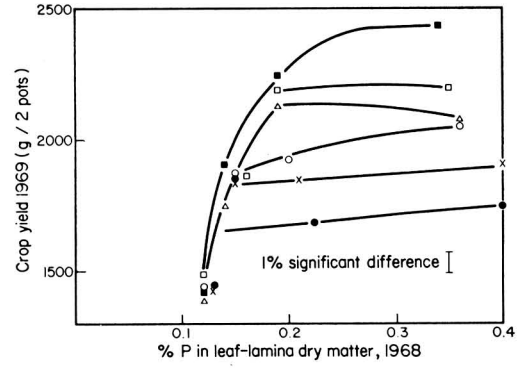

Figure 3. Relationship between leaf-lamina P (August 6), at different levels of nutrient-N supply and crop yield in the following season. (●) = 10; (×) = 12; (○) = 14; (△) = 16; (□) = 18; (■) = 20 mequiv. of N/l.

3.2. Flower number

3.2.1. Nitrogen

Flower number increased (**) almost linearly with initial nitrogen level (Table 2). Additional nitrogen applied in July had no significant effect on flower number thus indicating that it was applied too late to affect flower initiation. (Additional nitrogen was absorbed, as indicated by leaf nitrogen changes, especially at the higher levels of P supply.)

3.2.2. Phosphate

There was a suggestion of a decrease (*) in flower number with increasing initial-P level. This may have been associated with the effect of initial-P level on nitrogen uptake (as reflected in leaf-N). Additional-P in July gave an average increase (**) in flower number. The largest number of flowers was obtained with low initial-P plus extra P in July.

TABLE 2. Treatment effects on flower number and fruit set (5 sprigs from each of 2 shoots) of black currant (means of 10 sprig totals)

Initial N-means	Number of flowers	Initial P-means		Additional N- and P-means	
N10	195	P½	212	O	204
N12	202	P1	213	+N	205
N14	205	P2	203	+P	215
N16	209	P4	203		
N18	211				
N20	223			+N	n.s.
Level of significance	**		n.s.	+P	**
S.E.	4.7		3.8		3.0

	Number of fruits							
	No.	% set		No.	% set		No.	% set
N10	123	63	P½	117	55	O	126	61
N12	125	62	P1	126	59	+N	126	61
N14	130	63	P2	133	65	+P	132	61
N16	127	60	P4	134	66			
N18	129	61						
N20	133	60				+N	n.s.	
Level of significance	**			**		+P	*	
S.E.	3.4			2.8			2.2	

3.3. Fruit numbers

3.3.1. Nitrogen

Response to initial nitrogen was approximately linear. This effect was related to initial flower number rather than to any effect on fruit-set (Table 2).

The effect of extra nitrogen varied according to initial-N level: it gave an increase (*) where initial-N was lowest (N10), but at other levels no consistent effect was detected.

3.3.2. Phosphate

Increasing levels of initial-P increased (**) fruit number; this increase was related to an effect of phosphate on percentage fruit set (Table 2).

Additional-P increased fruit number (*) greatest where initial-P was lowest ($P\frac{1}{2}$); this effect was reduced at higher initial-P levels.

3.4. Crop weight

3.4.1. Nitrogen

Average response (**) to initial-N was approximately linear (Table 3 and Figure 2). The increase was greater (**) with higher initial levels of P than with lower levels.

Additional N increased (**) average yield (Table 3). The effect of additional N was strongly dependent (**) on level of initial-P, being greatest at high initial-P levels and at low initial-N levels.

TABLE 3. Treatment effects on total fruit yield (g/2 pots) and berry weight (g/100 berries) of black currant

Mean yield g/2 pots					
Initial N-means		Initial P-means		Additional N- and P-means	
N10	1684	P½	1441	O	1773
N12	1752	P1	1845	+N	1848
N14	1822	P2	2002	+P	1895
N16	1840	P4	2006		
N18	1932				
N20	2001			+N	**
Level of significance	**		**	+P	**
S.E.	25		21		15.6

Berry weight g/100 berries					
Initial N-means		Initial P-means		Additional N- and P-means	
N10	124	P½	130	O	127
N12	124	P1	130	+N	121
N14	131	P2	124	+P	128
N16	128	P4	117		
N18	127				
N20	118			+N	n.s.
Level of significance	*		**	+P	n.s.
S.E.	3.8		3.1		2.4

3.4.2. Phosphate

Average yield response (**) to initial-P diminished as the supply increased (Table 3). Additional-P increased yields at the two lower levels of initial-P only.

The response to extra P was greater (**) than the response to extra N in plants having lower levels of initial-P, but the reverse was true for plants with the higher levels of initial-P.

3.5. Berry weight

3.5.1. Nitrogen

Response to initial N showed a maximum (*) weight at about level N14 (Table 3).

Additional-N in July had no significant effect on berry weight. The apparent reduction in berry weight to extra N was more marked at lower levels of initial-P.

3.5.2. Phosphate

There was a negative response (**) in berry weight to initial-P (Table 3). Additional-P in July had no significant effect.

3.6. Leaf analysis

3.6.1. Nitrogen

Results for samples taken on August 6 (first season, non-fruiting) are given in Table 4.

Average response (**) to N was approximately linear. The rate of increase in leaf-N was greater at higher levels of initial-P. Additional-N gave an average increase (**) in leaf N; the increase depended (*) on initial-N level, falling sharply between N18 and N20. It also tended to increase (*) with increasing initial level of P.

TABLE 4. Treatment effects on leaf nitrogen and phosphorus (sampled August 6, 1968) of black currant (as % dry matter)

	% Nitrogen (N)			% Phosphorus (P)	
	Initial N-means	Response to Additional-N		Initial P-means	Response to Additional-P
N10	2.099	0.166	P½	0.124	0.020
N12	2.272	0.162	P1	0.150	0.015
N14	2.293	0.145	P2	0.198	0.026
N16	2.360	0.195	P4	0.372	0.046
N18	2.496	0.161			
N20	2.515	0.008			
Level of significance	**	**		***	**
S.E.	0.030	0.046		0.0034	0.0021

	(Sampled July 3, 1968)		
Initial N-means	% Nitrogen (N)	Initial P-means	% Phosphorus (P)
N10	2.13	P½	0.13
N12	2.16	P1	0.16
N14	2.30	P2	0.20
N16	2.33	P4	0.35
N18	2.46		
N20	2.44		
Level of significance	**		***
S.E.	0.031		0.0036

3.6.2. Phosphorus

Results (for samples taken on August 6, first season) are given in Table 4. Response to initial-P was slightly curvilinear in terms of P units applied.

The rate of increase (per P unit) tended to be lower (**) at higher levels of initial-N. Additional-N had no significant effect on leaf-P, but additional-P gave a significant increase (**) in leaf-P.

3.6.3. Potassium

Initial level of nutrient-K was constant throughout, but leaf-K varied with varying initial levels of N and P. Leaf-K decreased (**) with increasing levels of P.

3.7. Leaf nutrient—plant performance relationships

Figure 1 shows the relationship (curvilinear) between leaf-P in August and the growth of shoots during the first season.

Growth continued to increase until leaf-P reached a level of about 0.3% P in dry matter (August).

There was no significant relationship between leaf-N and growth over the range of values observed.

3.7.1. Crop yield

The relationship between leaf-N (1968) and crop yield (1969) is shown in Figure 2, from which it is clear that the response to N varied with level of initial-P.

Increasing leaf-N values, at the lower levels of initial-P ($\frac{1}{2}$ and 1 mequiv./l), had no significant effect on crop yield. At higher levels of initial-P (2 and 4 mequiv./l) response to increasing leaf-N levels was almost linear between values of 2.0% N and 2.5% N in dry matter. From the shape of the upper curve (for P4) plants were all within the "poverty adjustment" range with respect to nitrogen.

The relationship between leaf-P and crop yield is shown in Figure 3. Response to leaf-P varied with initial-N supply; 10 to 18 mequiv./l of nitrogen all gave limiting response curves. The upper response curve (for N20 mequiv./l) was normal and similar in shape to the growth curve (Figure 1).

4. Discussion and conclusions

Initial nutrient-N level (10 to 20 mequiv. of NO_3/l: leaf-N range, 2.1 to 2.5% dry matter) had no significant effect on primary shoot length, thus confirming previous results for black currants grown in sand culture (Bould;[1] Bradfield).[4] Bradfield[4] noted that increased nutrient intensity (average 3.0% of N in leaf dry matter) increased the number of secondary shoots formed during the first growing season and that these secondary shoots were largely responsible for the observed increases in crop yield.

In the current work, increasing initial-N level increased the number of flowers per truss but had no significant effect on percentage fruit set, thus the effect of nitrogen on crop yield operated through increased flower initiation and total flower number. Additional-N, given in early July, had no significant effect on flower number per truss, or on percentage fruit set. Leaf-N (August, first season) showed a positive response to additional-N, given in early July, therefore it must have been absorbed but was too late to have had any significant effect on flower initiation (all plants were in the "poverty adjustment" range with respect to nitrogen status). Teaotia,[5] investigated flower initiation in five varieties of black currant (including cv. Baldwin) under Long Ashton conditions. Flower initiation was first noted on June 11 and formation of first primordia on June 25. Teaotia[5] concluded that flower initiation commences in June–July, though there is a considerable difference in the subsequent rate of flower development. By September 18 all flower primordia were well developed in the varieties investigated.

Berry weight in the current experiment was not unduly influenced by initial nitrogen levels or by additional-N. This confirms previous results from pot experiments (Bould;[1] Bradfield).[4]

Working under field conditions, Sandvad[6] found no significant effect of nitrogen [leaf-N values ranging from 2.55 to 3.03% (1962) and from 2.14 to 2.74% (1963)] on size of berry, or on the number of berries per cluster. He concluded that the yield component most affected by nitrogen supply is the size of bush and the amount of new growth, which in turn affects the total number of flower buds.

It is not clear from the present data how additional-N affected crop weight (in the absence of any significant effect on primary shoot growth, number of flowers per sprig or on berry weight). It had a significant effect on leaf-N (especially at higher levels of initial-P) which in turn may have influenced the amount of secondary growth in the first season (not recorded).

Initial-P level had a highly significant effect on total crop yield, the effect being greatest at high levels of initial-N. This effect resulted from increased fruiting wood coupled with increased percentage fruit set. The yield response curve (for N20) was curvilinear over the leaf-P range 0.1 to 0.3% P in dry matter, thus confirming previous results (Bould).[1]

It is clear from these and other results that crop yield in black currant is influenced by the number of flowers initiated per bud, which is related to the nitrogen supply prior to, or at, initiation (June onwards), the percentage fruit set (related to phosphorus status, prior to or during flowering and pollination) and to general cultural conditions and water supply after flowering.

The results also confirm that leaf analysis in early July may be used as an index of nutritional status and as a guide to fertiliser requirements (see Bould).[1]

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Metabolism of Citric and Malic Acids during Ripening of Tomato Fruit

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Tomato fruit at various stages of ripeness were injected with ^{14}C-labelled citric and malic acids. The $^{14}CO_2$ evolved by individual fruit was determined over a 72 h period and the fruit then analysed for the incorporation of ^{14}C into sugars, amino acids and individual non-volatile organic acids. In mature green tomato fruit citric and malic acids were found to be metabolised to a comparable extent. Appreciable interconversion occurred together with oxidation to CO_2 and some labelling of glutamic acid and glutamine. In red fruit, citric acid was not as readily oxidised to CO_2 and little conversion to malic acid was observed. On the other hand, malic acid was oxidised to CO_2 and converted to citric acid as rapidly as in green fruit with, in addition, appreciable conversion to glutamic acid. These results are discussed in relation to the changes in concentration of the endogenous acids which take place during the ripening of tomato fruit.

1. Introduction

The principal organic acids present in tomato fruit are citric, malic and glutamic acids, their relative abundance depending on the variety and nutritional status of the plant. They also show characteristic changes in concentration during the development and ripening of the fruit.[1] Thus the malic acid content and the malic acid:citric acid ratio decrease during ripening, while the glutamic acid content increases.[2]

Little seems to be known either of the origin of these acids which accumulate during fruit development or of their subsequent fate. Since they play an important role in determining the flavour of tomato fruit, knowledge of their formation and metabolism is highly relevant to the problems of palatability, keeping quality and the satisfactory preservation of processed tomato products.

In the present work the behaviour of citric and malic acids in tomato fruit has been studied with the aid of ^{14}C-labelled acids administered to detached fruit at various stages of ripeness. The appearance of the labelled carbon in various tissue components was then followed in an attempt to account for the changes which occur during ripening.

2. Experimental

The tomato variety used in this investigation, Craigella, was grown in heated glasshouses. All fruit were of good visual quality and each weighed between 50 and 60 g. Definitions of the stages of ripeness used have been given previously.[3]

Aqueous solutions of [1,5-^{14}C]citric acid and L-[U-^{14}C]malic acid, obtained from the Radiochemical Centre, Amersham, Bucks, were prepared and 50 μl containing approximately 1 μCi were administered to each fruit. Two methods of application were employed: vacuum infiltration using the technique of Barbour, Buhler and Wang[4] and direct injection into a locule. The former method failed to give adequate distribution of labelled material within the fruit and only a small proportion appeared in the locules.[5] In the experiments reported here the second procedure was used, the solutions being injected into a locule from the opposite side of the fruit. The puncture was sealed immediately with paraffin wax to prevent loss of labelled material.

After injection a steady stream of air (25 ml/min) was passed over the individual fruit in a 120-ml glass chamber at room temperature (21 to 24 °C) for varying periods of time

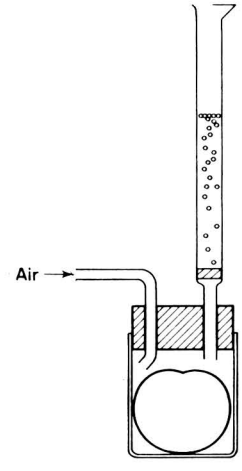

Figure 1. Apparatus for the collection of respired $^{14}CO_2$ from tomato fruit.

up to 72 h. The evolved $^{14}CO_2$ was absorbed in 8 ml of 12% (w/v) 2-phenylethylamine in methanol contained in a tube fitted with a sintered glass disc (see Figure 1), the absorbent solution being changed twice daily. On removal, the volume of the absorbent solution was made up to 10 ml with methanol and 5 ml aliquots used for ^{14}C assay. Quantitative absorption of $^{14}CO_2$ (>98%) was obtained by this procedure.

At the end of the respiration period the whole fruit was extracted with boiling water for 20 min, the extract filtered through glass wool and the residue washed repeatedly with hot water to give a total volume of 250 ml.

Preliminary experiments indicated that extraction of the fruit with hot ethanol to give a final concentration of approximately 80% was less satisfactory for our purpose than extraction with boiling water. If the alcohol was not removed before the ^{14}C assay then excessive quenching was observed owing to the presence of pigments. Removal of the alcohol *in vacuo*, on the other hand, produced residues which were relatively intractable to re-extraction with water.

The aqueous extract was passed successively through columns of Dowex 1 × 8

(200 to 400 mesh) in the acetate form and Zeo-Karb 225 (H^+) and the effluent (neutral fraction) collected. The dicarboxylic amino acids were eluted from the Dowex 1 column with 0.5 N-acetic acid (first acid fraction) and the remaining acids with 6 N-formic acid (second acid fraction). Elution of the basic components from the Zeo-Karb 225 was effected with 2 N-ammonium hydroxide. Each of the various fractions was concentrated under reduced pressure and made up to a volume of 25 ml prior to assay of the total ^{14}C content.

Samples (1 ml) of the aqueous fractions were mixed with 10 ml of a liquid scintillant consisting of 0.6% (w/v) 2-(4′-*t*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD)[6] in a toluene–Triton X-100 (2:1 v/v) mixture.[7] The resultant clear solutions were counted at ambient temperature in a Beckman LS-100 liquid scintillation counter. For the counting of the respiratory $^{14}CO_2$, 5 ml of 1.2% butyl-PBD in toluene were added to 5 ml of the absorbent solution. Counting efficiency of the aqueous samples ranged from 91.4% in unquenched samples to 83.6% in the most highly-quenched acid fractions. Where methanolic 2-phenylethylamine was present the counting efficiency was 85.1%.

The distribution of ^{14}C in the acid fractions was examined in more detail by paper chromatography followed by radiochromatographic scanning. The aqueous solutions after assay of total ^{14}C were evaporated under reduced pressure to small volume and the material streaked on to several three-inch ($\simeq 8$ cm) wide strips of Whatman 3MM paper. One-dimensional descending chromatography was carried out in the following solvents: (1) butan-1-ol–acetic acid–water (12:3:5, v/v) for 28 h for the first acid fraction and (2) propan-1-ol–ammonia (s.g. 0.880)–water (6:3:1, v/v) for 28 h for the second acid fraction. After being dried the papers were cut longitudinally into two equal strips and each strip examined for ^{14}C activity with a 4 π radiochromatogram scanner.

Chemical location of the acids on the chromatograms was carried out with a silver fluoresceinate spray reagent previously described for the detection of sulphonic acids,[8] or with ninhydrin in the case of the amino acids. The positions of the bands so obtained were then compared with the areas of ^{14}C activity obtained by scanning.

In a number of experiments the labelled components of the fractions were identified by cutting out the radioactive bands from chromatograms and eluting them with water. The solutions, after being concentrated under reduced pressure, were rechromatographed against a range of unlabelled reference acids in the following solvent systems: (1) butan-1-ol–acetic acid–water (12:3:5, v/v); (2) propan-1-ol–ammonia (s.g. 0.880)–water (6:3:1, v/v); (3) ethanol–ammonia (s.g. 0.880)–water (160:7:30, v/v); (4) pyridine–methanol–water (1:20:5, v/v); (5) methylethylketone–cineole–formic acid (90%)–water (50:50:21:15, v/v); (6) propan-1-ol–cineole–formic acid (90%)–water (50:50:22:3, v/v).

3. Results

3.1. Recovery of ^{14}C from injected fruit

The activity of the ^{14}C-labelled CO_2 evolved from each fruit, together with the water-extractable ^{14}C of the tissues, was determined after periods of up to 72 h and expressed

as percentages of the total ^{14}C applied. The results of 26 experiments are summarised in Table 1.

Almost complete recovery of the ^{14}C of labelled citric acid was obtained. Recoveries from fruit injected with ^{14}C-labelled malic acid were, however, significantly lower

TABLE 1. Mean recovery of ^{14}C from mature green and red tomato fruit injected with ^{14}C-labelled citric or malic acids. (Number of experiments given in brackets)

Compound injected	Initial Stage of Ripeness		
	Mature green	Red	Mean
[^{14}C]citric acid	93.3 (6)	100.9 (7)	97.4 (13)
[^{14}C]malic acid	80.8 (5)	82.0 (8)	81.5 (13)
Mean	87.6 (11)	90.8 (15)	

L.S.D. ($P < 0.05$) between mean values for compounds injected ±3.9.

($P < 0.001$). These results would suggest that nearly 20% of the ^{14}C administered as malic acid was incorporated into the water-insoluble fraction of the fruit. The stage of ripeness at injection had no significant effect on the recovery of ^{14}C. The interaction between initial stage of ripeness and the compound injected just failed to attain significance at $P < 0.05$.

3.2. $^{14}CO_2$ production by injected fruit

The course of $^{14}CO_2$ output from both mature green and red tomato fruit injected with either ^{14}C-labelled citric or malic acids was followed over 72 h periods. Approximately 30% of the ^{14}C applied as malic acid was recovered as $^{14}CO_2$ after 72 h, some 80% of this being evolved during the first 30 h after injection. Both mature green and red fruit behaved similarly [Figure 2(a)]. Mature green fruit injected with labelled citric

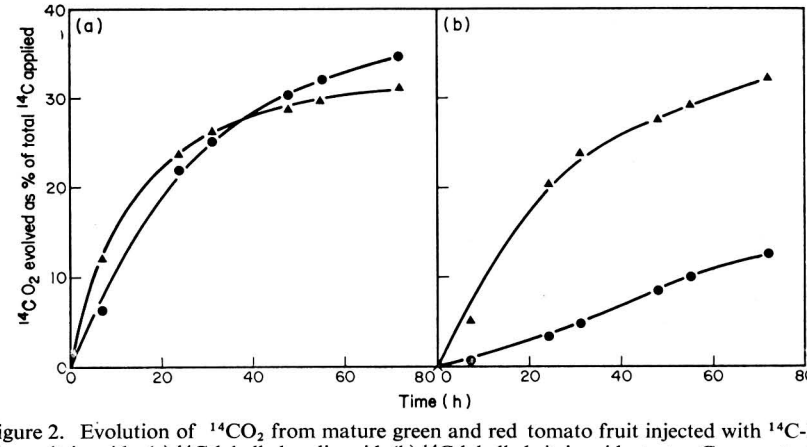

Figure 2. Evolution of $^{14}CO_2$ from mature green and red tomato fruit injected with ^{14}C-labelled malic or citric acids. (a) ^{14}C-labelled malic acid, (b) ^{14}C-labelled citric acid.–▲–▲–, Green; –●–●–, red.

acid showed a similar pattern of $^{14}CO_2$ production. On the other hand, evolution of $^{14}CO_2$ from red fruit supplied with labelled citric acid was markedly slower [Figure 2(b)], only 12.8% of the applied ^{14}C being recovered as $^{14}CO_2$ after 72 h.

Statistical analysis of the four groups of data was carried out using the technique of principal components.[9] Four separate comparisons were made, namely, green malate-treated fruit against red malate-treated, green citrate against red citrate, green malate against green citrate and red malate against red citrate. Common principal axes were taken for each test and significant differences in the first principal component were found between red citrate and green citrate ($t_{(8)} = 7.6$, $P < 0.01$), and between red malate and red citrate ($t_{(7)} = 11.2$, $P < 0.01$). Thus the red citrate group is significantly different from the other three groups while there is no evidence for important differences between the other three.

3.3. Distribution of ^{14}C in aqueous extracts of injected fruit

The distribution of ^{14}C in the neutral, basic and acid fractions of the aqueous fruit extracts 72 h after injection with either ^{14}C-labelled citric or malic acids is shown in Table 2. The combined recovery of ^{14}C in the different fractions was virtually complete,

TABLE 2. Distribution of ^{14}C in aqueous extracts of tomato fruit injected with ^{14}C-labelled citric or malic acids

Compound injected	Initial stage of ripeness	^{14}C distribution (as percentage of activity of aqueous extract)				
		Neutral fraction	Basic fraction	1st Acid fraction	2nd Acid fraction	Total recovery
[^{14}C]Citric acid	Green[a]	2.9	7.2	4.1	84.5	98.7
	Red[b]	1.0	2.1	7.1	86.8	97.0
[^{14}C]Malic acid	Green[a]	5.3	8.9	4.6	78.6	97.4
	Red[a]	7.9	5.6	25.2	60.0	98.7

[a] Mean of values from four fruit.
[b] Mean of values from three fruit.

representing on average 98.0% of the total activity of the aqueous extracts. Analysis of variance revealed significant interactions between the stage of ripeness and the compound injected for each of the fractions examined except the basic fraction. When ^{14}C-labelled citric acid was injected the ^{14}C was confined largely to the second acid fraction irrespective of the initial stage of ripeness, whereas on injection of labelled malic acid, significantly less ^{14}C remained in the second acid fraction from red fruit than from green (significance of interaction: $P < 0.05$; L.S.D. at 5% level $\pm$ 10.9). At the same time significantly increased labelling of the first acid fraction from red fruit was observed when ^{14}C-labelled malic acid was injected but no such effect was noted when ^{14}C-labelled citric acid was used (significance of interaction: $P < 0.001$; L.S.D. at 5% level $\pm$ 6.1). Significantly more ^{14}C appeared in the neutral fraction from green fruit than from red after injection with ^{14}C-labelled citric acid, the converse being observed with ^{14}C-labelled malic acid (significance of interaction: $P < 0.01$; L.S.D. at 5% level $\pm$ 1.2). Significantly higher amounts ($P < 0.05$) of ^{14}C appeared in the basic

fraction from green fruit than from red, irrespective of which of the two ^{14}C-labelled acids was injected.

3.4. Labelling of the acid fractions of aqueous extracts

One-dimensional paper chromatography was carried out on the two acid fractions and the dried papers scanned for ^{14}C activity. Chromatograms of the less strongly labelled first acid fraction from red or green fruit injected with ^{14}C-labelled citric acid each gave a peak of activity coinciding with a ninhydrin-positive band identified as glutamic acid. No detectable activity was found to be associated with aspartic acid, the other ninhydrin-positive component of the fraction.

In the case of fruit injected with ^{14}C-labelled malic acid, however, activity was present in both the aspartic and glutamic acid bands. Integrated scanning of the two peaks obtained showed that there was approximately equal labelling of the two acids in green fruit, whereas with red fruit about 80% of the activity of the fraction was found in the glutamic acid band. No other radioactive components could be detected in this fraction.

Chromatograms of the strongly labelled second acid fraction gave two peaks identified as citric and malic acids, the larger of the two corresponding to the acid which had been injected initially into the fruit. In the fractions from green fruit injected with either citric or malic acids, there was a further small peak corresponding to a fast-running acid, which was detected visually on the chromatograms with the silver fluoresceinate reagent. Chromatography of this acid against a range of authentic marker acids in five solvent systems and a study of the sequence of colour changes it underwent when sprayed with the silver fluoresceinate reagent and also with the hydroxylamine–ferric chloride reagent of Ramakrishna and Krishnaswamy,[10] revealed it to be pyrrolidonecarboxylic acid, formed as an artifact from glutamine during the hot water extraction of the fruit.

3.5. Distribution of ^{14}C in individual acids

The relative activities of the labelled components in the second acid fractions from the different experiments were determined by integrated scanning of the peaks on chromato-

TABLE 3. Distribution of ^{14}C in the acids present in aqueous extracts of tomato fruit injected with ^{14}C-labelled citric or malic acids

Compound injected	Initial stage of ripeness	^{14}C distribution (as percentage of activity of total acid fraction)				
		Citric acid[a]	Malic acid[a]	Aspartic acid	Glutamic acid[b]	Glutamine
[^{14}C]Citric acid	Green[c]	57.2	35.0	0	3.4	4.4
	Green/yellow[c]	74.9	16.1	0	5.8	3.3
	Red[d]	88.6	5.1	0	7.5	0
[^{14}C]Malic acid	Green[c]	33.3	56.2	3.1	3.1	4.4
	Green/yellow[c]	30.6	61.1	2.4	2.4	3.5
	Red[d]	29.8	40.2	6.3	23.2	0.5

[a] Interaction significant at $P < 0.001$.
[b] Interaction significant at $P < 0.01$.
[c] Mean of values from two fruit.
[d] Mean of values from four fruit.

grams. The values were combined with those of the first acid fractions to give the distribution of ^{14}C among the five acids which became labelled, as summarised in Table 3.

In green fruit, citric and malic acids were metabolised to a comparable extent. This contrasts with their behaviour in red fruit, where malic acid was more actively metabolised and gave rise to substantial amounts of citric and glutamic acids and some aspartic acid. Significant labelling of aspartic acid was only obtained when malic acid was injected and labelling of glutamine (detected as pyrrolidonecarboxylic acid) occurred from either acid only in green or green-yellow fruit.

4. Discussion

Previous work has shown that malic acid concentrations in tomato fruit decrease with ripening,[2, 11, 12] while citric acid increases up to the green-yellow stage of ripeness and then either falls[11–13] or shows no further significant change.[2] At the same time glutamic acid concentrations rise sharply and aspartic acid concentrations also increase, although to a much lesser extent.[2, 14, 15] These trends clearly imply significant changes in the rates of synthesis and/or breakdown of the acids during ripening.

The present experiments with ^{14}C-labelled citric and malic acids showed that in mature green tomatoes the two acids were metabolised to a comparable extent over a 72 h period. Appreciable interconversion of the two acids occurred, together with the oxidation of about 30% of either compound to CO_2. A small fraction of the labelled carbon was recovered in glutamic acid and glutamine and also in aspartic acid with malic acid-injected fruit. Only slight conversion of either acid to sugars took place, but about 20% of the carbon of malic acid became associated with the water-insoluble fraction of the fruit.

Divergencies in the behaviour of the two acids were noted as the fruit ripened. Thus, as the fruit changed colour the extent of the oxidation of citric acid to CO_2 decreased markedly and in red fruit was less than half of that in green fruit (Figure 2). At the same time conversion to malic acid was reduced, as was the incorporation of its carbon into sugars, amino acids and glutamine, while there was a slight increase in the incorporation into glutamic acid (see Table 3). Malic acid, on the other hand, was oxidised to CO_2 and converted to citric acid as readily in red as in green fruit. With increasing ripeness glutamine formation was reduced, but conversion to aspartic and glutamic acids was increased, that of the latter over seven-fold.

The results obtained show that during the later stages of ripening there was a significant decrease in the overall metabolism of citric acid, whereas that of malic acid was largely unaffected and was even enhanced in the direction of aspartic and glutamic acid synthesis. These findings are in agreement with the changes in concentration of the non-volatile acids previously observed in tomato fruit during ripening. They indicate that the injected ^{14}C-labelled acids were freely available to the sites of metabolic activity in the fruit and that the subsequent behaviour of these acids closely paralleled that of the corresponding endogenous compounds.

The site of production of the major organic acids in fruits has been taken to be similar to that of the sugars. It is believed that they are formed in the leaves and then

translocated[16,17] and arguments supporting this contention have been reviewed by Ulrich.[18] There is, however, evidence that in some fruits a proportion of the major fruit acids is formed *in situ*. Thus in the grape the formation of malic and tartaric acids from $^{14}CO_2$[19] and from glucose and fructose[20] has been demonstrated. Comparable results have been obtained for strawberries[21] and apples.[22,23] Similarly in the tomato, experiments with labelled precursors point to synthesis within the fruit as a source of citric and malic acids. Thus Wang and his co-workers[24,25] have shown the entry of ^{14}C into citric and malic acids from labelled CO_2, acetate and pyruvate. Tishel[23] has also shown that glucose is a precursor of the two acids in detached green tomatoes. He showed that both citric and malic acids became increasingly labelled up to 24 h after the injection of [U-^{14}C]glucose. Over the next three days, however, the activity of the malic acid declined rapidly while that of citric acid was little changed. Sakiyama[26] has since reported that both the titratable and total acidities of tomatoes detached when green increased during storage, whereas the acidity of those detached at the first appearance of colour declined on subsequent storage. Thus, it would appear that a proportion of the acids of tomato fruit is synthesised *in situ*, but the extent of this contribution in relation to that made by the leaves is unknown and will be the subject of further investigation.

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Potato Starch Factory Waste Effluents
III. Recovery of Organic Acids and Phosphate[a]

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The use of anion-exchange resins for removal of acids from potato starch factory waste-water (after prior removal of proteins and amino acids) was studied. Over 99% of the acids were removed by the process. Data are given on the effect of influent temperature and flow-rate on column efficiency during the acid adsorption step. Data are also given on methods of increasing the concentration of acids in the eluate by increasing the alkalinity of the eluting agent or by recycling the eluate. Possible uses for the eluate are discussed.

1. Introduction

The liquid waste effluents of potato starch factories have an extremely high biochemical oxygen demand (b.o.d.) and therefore require an efficient type of sewage or other waste treatment. However, the potato starch industry operates on a narrow margin of profit and probably could afford added waste treatment costs only if this treatment recovered useful materials from the waste.

Previous publications from this Laboratory have discussed the reverse osmosis concentration of the effluent,[1] the recovery of protein,[2] the recovery of potassium and other cations,[3] the laboratory-scale removal of amino compounds by cation-exchange[4,5] and the operational data required for evaluating the commercial possibilities of the latter process.[6] After these treatments, however, phosphoric acid and organic matter, including sugars and several organic acids, are left in the waste. It thus could still cause problems of oxygen depletion or eutrophication if allowed to flow into streams or lakes. The present paper gives data on the use of anion-exchange resins to remove the acids. It also discusses possible uses for the recovered acids and the nature of the unidentified acids present.

2. Experimental

The wastes used for this study were the effluents resulting from the amino-acid recovery step.[6] These effluents were frozen until needed.

Ion-exchange studies were performed using water-jacketed glass-pipe columns [1-in (25.4 mm) × 48 in (1.21 m) long][b] containing 317 ml [24.3 in (≃0.617 m)] of wet

[a] Part II in this series is *J. Sci. Fd Agric.* 1972, **23**, 745.

[b] Fischer & Porter Company. Mention of company or trade names does not imply endorsement by the Department over others not named.

resin. Two types of weak-base anion-exchange resin[a] were studied—IRA-68, a standard "gel"-type resin and IRA-93, a macroreticular resin. Water from a controlled-temperature bath was circulated through the jackets. Constant-flow pumps were used for all streams—waste input, wash water and regenerant. The pH of liquids at the column outlet was continuously recorded using Corning combination electrodes set in glass flow-cells. Total acidities were determined by titrating to a phenolphthalein end-point; individual acids were determined by the ion-exchange method of Schwartz, Greenspun and Porter.[7] The dichromate reflux method[8] was used for estimating chemical oxygen demand (c.o.d.). Reducing sugars were determined by the Shaffer–Somogyi micro method,[9] solids by heating at 90 °C in a forced-draft oven.

For phosphorus analysis the sample, plus 50 mg of magnesium acetate, was evaporated to dryness in a platinum dish (steam bath). After four drops of olive oil were added the sample was ashed at 600 °C for 1 h. The residue was dissolved in hot 1:4 HCl and transferred to a 100-ml volumetric flask. Phosphorus was then determined by the method of Scroggins.[10] All apparatus was first rinsed with hot 1:4 HCl to remove traces of phosphorus from detergents, etc.

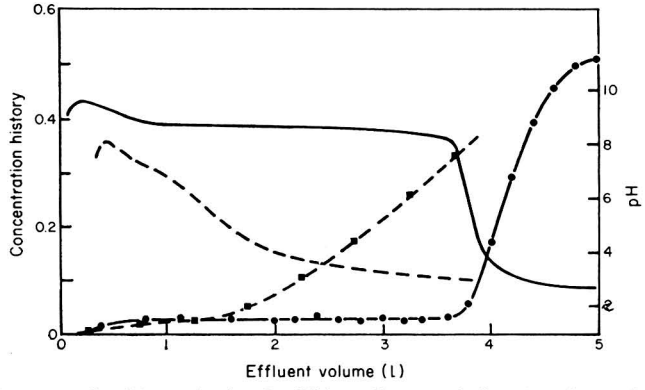

Figure 1. Concentration history (ratio of acidities, effluent to influent) and pH of anion-exchange effluents. ——, pH, IRA-68; – – –, pH, IRA-93; ■—■, IRA-93; ●—●, IRA-68.

3. Results and discussion

3.1. Adsorption of acids

For acid adsorption the two resins were used in the free-base form. Figure 1 shows the effluent pH and concentration history (ratio of acidities, effluent to influent) for two runs. For the run with the macroreticular resin (IRA-93) influent acidity was 0.12 mequiv./ml and flow-rate 15.1 b.v. (bed volumes)/hour. For the run with the "gel"-type resin (IRA-68) influent acidity was 0.16 mequiv./ml and flow-rate 12.0 b.v./h.

The superiority of the IRA-68 in this application is evident in the Figure. When the flow-rate for the IRA-93 was reduced from 15.1 to 7.0 b.v./h its performance, though improved, still did not meet that of the IRA-68 at 12 b.v./h. For this reason the latter was used in all further studies.

[a] Kindly furnished by Rohm and Haas Company, Philadelphia, Pa.

In the preceding runs, influents were kept at 30 °C during the adsorption step. To examine the effect of changes in influent temperature and flow-rate, a series of runs was made in groups of three—the members of each group using the same influent batch. The first run was made under conditions taken as standard—influent temperature 30 °C, flow-rate 16.2 b.v./h. For the second run the temperature was kept at 30 °C, but the flow-rate was decreased to 12.0 b.v./h; for the third the flow-rate was kept at 16.2 b.v./h., but the temperature was increased to 50 °C. Milliequivalents of acid adsorbed by the column up to the acid break-through point was taken as the measure of column efficiency. This was determined by measuring, as accurately as possible, the volume of influent put through the resin and titrating both influent and effluent. The break-through point was taken as the point where the slope of the pH curve increased to 45 °C.

TABLE 1. Effect of influent temperature and flow rate on acid adsorption

Adsorption in standard runs[a] (mequiv.)	Change in adsorption Slow flow, 12.0 b.v./h (%)	High temperature 50 °C (%)	Influent concentration[b]
470	+11.1	+1.0	High
468	−1.0	+2.5	High
453	+8.3	+6.4	High
447	+3.6	+5.1	High
458	+9.2	—	High
420	+3.6	—	Low
448	+4.1	−2.2	Low
404	—	+2.6	Low
Average	+5.6	+2.6	

[a] 16.2 b.v./h, 30 °C.
[b] High = 0.11 to 0.16 mequiv./ml; low = 0.06 to 0.07 mequiv./ml

Table 1 gives the results. A correction of −0.7% has been added to the higher-temperature runs to allow for the expansion of water between 30 and 50 °C. Slower flow gave an average increase of 5.6% in amount of acid adsorption, higher temperature an increase of 2.6%. The effects of these variables, however, might be expected to show up more with influents of higher concentration. If runs with less-concentrated influents are omitted from the averages, the increase in acid adsorption goes up to 6.2% for slow-flow runs and 3.7% for high-temperature runs.

No pattern could be detected in effluent acid concentrations from these runs. They ranged from 12 to 86×10^{-5} mequiv./ml and averaged 41×10^{-5}. In all cases the decrease in acidity was over 99%; it averaged 99.6%. Reduction in phosphorus averaged 97.9% for the standard and slow-run flows, 98.6% for the 50 °C runs. Phosphorus data are given in Table 2; it can be seen that its concentration, in both influent and effluent, varies directly with the overall acidity of the influent.

Table 3 shows the effect of the anion-exchange process on c.o.d. and solids concentrations. Runs are listed in order of decreasing influent acidity. In runs two through four, acid adsorption proceeded at the standard flow-rate of 16.2 b.v./h. The flow-rate

TABLE 2. Effect of influent temperature and flow-rate on phosphorus removal

Phosphorus in influent (mg/l)	Phosphorus in effluent: Standard runs, 16.2 b.v./h, 30 °C (mg/l)	Slow flow, 12.0 b.v./h (mg/l)	High temperature, 50 °C (mg/l)	Total acid in influent (mequiv./ml)
272	4.0	4.6	2.3	0.157
244	5.5	3.5	4.8	0.151
244	4.9	4.7	4.7	0.150
182	6.9	6.1	—	0.114
85	1.3	—	1.8	0.072
60	0.8	0.6	0	0.065
58	1.4	2.1	—	0.064

TABLE 3. Effect of anion exchange on c.o.d. and solids

Acidity of influent (mequiv./ml)	c.o.d. Influent (mg/l)	c.o.d. Effluent (mg/l)	Decrease in c.o.d. (%)	Solids Influent (%)	Solids Effluent (%)	Decrease in solids (%)
0.156	11 210	7780	31	1.26	0.69	45
0.097[a]	10 380	5520	47	0.95	0.43	54
0.097[a]	10 380	5460	47	0.95	0.43	55
0.088	8640	4420	49	0.88	0.35	60
0.063	5270	3650	31	0.73	0.34	54
0.062	6330	3390	46	0.65	0.29	55
0.058	7030	4380	38	0.62	0.30	52

[a] Two runs on the same influent.

TABLE 4. Effect of waste treatment on c.o.d., solids and reducing sugars

Treatment stage	c.o.d. (mg/l)	Decrease in c.o.d. (%)	Solids (%)	Decrease in solids (%)	Reducing sugars (%)
Untreated waste	23 710		2.48		0.17[a]
After protein and amino-acid removal	9800	59	0.93	63	0.24
After anion removal	5130	48[b]; 78[c]	0.40	57[b]; 84[c]	0.19

[a] Determined on deproteinised waste.
[b] Decrease produced by anion exchange.
[c] Overall decrease.

for the others was 12.0 b.v./h. For the three standard runs, data were also obtained for c.o.d., solids and reducing sugar concentrations in untreated waste and after amino-acid removal. Average values are given in Table 4. Anion-exchange resulted in a 48% decrease in c.o.d. and a 57% decrease in solids; decreases for the entire purification process (removal of protein, cation, amino acids, organic acids and phosphorus)

were 78 and 84%, respectively. Non-reducing sugars were absent in the waste after amino-acid removal—probably as a result of hydrolysis caused by the low pH of the waste (1.5 to 1.9) after cation-exchange. This hydrolysis may be reflected in the increase in reducing sugars shown in Table 4; the subsequent decrease in reducing sugars is unexplained.

3.2. Elution of acids

For the early runs, acids were eluted (as ammonium salts) with 780 ml of 1 N-NH_4OH—a little over 50% in excess of the calculated column capacity. This also served to regenerate the free-base form of the resin. Flow-rate for this step and for the first bed

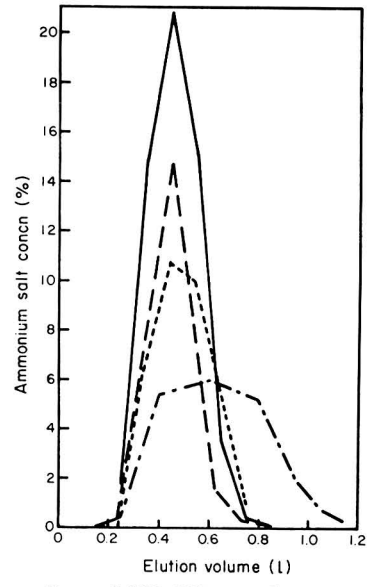

Figure 2. Effect of eluate recycling and NH_4OH strength on concentration of ammonium salts. ——, 4.5 N-NH_4OH recycled; — —, 4 N-NH_4OH; – – –, 2 N-NH_4OH; — · — ·, 1 N-NH_4OH.

volume of the following water-rinse (salts and NH_4OH still being eluted) was 3.6 b.v./h. For later runs, including those made for the influent flow-rate and temperature studies, the regenerant was 390 ml of 2 N-NH_4OH. The same low flow-rate was used for all regenerant concentrations.

In a study of conditions for obtaining the maximum concentration of salts, runs were made using 1 and 2 N-NH_4OH in the amounts given above. Runs were also made with 320 ml of 4 N-NH_4OH. After one 4-N run, 400 ml of that part of the eluate and rinse most concentrated in acids was mixed with 102 ml of concentrated NH_4OH—enough to make the solution approximately 4.5 N in free NH_4OH. Of this 320 ml was used as regenerant for the next run. This recycling was repeated three times. Eluate fractions were dried to constant weight to determine salt concentrations. Figure 2 shows typical curves for ammonium salt concentration at each regenerant strength

and the curve for the final recycling. Figure 3 graphs the progress of salt concentrations and the total weight of salts recovered for all the recyclings; it is apparent that after three recyclings the curves are beginning to level off. The curve for average concentration is stepped because the third point was determined on a larger volume of eluate than the second. Similar data for the non-recycled runs are given in Table 5. Going from 1 to 4 N-ammonia increased average salt concentration from 4.3 to 7.8%; recycling four times increased it to 11.1%. Although it would appear from Table 5 that regenerants above 4 N in strength might give even higher salt concentrations, the hazards of using strong ammonia must be considered. There is also an indication that at the higher NH_4OH concentrations the total amount of salts collected decreases somewhat.

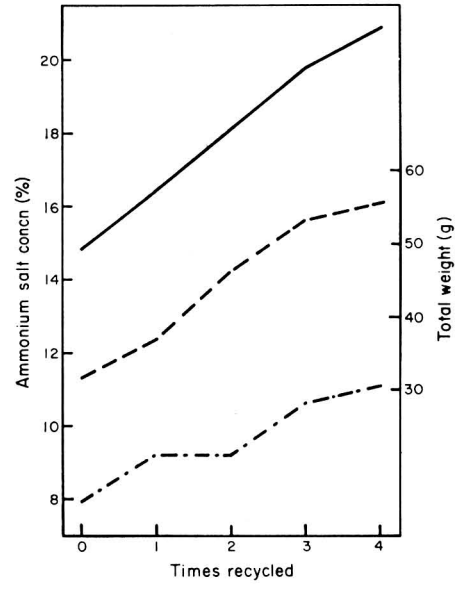

Figure 3. Effect of eluate recycling on concentration and weight of ammonium salts. ——, Maximum concn; – – –, total weight; — · — ·, average concn.

3.2.1. Composition of eluate

Analyses of eluates from two runs gave an average of 4% pyroglutamic acid, 12% malic acid, 57% citric acid, 6% phosphoric acid, 3% oxalic acid and 18% of unidentified acids. One eluate also contained about 1% aspartic acid. Relative amounts of acids are similar to those previously obtained for potato extracts[11] provided allowances are made for the nearly complete removal of glutamic and aspartic acids by the cation-exchange process. Pyroglutamic acid is probably an artifact derived from glutamic acid.

Although the unidentified acid came off the analytical anion-exchange column in one peak, paper chromatography revealed it to be a mixture of at least three or four components. The use of 3:1 acetone–25% CCl_3COOH as eluting solvent[12] produced

two major spots with R_F 0.47 and 0.61. The elution volume of the material from the analytical column and the fact that it reduced the molybdate spray reagent of Hanes and Isherwood[13] indicated it might contain organic phosphates such as sugar phosphates, nucleotides or phytic acid. Also, its infrared curve showed no carboxyl and only slight C–H absorption compared to the highly polar groups present; one broad band at 2900 to 3100 cm^{-1} could be OH on either phosphorus or sulphur. In spite of this, however, analysis of the material showed only 0.3% phosphorus and there was very little chromatographic evidence for the above phosphates. Some indication of the presence of nucleotides was the fact that a "beard" under the lower major spot gave a pale-brown colour reaction with *p*-anisidine spray reagent[14] and absorbed light from a 254-nm lamp.

TABLE 5. Concentration and total weight of salts in NH_4OH eluates

NH_4OH concentration (normality)	Salt concentration Average [a] (%)	Maximum (%)	Total weight of salts [a] (g)	Volume of eluate [b] (ml)
1	4.0	6.7	37.7	950[c]
1	4.5	6.0	36.0	800
2	—	11.5	—	—
2	—	10.9	—	—
2	6.8	10.8	34.2	500
2	6.0	10.5	30.2	500
4	7.7	14.0	31.0	400
4	7.9	15.4	31.6	400
4	7.9	14.8	31.4	400

[a] In the volume of eluate given in the last column.
[b] 100-ml fractions. Includes only fractions containing at least 0.5% salts.
[c] Major fractions 200 ml.

In addition to the non-volatile acids determined the eluate must contain sulphite, since $NaHSO_3$ had been added in preparing the original waste-water so that recovered products would be light-coloured. Anion-exchange influents were estimated to contain about 0.007 mequiv./ml of sulphite. It is possible that the unidentified material may consist in part of artifacts produced by sulphite reactions—but not in entirety, since analyses of acids in unsulphited potato extracts also show an unidentified peak.

3.3. Rinse procedures

For standard runs, the water-rinse after acid adsorption consisted of a 120-ml downwash (to clear piping), a 2-l backwash at 75% bed expansion and then a drainage of 140 ml to reduce the head of water. Elution of acids was followed by a 2-l downwash (at 12.0 b.v./h after the first bed volume). These relatively large volumes of water were used to insure that impurities did not accumulate on the column during the various studies. In some runs the backwash was reduced to 634 ml (2 b.v.) and the final downwash to 951 ml (3 b.v.); this did not affect the column capacity.

3.4. Commercial possibilities

For plant operation, a three-column, continuous operation system could be set up such as previously described for amino-acid removal.[6] In this system, while one column is being loaded, a second is acting as scavenger to reduce acid leakage and the third is being eluted. If it is assumed as before[6] that the plant produces 6760 gal[a]/hr of waste, each column would require a bed of 417 gal (55.7 ft^3[b]) of resin to achieve the 16.2 b.v./h flow-rate of the standard laboratory runs. If the slower rate (12.0 b.v./h) is desired, 562 gal (75.1 ft^3) would be needed.

The acid mixture recovered from the wastewater was tested as a replacement for phosphate detergent builders after first removing excess NH_4OH from the eluate and converting to sodium salts by passing through a cation-exchange resin in the sodium form. A few drops of hydrogen peroxide were added to decolorize the solution and prevent turbidity which otherwise formed on standing. Its pH was adjusted to 7.0 and portions were tested with four different detergents. With three of these, the solution performed about as well as the control phosphate builder in washing ability, but there was considerable soil redeposition on clean test-swatches. When the solution was first adjusted to pH 10, a pH at which detergents are usually more effective, there was improved washing ability with only one of the detergents and there was still redeposition of soil in spite of the addition of carboxymethylcellulose as a suspending agent. A further difficulty at pH 10 was the fact that the pH of the solution decreased on standing. This may have been caused by alkaline hydrolysis of the unidentified material.

For testing as a detergent builder or for other uses such as food uses, it may be better to obtain individual acids or more restricted mixtures, such as a mixture of citric and malic acids. This could be done by using a volatile acid to elute the potato acids from the column in a manner similar to that used for determining the individual acids.[7] However, this would introduce an extra step in the process, since the resin would still have to be eluted with ammonia to restore it to the free-base form.

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[a] Throughout this paper 1 gal $\simeq$ 3.79 l.
[b] Throughout this paper 1 ft^3 $\simeq$ 0.0283 m^3.

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Location of Lipid-soluble Selenium in Marine Fish to the Lipoproteins

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It has been established that a lipid-soluble selenium compound is enriched in high molecular weight (m.w. ⩾5000) extracts from fish. The selenium compound is also enriched in oil extracted from fish by a mixture of non-polar and polar organic solvents (hexane–isopropanol) as compared to the oil extracted with non-polar solvents (hexane) only. When raw fish material is stored it gradually deteriorates and the selenium content in oil produced by boiling of the raw fish material, increases with time. The results indicate that at least part of the lipid-soluble selenium is bound as a lipoprotein.

1. Introduction

It has been shown that marine oils contain 0.1 to 2.0 parts/million of selenium[1] and that the selenium is present as a lipid-soluble organic selenium compound. Some of the oils analysed were produced industrially and some were isolated under controlled conditions in the laboratory either by treatment with hot water, or by extraction with chloroform–methanol mixtures. It was also shown that the lipid-soluble organic selenium compound has a polar character. Chromatographed on silicic acid, most of the lipid-soluble selenium compound was eluted in front of the bulk phospholipids by mixtures of chloroform and methanol.

When solutions produced from marine raw materials either by boiling or by enzyme hydrolysis are subjected to gel filtration, the selenium content is enriched in the high molecular weight fraction as compared with the original material.[2] The conclusion drawn is that the selenium very probably exists as compounds other than seleno amino acids (present in the protein phase analogous to the sulphur amino acids).

The purpose of this work was to study in more detail whether the lipid-soluble selenium compound shown to be present in marine oils is bound to the lipid phase in the high molecular weight fraction, i.e. whether it is part of a lipoprotein complex. Assuming that the lipid-soluble selenium compound is bound to proteins, it is also of interest to determine whether it may be enriched by means of selective extractions. The neutral oil may be extracted by a non-polar solvent, such as hexane. The lipids which

are bound to the proteins may then be extracted by a polar solvent, such as an alcohol, in addition to the hexane.

If the organic selenium compound is present in a lipoprotein complex, it is possible that the selenium content will be dependent upon the way in which the oil is isolated. When the oil is extracted from the raw material by chloroform–methanol, the lipid-soluble selenium compound will most likely be quantitatively extracted together with the neutral oil. On the other hand, if the oil is isolated by treatment of the raw material with water at different temperatures, the selenium organic compound may well behave like the phospholipids. Consequently, it will depend upon the conditions under which the isolation occurred and also upon the quality of the raw material used for extraction.

2. Experimental

2.1. Preparation of samples

Raw materials with different fat contents were selected for the analysis. Here cod fillet, cod liver and whole herring were used. Some samples of industrially produced glue water from herring and mackerel were also included.

The glue water is prepared by boiling homogenised raw material in glass apparatus for 20 min. Distilled water is added as the boiling proceeds. Oil produced in this process was centrifuged off and the glue water (the water phase) was then filtered and the filter washed once with distilled water. The glue water from cod fillet contained no visible traces of oil. The samples of glue water were concentrated to 4 to 5% dry matter, and stored at −20 °C for subsequent molecular gel fractionation.

The fraction which was insoluble in water after the preparation of the glue water, i.e. the fish meal, was hydrolysed by incubation with a protease (Bioprase; Nagese and Co Ltd, Japan). This procedure has been described earlier.[2] The water-soluble part was filtered off and treated in the same manner as the glue water. Samples of homogenised fresh raw material from herring were similarly hydrolysed using the same enzyme. Because of the natural enzyme activity in this material, incubation time was held to 3 h; i.e. after considerably less time than for the material prepared by boiling with water.

The enzyme-hydrolysed samples and the glue water were subjected to molecular gel filtration on a dextran resin (Sephadex G25 medium, Pharmacia Fine Chemicals, Sweden). To ensure sufficient amounts of material in the fractions which were to be further analysed, 300 ml of solution holding 15 g of dry material was used in each fractionation experiment. In all the elutions a column of the type K 100/100 (Pharmacia Fine Chemicals Sweden) was used. As elution agent a weak aqueous ammonia solution with a pH value of about 9 was employed.

The absorption of the eluate at 254 nm was registered using a Uvicord spectrometer (LKB, Sweden). The various fractions were divided into a high molecular weight fraction, the protein fraction—(fraction 1), and intermediate molecular fraction, the peptide fraction—(fraction 2), and a low molecular fraction which mainly consisted of amino acids and salts—(fraction 3), see Figure 1.

The fractions were evaporated to a suitable volume and the lipids were extracted with chloroform–methanol (2:1) for about 2 h at room temperature under constant stirring. More water was added and the chloroform phase was separated off. For some of the fractions, the oil yield was small and it was difficult to continue the analysis without taking extra precautions. In such cases 1 ml of olive oil was added as a carrier. A previous analysis had shown that the olive oil contained no selenium. The mixture of olive oil and oil extracted from the gel-filtration fractions was then treated in the same way as the lipids extracted from the other fractions.

The extraction using hexane and thereafter hexane–isopropanol was applied to both raw material and to material where a part of the oil had been isolated by boiling with water. The conditions for these experiments are given in Table 2. Cod liver and herring were used in these experiments. For the extraction process, the ratio between the extracting medium and the raw material was 2 to 1. This mixture was shaken for about

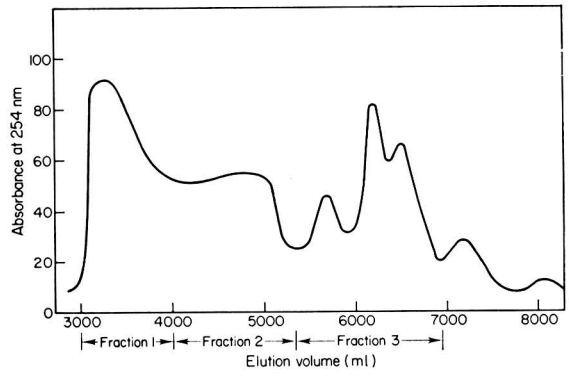

Figure 1. The u.v. absorbancy at 254 nm of the eluate from gel filtrated (Sephadex G25 medium) enzyme-hydrolysed cod liver residue. The protein, the peptide and the amino-acid fractions are indicated.

2 h and the hexane phase was separated off and the hexane evaporated. Prior to the determination of selenium the oils were washed twice with distilled water.

Preliminary experiments were performed on raw material which had been stored from 2 to 10 days at 4 to 8 °C (see Table 3). From these samples the oil was isolated by boiling with water.

All oil samples produced were analysed by neutron activation. The lipid fractions were transferred to quartz ampoules, sealed and irradiated for 24 h together with selenium standards in a neutron flux of approximately 1×10^{13} n/cm^2 s. After a "cooling off" period of about two weeks, the irradiated samples were transferred to inactive glass vials and their activity determined on a multichannel γ-spectrometer with a 2×2 in NaI crystal. A detailed description of the procedure has been given elsewhere.[3] For samples with a low phosphorus content it is usually possible to carry out the registration of selenium after a two week period without any prior chemical treatment. Figure 2 shows the γ-spectra of neutron-irradiated oil extracted from the high molecular weight fraction from enzyme-hydrolysed cod liver residue; Figure 3 shows the selenium standard. The spectra were recorded about 2 weeks after irradiation.

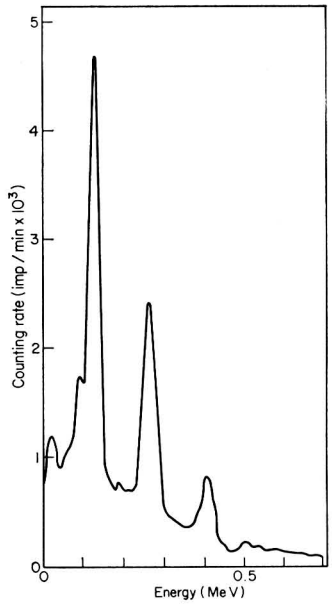

Figure 2. Gammaspectrum of neutron-activated oil extracted from the protein fraction of enzyme-hydrolysed cod liver residue.

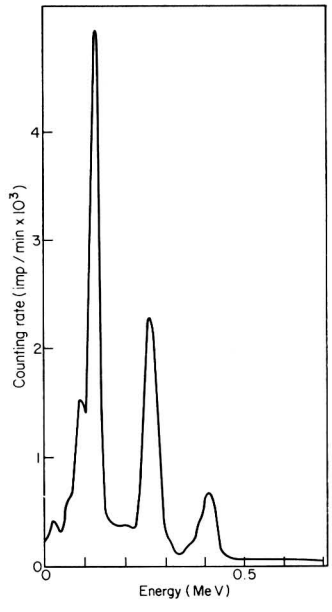

Figure 3. Gammaspectrum of the neutron-activated selenium standard.

TABLE 1. Yield and selenium content of oils extracted from fractions produced by molecular gel filtration of glue water and enzyme-hydrolysed presscake and fresh fish

Sample	Treatment	Fraction 1		Fraction 2		Fraction 3	
		Yield of oil (%)	Se	Yield of oil (%)	Se	Yield of oil (%)	Se
Mackerel	Factory prod. glue water (high quality)	0.8	15 parts/million	0.50	0.1 parts/million	0.3	3.0 parts/million
Herring	Factory prod. glue water (low quality)	5.4	0.41 parts/million	0.71	0.6 parts/million	0.2	0.1 parts/million
Cod liver	Laboratory prod. glue water	2.9	1.0 parts/million	0.48	3.0 parts/million	0.4	1.4 parts/million
Cod liver	Enzyme-treated presscake	0.2	33 parts/million	0.2	2.8 parts/million	1.1	1.2 parts/million
Cod fillet	Laboratory prod. glue water	—	0.025 μg	—	0.012 μg	—	0.04 μg
Cod fillet	Enzyme-treated presscake	—	0.040 μg	—	0.013 μg	—	0.03 μg
Herring	Laboratory prod. glue water	—	0.07 μg	—	0.008 μg	—	0.03 μg
Herring	Enzyme-treated presscake	—	0.11 μg	—	0.030 μg	—	0.02 μg
Herring	Enzyme-treated fresh fish	—	0.06 μg	—	0.07 μg	—	0.04 μg

TABLE 2. The selenium content (parts/million) in oils produced by successive water treatment, hexane and hexane–isopropanol extractions of cod liver and herring

Sample	Conditions	Water treatment		Hexane extraction		Hexane/isopropanol extraction	
		Yield of oil (g)	Se (parts/million)	Yield of oil (g)	Se (parts/million)	Yield of oil (g)	Se (parts/million)
Cod liver[a]	−20 °C	69	0.15	9	0.22	1.0	0.75
Cod liver	60 min 100 °C	74	0.22	7	0.20	1.4	0.65
Cod liver	4 h 100 °C	77	0.24	5	0.24	1.4	0.50
Herring[b]	+20 °C	—	—	15	0.065	40	0.16
Herring	4 h 60 °C	27	0.05	25	0.05	6	0.43
Herring	20 min 100 °C	1.0	0.06	45	0.09	13	0.21
Herring	1 h 100 °C	1.3	0.13	44	0.10	13	0.21
Herring	4 h 100 °C	17	0.075	27	0.09	14	0.064

[a] The cod liver contained ~40% oil.
[b] The herring contained ~5% oil.

3. Results and comments

Results presented in Table 1 indicate that lipid-soluble selenium is enriched in the high molecular weight fraction (fraction 1 in Figure 1), (m.w. 5000 or more) and hence is part of a lipoprotein complex. In particular this is evident in the samples of mackerel and cod liver glue water, of hydrolysed cod liver residue and in some of the samples of herring. Unfortunately, the amount of lipids in some of the samples prepared in the laboratory was too low to allow the yield to be determined. The selenium contents of the other samples do not give such definite results, although selenium is present in the oil isolated from the high molecular weight fraction in all samples.

The results of Tables 2 and 3 show that a certain amount of the selenium compound is present also in oil samples extracted with hexane or by treatment with water at 60 °C. This indicates that at least some of the selenium compound is either bound relatively weakly or exists free in the lipid phase. There is also the possibility that different selenium compounds are present and that more than one type of association, possibly to different proteins, may exist.

TABLE 3. The selenium content (parts/million) in marine oils from raw materials of different age

Sample	Storage time (days)	Se (parts/million)	Storage time (days)	Se (parts/million)	Storage time (days)	Se (parts/million)
Capelin	2	0.05	5	0.08	7	0.11
Herring	3	0.11	6	0.17	8	0.21
Mackerel	2	0.09	5	0.22	7	0.28

The results presented in Table 2 indicate that as the storage time increases and the raw fish samples gradually deteriorate, more of the selenium will follow the oil when this is isolated by boiling with water. The phospholipids behave in the same way. The content of phospholipids was measured by registration of the phosphorus isotope, ^{32}P, produced by the neutron activation.

The extraction experiments show that selenium is enriched in oil extracted by a mixture of hexane and isopropanol. The latter mixture is capable of splitting off the lipids in the lipoproteins. This effect may be observed particularly for samples where the majority of the neutral lipids were first removed by water treatment at temperatures of less than 100 °C. The results indicate that a certain amount of the selenium compound is bound in the lipid phase and that it is liberated in a way similar to the phospholipids.

For herring in particular, there seems to be an increase of the selenium content in the oil with boiling time up to about 1 h, then a decrease is observed (4 h). It may be that the heat treatment results in the transformation of the selenium compound into new substances having different solubility characteristics, or that the selenium compound itself could react with other components.

The evidence is that the lipid-soluble selenium compound occurs in the high molecular fractions produced from the different solutions as part of a lipoprotein complex. This indicates that the compound can probably be localised to the cell membrane and should be of interest in connection with the theory which proposes that selenium has a function related to the protection of the cell membrane.[4]

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The Basis of Meat Texture

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The tenderness of meat is probably the most important single factor in the consumer's assessment of its quality. This review describes the fundamental factors believed to be involved in determining the tenderness of meat. The contribution due to the pre-rigor contraction of the myofibrils and the possible control of this phenomena is discussed. The role of the connective tissue proteins is reviewed, particularly the effect of the age-related changes in the properties of collagen.

1. Introduction

Although man has appreciated the quality of animal muscle as meat from the very earliest times, an objective definition of what is meant by quality is still a major problem of the present meat industry. Hammond[1] defined it as "meat for which the consumer is consistently prepared to pay the most". Certainly the most important person assessing the quality of the meat we produce is the ultimate consumer. His assessment will be subjective and confined to tenderness, flavour and colour. It is conceivable that in the future additives may be developed to flavour and colour meat, but unfortunately, no matter how attractive the meat looks or tastes, if it is very tough it is objectionable to the consumer. Tenderness is, therefore, probably the most important single factor in the assessment of the quality of meat. The texture of meat has been studied for very many years and the subjective taste panel assessments have been correlated with objective methods depending on the measurement of the shear force required to rupture the meat fibres.[2] However, it is only in the last decade that any comprehensive theories at the molecular level have emerged to explain the textural changes observed at the consumer level.

2. Structure of meat

The animal body consists of some 300 muscles which in general terms may be considered to be similar in composition. However, we all know that certain cuts of meat such as the shin are tough whilst others, such as the fillet steak, are nearly always the most tender. It is generally agreed that the best quality meat, that is meat suitable for grilling or roasting, is obtained from the proximal parts of the hind limbs and the dorsal area posterior to the fifth rib. The "toughness rating" of meat can be determined

subjectively using a taste panel or objectively employing various mechanical devices. Basically these two techniques agree. At the Meat Research Institute the texture is measured on an Instron machine,[3,4] using the Warner–Bratzler or the Volodkovitch technique and Figure 1 shows the trace of a typical experimental sample. The height of the first peak (A) gives the resistance resulting from the muscle fibres and the last peak (B) is due to the connective tissue fibres. The object of this paper is to discuss the basis of the contribution of both these components to the toughness of meat.

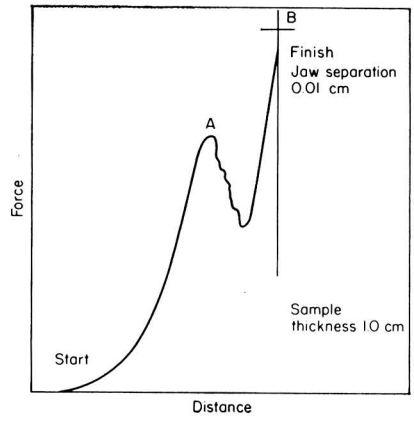

Figure 1. Typical force–extension curve of cooked muscle using the Volodkovitch jaws on the Instron. A represents the resistance due to the muscle fibres and B that due to the connective tissue fibres. Courtesy of B. B. Chrystall and D. N. Rhodes.

2.1. Structure of muscle fibres

First I shall describe the molecular structure of muscle and then attempt to discuss the part played by these elements in the texture of meat and their possible modification.

It must be remembered that muscle has to perform two functions, first, to support loads and, second, to contract or relax so as to perform movements. The structure of muscle has therefore evolved to carry out these functions as efficiently as possible and any study of the structure of meat is really a study of the structure of muscle—albeit in the *post-mortem* state. The structure of a typical muscle is best demonstrated by analytically breaking down the entire structure into its ultimate components to reveal the structure at the molecular level.

Muscle contains about 75% water, only about 10 to 15% of which is directly bound to the proteins and the rest is termed "free water". This free water is however immobilised in the muscle by the physical structure of the proteins, that is it is not released on squeezing the meat. If this configuration is altered the amount of water immobilised is changed and in certain circumstances it can change so much that large amounts of water are released from the meat on squeezing, as in the phenomena known as pale–soft–exudative (p.s.e.) pork.[5,6] The solid material of the muscle is made up of about 70% protein, about 10% fat, 3% carbohydrates and 5% salts.[7] The complex mixture of proteins present in muscle consists predominantly of fibrous proteins and it is these

proteins that give it its structure. Furthermore, it is the nature of these proteins and their complex arrangement in muscle that determine the tenderness of muscle as meat.

The muscle contracts as a whole and on sectioning the muscle to reveal the contractile elements involved it can be seen to be made up of bundles of fibres surrounded and separated from each other by a white connective tissue sheath (Figure 2). The structure of this latter element will be considered in the second half of the paper.

Examination of the large muscle fibre bundles shows them to be made up of finer muscle fibres, again surrounded by a very fine connective tissue sheath (Figure 2). These fibres run more or less parallel to the long axis of the muscle, but in some muscles such as the back muscle the fibres run at an angle to the long axis of the muscle. The fibres may vary from a few mm in length up to several cm, e.g. fibres 34 cm long have been observed in adult human sartorius muscle, but they may have actually extended the full length of the muscle (52 cm). The diameter in an adult averages about 60 μm. Thus, depending on the particular function of the muscle, the shape and internal arrangements of the fibres are many and varied.

These fibres are seen to be made up of finer fibrillar structures of about 1 μm diameter, the myofibrils, which function as the contractile units within the muscle. On examination at a higher magnification using the electron microscope the myofibrils are seen to be bound together by the sarcoplasmic reticulum. This membrane is involved in the transfer of the nervous impulse from the surface of the fibres to its constituent contractile units.[8,9] The transverse elements of this reticulum are continuous with the sarcolemma surrounding each fibre. In each muscle fibre of 60 μm diameter there are about 2000 myofibrils of 1.0 μm diameter. The structure of the myofibrils can be seen to consist of parallel aligned myofilaments, these being composed of the macromolecules of myosin and actin. There is a transverse periodicity of the fibres and the repeat period segments, termed sarcomeres, are separated by a structure referred to as the Z-disc. Within each sarcomere there is an array of thick myosin filaments and thin filaments of actin. The actin filaments are attached to the Z-discs and the myosin filaments occupy the centre portion of the sarcomere [Figure 2(c)]. These thick and thin filaments interdigitate with each other in such a way that each myosin filament is surrounded by six thin actin filaments—further, each thin filament is surrounded by three thick filaments. This arrangement leads to a variation in the density of the filaments which must obviously change as the muscle contracts and the protein filaments interdigitate. The sarcomere length varies by about 10 to 15% during contraction. This movement of the two sets of filaments in relation to each other during contraction and relaxation is now referred to as the "sliding filament" hypothesis, proposed by Huxley and Hanson some 20 years ago (for recent review see ref. 10).

3. Relationship to the tenderness of meat

The structure of meat has been discussed at the molecular level in some detail as a knowledge of these structural elements is essential for any adequate explanation of textural variation between different muscles of the same animal and between animals and breeds. An excellent example of the use to which a fundamental understanding can be put is the explanation of the changes that can occur due to the way the meat is handled *post-mortem*.

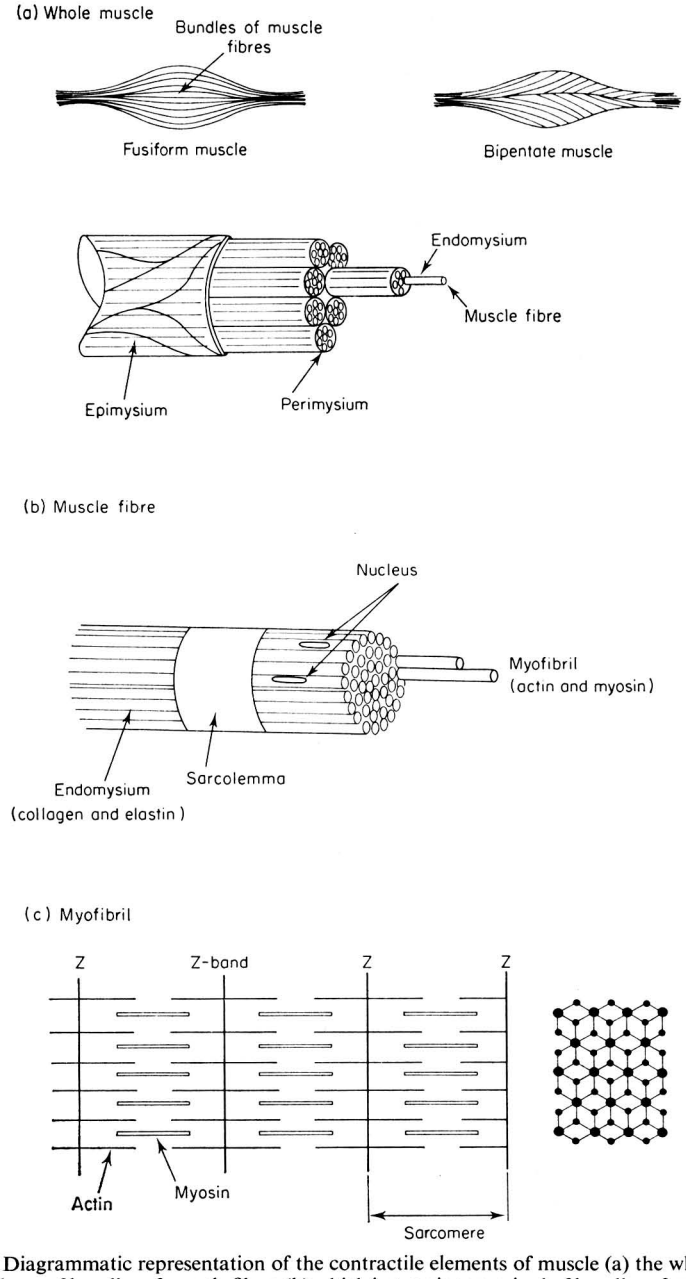

Figure 2. Diagrammatic representation of the contractile elements of muscle (a) the whole muscle, which is made up of bundles of muscle fibres (b) which in turn is comprised of bundles of myofibrils (c). The myofibrils are made up of the two fibrous proteins, actin and myosin, which interdigitate during contraction.

Before the actin and myosin filaments are fixed in position during rigor mortis these elements can slide passively past each other. Some ten years ago during a detailed structural analysis of post-rigor muscle fibres Locker[11] noted considerable variation in the sarcomere length of different muscles and demonstrated a direct correlation between the tenderness of the meat and the sarcomere length of the different muscles (Figure 3). Locker attributed these variations to the strains induced in the muscles when the carcasses were hung vertically by the achilles tendon during the process of rigor. Further work by Marsh and Leet[12] showed that the relationship was complex, up to 20% contraction had little effect, maximum toughness was obtained at 40%

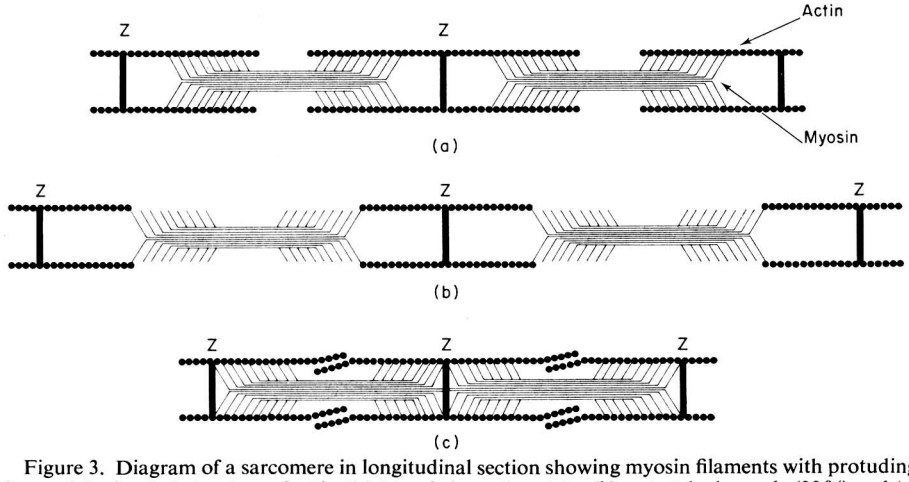

Figure 3. Diagram of a sarcomere in longitudinal section showing myosin filaments with protuding feet and the beaded structure of actin. (a) Muscle in resting state, (b) a stretched muscle (30%) and (c) contracted muscle (40%).

contraction, above 40% the meat became tender again and at 60% contraction the tenderness is equivalent to 20% contraction. Locker suggested that hanging the carcass differently would lead to the stretching of a greater proportion of the muscles or the more important muscles, e.g. the *longissimus dorsi*. A number of workers have followed this up and demonstrated that the horizontal position[13] or hanging by the aitch-bone[14] result in a higher proportion of tender meat (Figure 4).

Locker and Hagyard[15] later showed that temperature after slaughter was important. It is now accepted that the minimum contraction occurs at 15 °C, at temperatures less than 10 °C extensive contraction occurred, above 25 °C the contraction due to rigor was less rapid. The contraction occurring at temperatures less than 10 °C is now generally referred to as "cold-shortening". The New Zealand producers, in the interests of speed and efficiency attempted to accelerate their processing plants by blast freezing the meat pre-rigor. Unfortunately the process led to extremely tough meat. The scientists at the New Zealand Meat Research Institute were therefore able to explain that this process induced rapid contraction of the muscle fibres thus causing the meat to appear tough on cooking. On the basis of their knowledge of the fundamental structure of muscle they were able to instruct the producers as to the most efficient cooling procedures to be followed during rigor.[16]

Precisely why the muscle contracts under the influence of low temperature is not known, though it is probably triggered by the release of Ca^{2+} ions from the sarcoplasmic reticulum; nor is it really clear why shortened muscle is tougher. Possibly the altered structure permits more disulphide bonds to form, or to reform more stable ones, during cooking of the contracted muscle. The fact that the muscle has shortened does not in itself result in tough meat since muscle cooked pre-rigor contracts but has been reported to be extremely tender.[17] A better understanding of the biochemical changes during rigor resulting in the contraction of the myofibril will almost certainly eliminate this problem.

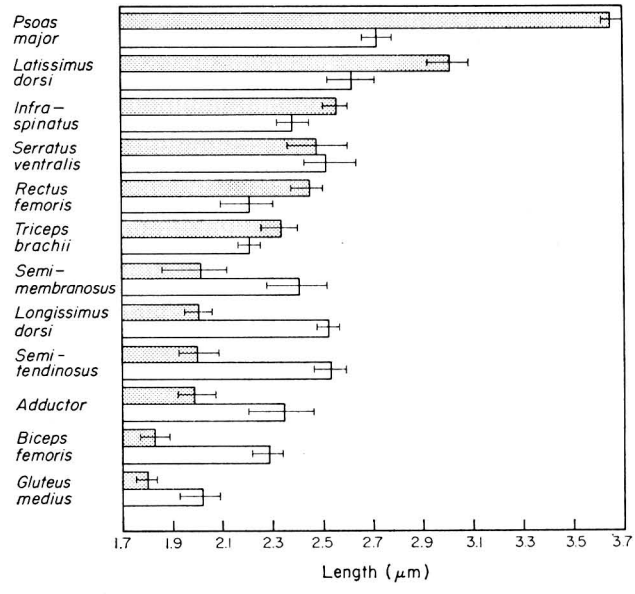

Figure 4. Comparison of the ultimate sarcomere length of different muscles from carcasses placed in the vertical and horizontal positions pre-rigor. Note decreased variation when rigor allowed to proceed in the horizontal position. ▒,vertical; □ horizontal. (Courtesy of Herring, Cassens and Briskey[13] and the Institute of Food Technologists.)

In post-rigor meat increased tenderness can be achieved by conditioning at chill temperatures for a certain time.[18] Again analysis at the molecular level has demonstrated that fragmentation of the myofibrils occurs primarily at the Z-discs.[19,20] Little is at present known about the structural proteins of the Z-discs but an extension of these fundamental studies could possibly lead to a resolution of the problem.

4. Structure of the connective tissue fibres

The myofibrils just described are surrounded by the endomysium, the muscle fibres by the perimysium and the whole muscle by the thick epimysium (Figure 2).[21] These connective tissue sheaths are comprised mainly of collagen[22] and to a lesser extent of another fibrous protein, elastin.[23] They form a supporting network throughout the whole muscle and come together at the ends of the muscle to form thick tendons.

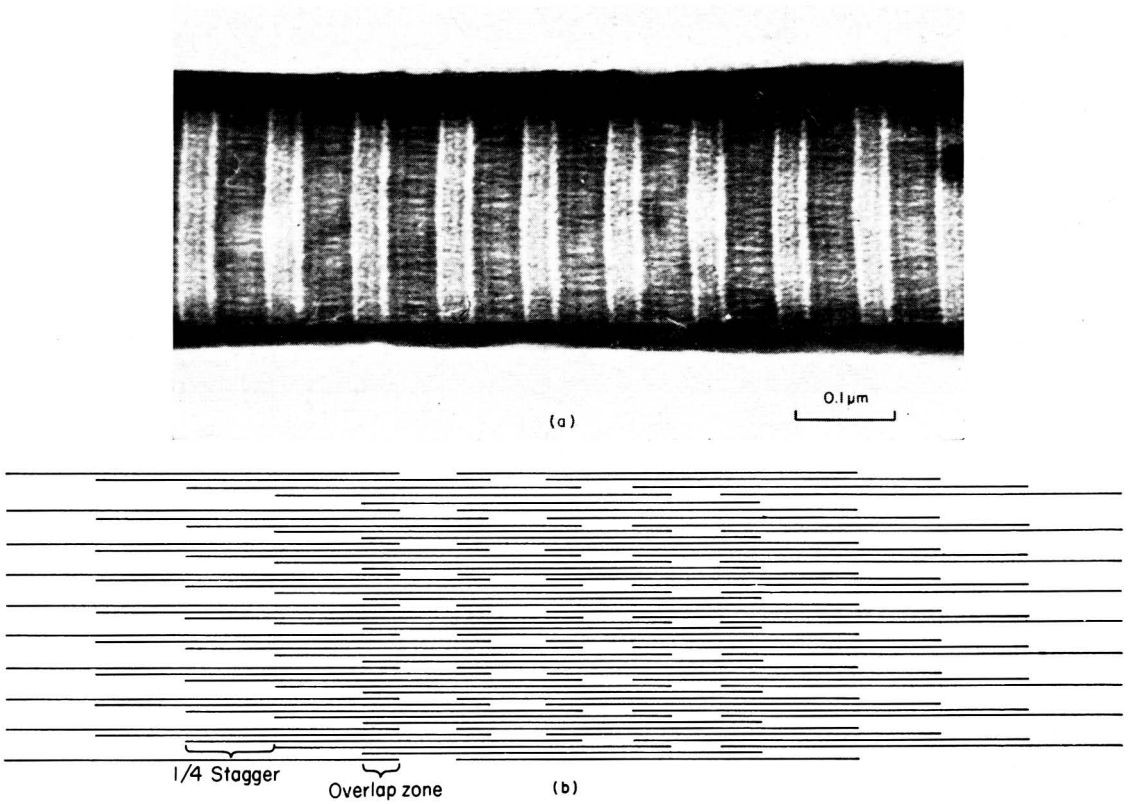

Figure 5. (a) Electron micrograph of native collagen fibrils negatively stained with phosphotungstic acid. (b) Schematic representation of the alignment of the tropocollagen molecules in the native fibril. Length of molecule 280 nm, axial periodicity of native fibril 68 nm.

Normal muscular contraction occurs by interdigitation of the actin–myosin elements and movement is achieved by transmission of the contractile force through the tendons to the skeleton. Since this must be achieved with minimum loss of energy the tendon fibres must be virtually inextensible and it is the fibrous protein collagen that provides this property. Since this strong inextensible fibre comprises a high proportion of the connective tissue sheaths around all muscles it is not surprising that it has been implicated in the toughness of meat.

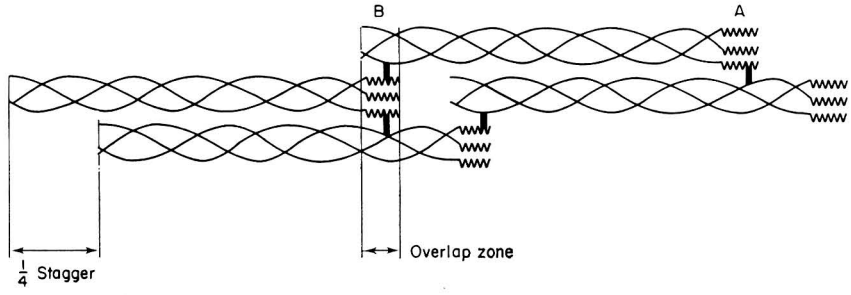

Figure 6. Possible location of the intermolecular crosslinks between the tropocollagen molecules aligned in the quarter stagger fashion in the fibre.

In complete contrast to collagen, elastin as its name implies, is a highly extensible fibrous protein. It has a completely amorphous structure, the protein chains are randomly coiled, kinetically free for most of their length and crosslinked at intervals by thermally stable bonds[2,3] to give it its rubber-like elasticity. Although a tough element, muscle contains only a very small proportion of elastin and most of this is confined to the capillaries and it is therefore unlikely that elastin makes a significant contribution to the toughness of meat.

4.1. Structure of collagen fibres

As we analysed the structure of the muscle we will now examine the structure of the collagen fibre down to the molecular level.

High resolution electron microscopy has again helped in the elucidation of the structure of the molecules. The fibres are seen to have a repeat pattern of 70 nm and on close examination it is possible to see the single molecules running axially along the fibre.[24] A small proportion of the collagen can be dissolved in dilute acetic acid and if reprecipitated with ATP forms single segments 280 nm in length. If the molecule is actually this long and the repeat pattern in the native fibre is 70 nm then the molecules in the fibre must be aligned in a quarter-stagger fashion, rather like building bricks (Figure 5). This arrangement of the molecules in the fibre is now generally accepted so we can now progress to the structure of the tropocollagen molecule. X-ray analysis has revealed that each of the long rod-like molecules, 280×1.5 nm, is made up of three peptide chains. Each of the three chains is in a helical structure—not the α-helix of most proteins but a unique polyproline helix. The three chains in this form are then wound round each other to produce an extremely compact and strong molecule.[22]

From the knowledge acquired about the basic structure of collagen at the molecular level it becomes obvious that despite the fact that the molecule is strong, in the native fibre the molecules would be capable of sliding past each other unless some means were provided to prevent it. This is achieved by a unique mechanism in which crosslinks are introduced between the molecules and so prevent slippage (Figure 6).

The importance of these crosslinks can be demonstrated very dramatically when their formation is inhibited. During the famines in India the populace ate sweet pea leaves. Unfortunately this contains a chemical capable of inhibiting the formation of crosslinks and many people suffered from weak joints and spines, particularly among the rapidly growing young. The active factor was later isolated and found to be β-aminopropionitrile

$$\begin{array}{l} CH_2—CH_2—CN \\ \quad | \\ \quad NH_2 \end{array}$$

Injection of this pure compound into fertile hen's eggs renders the chick completely lathyritic—although perfectly formed all its tissues are extremely fragile, in fact the head separates from the body with very little effort.[25] Analysis of the chick collagen reveals that the only defect is the absence of crosslinks between the collagen molecules.[26] Our own studies over the past few years have been concerned in the biosynthesis of these important crosslinks. We have attempted to isolate the crosslinks and characterise their structure.

The studies of Bornstein and Piez[27] demonstrated that only one amino acid in the short non-helical region at the N-terminal end of the molecule was involved in the formation of the crosslinks, but the recent studies of Kuhn and his colleagues[28] indicate a similar region at the C-terminal end of the molecules although its involvement in the crosslinking process has not yet been demonstrated. The first step in the biosynthesis of the crosslink is the oxidation of the specific lysine residues by an amino oxidase present in the growing tissues. Studies on the characteristics of the enzyme have demonstrated that copper is an essential co-factor.

The subsequent formation of the stabilising crosslinks involves the interactions of aldehyde groups to form covalent bonds between the tropocollagen molecules. Some of these bonds are labile aldimine bonds (Schiff bases), which are easily ruptured by pH changes, heat or denaturing agents, and others are relatively more stable aldimine crosslinks (Figure 7).[29] Furthermore, the nature of the bonds changes with age, the aldimine bonds being replaced by thermally stable bonds but the structure of these bonds has not yet been elucidated.[30]

4.2. Relationship of connective tissue fibres to meat tenderness

We have already seen how a knowledge of the structures of the contractile elements of meat has led to an understanding and at least partial correction of the deleterious effect of excessive shortening of the muscle fibres during *post-mortem* handling. It is now therefore of some interest to see if we can apply our basic knowledge of the chemistry of collagen to the tenderness of meat.

Analyses have shown that collagen is a strong inextensible fibre whose properties vary considerably with age of the animal; the collagen becomes more thermally stable

and much less soluble. We have now explained this on the basis that the Schiff bases initially formed are unstable to heat and the collagen fibres therefore fall apart. With increasing age these crosslinks are stabilised in an as yet unknown manner so that on thermal denaturation the fibres do not fall apart but remain as integral fibres although now in an elastic state. Although they are soft and extensible compared to native

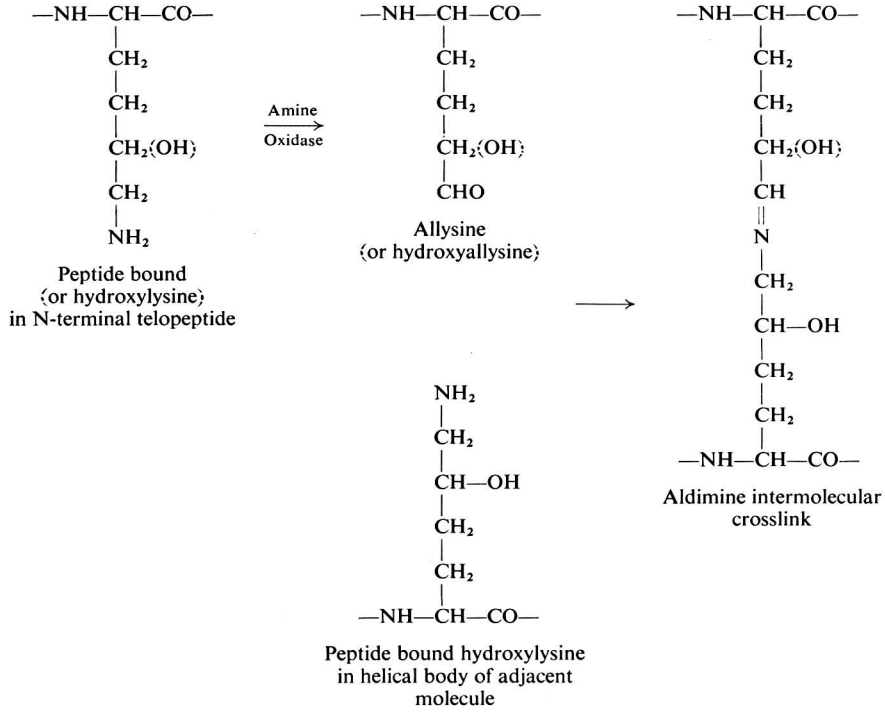

Figure 7. Biosynthesis of the aldimine crosslinks from peptide bound lysine residue. Oxidative deamination of hydroxylysine is also indicated by the OH in parenthesis.

collagen they are still tough or better "chewy", i.e. they might be considered to be not unlike chewing an elastic band and can therefore still make a contribution to the toughness of meat. This change in "quality" of the collagen is readily seen on cooking veal; the collagen is denatured and exuded from the meat and on cooling sets to a gelatin gel, whilst the same muscle from a 14 to 18 month old animal, although containing the same amount of collagen, exudes little if any gelatin. It is also evident on eating, or analysis by shear values, that the meat is tougher to eat. Similar studies on much older animals shows the meat to be even tougher so that there is a direct relationship between toughness and age of the animal.[31,32]

Only collagen could be responsible for these age changes—the contractile elements actin and myosin cannot account for these changes since their metabolic turnover time is about 12 days, and therefore even in an old animal the actin and myosin is not "old".

In contrast collagen has a very long turnover time, thus giving time for the crosslinks to accumulate and then stabilise themselves. These changes certainly provide a reasonable explanation of the variation in tenderness of meat with age.

It has always been a reasonable first hypothesis to suggest a relationship between the content of the tough connective tissue fibres and the toughness of meat and at a first glance this appears to be true viz. *psoas major* contains about 1% collagen, the *biceps femoris* 4% and the *sternomandibularis* 10% and these muscles as well as being in order of increasing collagen content are also in order of increasing toughness. However, on closer, more accurate analysis the relationship falls down. It is now obvious that determination of the total collagen content is meaningless—it is the quality of the collagen that matters, i.e. the proportion of thermally stable bonds to labile Schiff bases.

In this connection it is also worth including the possibility of an animal putting on this young "labile" collagen later in life, e.g. Irish store cattle may be fattened up at the right age and it is possible that new collagen could be laid down—this new collagen would be labile and thus permit a proportion of the collagen to fall apart on cooking. However, this has not yet been demonstrated to be the case in practice.

The effect of age changes in the chemistry of the crosslinks on the tensile strength of the native fibre cannot be assessed as readily although increases in strength have been reported.[22] This is important for prime beef which is generally destined for "rare" steaks in which the collagen remains in the native state. In these cases the total amount of collagen must become a more important factor than in the case of cooked meat.

Unfortunately in any analysis of texture, whether by taste panel or by instrument, it is impossible to eliminate the effect due to sarcomere length, i.e. contraction and stretching during *post-mortem* hanging. In certain extreme cases, e.g. in cold-shortening as described earlier, this effect can override the effect of the collagen. The true contribution due to the connective tissue is consequently difficult to assess.

Clearly one can avoid the effect of aged collagen by using animals between 12 and 18 months old. Indeed, over the past decade a much higher proportion of cattle are slaughtered at this age than the old traditional age of $2\frac{1}{2}$ to 3 years. However, a high proportion (55%) of cattle used for beef are from dairy herds and of these animals a significant proportion (40%) tend to be older cows and the meat is consequently tougher. Furthermore, not all cuts of meat from beef or dairy animals are ideal and many of the tougher cuts have to be used in manufactured meat products.

Based on a sound knowledge of the structure of the connective tissue fibres it may be possible to devise a method of treating the meat in such a way as to render it more acceptable from a textural point of view. The most obvious way to achieve this is by the use of enzymes. The use of papain is well known.[33, 34] Injection is carried out 1 to 30 min prior to slaughter in order to distribute the enzyme around the animal body. However, there are serious disadvantages to the technique, the enzyme appears to accumulate in certain tissues, e.g. the liver and tongue, which then disintegrate on cooking. For the technique to be truly effective it will be necessary to tenderise the muscle of the older animals and the tougher cuts, but to date the technique has only been carried out with prime beef cattle. The major disadvantage of this technique is that the enzymes only act on the proteins on cooking, i.e. after thermal denaturation.

A better alternative would be to stimulate the process of conditioning, i.e. storage of meat at chill temperatures, which is a well known technique for tenderising meat. If we knew the processes involved, possibly the catheptic enzymes released from the lysosomes, we might be able to accelerate the process. An ideal solution would be to have an enzyme capable of specifically attacking native collagen in raw meat rather than all the proteins denatured during the cooking process. In this way the control of the enzymically produced texture changes would be in the hands of the producer rather than the consumer and could therefore be kept under more strict control. Collagen under these conditions would still be in the native state and would not be attacked by enzymes except in the non-helical telopeptide region. Fortunately, these regions contain the crosslinks and the enzymes would therefore effectively reduce the strength of the fibres and this would be more evident on thermal denaturation during the cooking process.

Clearly, collagen is not something one does not want in meat; if no crosslinks were present, as in the lathyritic animals, the meat would fall apart on cooking and it would only be suitable as soup. Thus collagen with the optimum number of crosslinks is essential to give meat an acceptable texture; too few crosslinks and it disintegrates and too many crosslinks makes the meat tough.

At the present time chemical control of the crosslinks is not possible; we need to know a lot more about their chemistry and their location, in particular the mechanism by which the labile Schiff base crosslinks are stabilised during the ageing process. Certainly, if we could keep the collagen in its "young" state, i.e. with a predominance of Schiff base crosslinks, these bonds would rupture on heating and the meat would be tender. Further knowledge of the telopeptide regions and the effect of enzymes on collagen will also be helpful in future attempts to overcome the problem of toughness.

Extension of the fundamental knowledge of the myofibrillar proteins will almost certainly lead to a resolution of the problem of muscular contraction during rigor, possibly by ionic treatment and better handling procedures. This will then leave the problem of the "background toughness" due to the connective tissue. A full understanding of the biosynthesis, fibrogenesis and crosslinking of collagen and elastin at a fundamental level is essential. Although we are making some progress in the nature of the crosslinks there is still a great deal to be learnt on all aspects.

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Studies on Chicken Actomyosin

I. Effect of Storage on Muscle Enzymic and Physico-chemical Properties

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Experiments were conducted to study the effect of storing broiler chicken muscle at 25 °C on certain properties of actomyosin of the *pectoralis major*.

Storage for 3 h or more resulted in the Mg^{2+}-activated ATPase of actomyosin becoming less sensitive to inhibition by increasing concentrations of KCl and in an increased rate of superprecipitation of the complex. The analytical ultracentrifuge pattern of the actomyosin of stored muscle differed from that of fresh muscle. Some evidence was obtained for the observed changes being partly dependent upon the pH of the muscle used as the source of actomyosin.

1. Introduction

The skeletal muscles of a living animal are soft and freely extensible. Some hours after death, however, the muscles stiffen and become inextensible, i.e. rigor mortis occurs.[1] Rigor is resolved after a further period of time when the muscles soften. They do not, however, regain their extensibility. The exact mechanisms involved in the onset of rigor and its subsequent resolution have not been fully elucidated despite investigations in many laboratories.

In vivo, the principal myofibrillar proteins, myosin and actin, are present as interdigitating filaments which, in resting muscle, are prevented from interacting by the presence of a magnesium–adenosine triphosphate complex.[2] During muscular contraction this complex is dissociated and the actin filaments pass between the myosin filaments with the concomitant splitting of ATP to ADP. At the completion of contraction the magnesium–adenosine triphosphate complex is reformed, breaking the myosin–actin interaction, as a consequence of which the muscle relaxes.

Myosin and actin together with some minor myofibrillar components involved in muscle contraction and relaxation may be isolated as a complex, actomyosin. The most obvious of site protein changes accompanying the process of rigor mortis and its resolution is therefore the actomyosin complex. While the biological and physicochemical properties of actomyosin isolated from fresh (at-death) and *post-mortem* rabbit and bovine muscle have been widely investigated[3–7] there have been few intensive studies of the complex isolated from chicken muscle.

This study was therefore designed to investigate the adenosine triphosphatase

(ATPase) activity and physico-chemical properties of actomyosin from at-death and *post-mortem* chicken muscle. A preliminary report has been published.[8]

2. Materials and methods

2.1. Reagents

Tris(hydroxymethylaminomethane) was obtained from Boehringer Corporation (London) Ltd. Disodium ATP (Boehringer Corporation) was converted to the Tris form[9] and stored frozen as 50 mM solutions. Ethylene glycol bis(aminoethyl)tetra-acetic acid was purchased from Koch-Light Laboratories Ltd, Colnbrook, Bucks. All other reagents used were "Analar" grade. Two-times glass distilled water, prepared from deionised water, was used throughout these studies.

2.2. Experimental material

Broiler chickens (Cobb AH Cln) were killed by a blow to the head and bled at room temperature. The breast muscle (*pectoralis major*) was used as the source of experimental material. For the preparation of actomyosin from fresh muscle (fresh actomyosin) the muscle was removed from the unplucked bird immediately after death. When the carcasses were to be stored the birds were killed and dry-plucked but not eviscerated. The carcasses were stored at 25 °C. In this paper actomyosin isolated from the muscle of stored carcasses will be termed 2 h actomyosin, 4 h actomyosin etc., the numbers denoting the period of storage at 25 °C.

One pectoral muscle was taken as the source of fresh material and the other as that of stored material. In no instance was a single muscle used as the source of more than one preparation of actomyosin.

2.3. Preparation of actomyosin

All preparative procedures were carried out at 1 °C. Minced muscle (50 g) was suspended in 3 vol. of Weber–Edsall solution. After 20 h stirring the suspension was centrifuged (23 000 **g** for 1 h) and the supernatant diluted with 2 vol. of 0.2 mM-$NaHCO_3$. The resulting precipitate was collected by centrifugation (23 000 **g** for 20 min) and dissolved by the addition of an equal vol. of 2.4 M-KCl followed by 2 vol. of 0.2 mM-$NaHCO_3$. The purification step of precipitation and dissolution was repeated two more times. The protein solution was finally clarified by centrifugation (40 000 **g** for 1 h).

For use in enzymic and superprecipitation studies, actomyosin was suspended in 50 mM-KCl.

2.4. Assay of ATPase activity

The method of assay used was based on that of Perry[10] and the details are given in the legends to the Figures. ATPase activity was expressed as μg of phosphate liberated by 1 mg of protein in 5 min at 25 °C.

2.5. Superprecipitation

Superprecipitation was measured at 20 to 30 °C by the method of Ebashi[11] using the Unicam SP 500 spectrophotometer at 660 nm. The compositions of the assay media are given in the Figure legends.

2.6. Measurement of muscle pH

1 g of muscle was homogenised with 10 vol. ice-cold 5 mM-sodium iodoacetate, pH 7.4, containing 0.15 M-KCl. The pH value of the homogenate was measured with a glass electrode at 0 °C.

2.7. Protein concentration

Protein concentrations were determined by the biuret method[12] standardised against dry bovine serum albumin (Sigma London Chemical Co., Ltd. London, S.W.6).

2.8. Analytical ultracentrifugation

The Spinco Model E analytical ultracentrifuge was used at 20 °C. A standard cell with a 30-mm centre piece was used.

The sedimentation coefficients, $s_{20,0.6M-KCl}$, were calculated from Schlieren diagrams using the method of Markham.[13] The bar angle was 55 °.

3. Results

3.1. Effect of storage on the ATPase of muscle actomyosin

The Ca^{2+}-stimulated ATPase activities of both fresh and 4 h actomyosins were found to increase with increasing KCl concentration. It will be seen from Figure 1, however, that the enzymic activity was lower in the 4 h actomyosin than in the fresh. The results

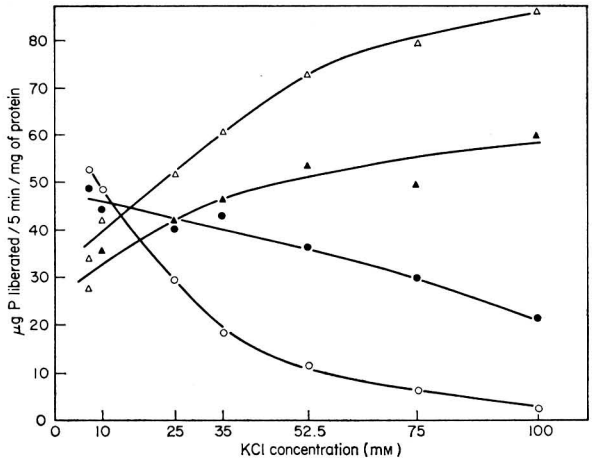

Figure 1. Effect of KCl on Ca^{2+}- and Mg^{2+}-stimulated ATPase of fresh and 4 h actomyosins. Assay media contained 25 mM-Tris–HCl, pH 7.6, 2.5 mM-Tris–ATP, 2.5 mM-$CaCl_2$ or $MgCl_2$, 0.62 mg of protein and concentrations of KCl indicated.

–△–△–, Fresh actomyosin Ca^{2+}-stimulated ATPase; –▲–▲–, 4 h actomyosins Ca^{2+}-stimulated ATPase; –○–○–, fresh actomyosin Mg^{2+}-stimulated ATPase; –●–●–, 4 h actomyosin Mg^{2+}-stimulated ATPase.

of parallel experiments relating KCl concentration to Mg^{2+}-stimulated ATPase activity are also shown in Figure 1. In contrast to the enhancing effect noted with the calcium-stimulated ATPase as the KCl concentration was increased, the Mg^{2+} stimulation was progressively inhibited as the salt concentration was increased from 7.5 mM to 100 mM, the effect being more pronounced in fresh actomyosin than in 4 h actomyosin. The Mg^{2+}-stimulated ATPase of actomyosin from muscle which had been stored for periods up to 1 h resembled fresh actomyosin in being readily inhibited by increasing concentrations of KCl, while actomyosin from muscle stored for periods of 3 to 24 h was less sensitive to the effects of salt concentration as may be deduced from the data presented in Table 1.

TABLE 1. Relationship between muscle pH and enzymic activity

Prep. no.	Storage time (h)	pH	Mg^{2+}-ATPase activity[a] 7.5 mM-KCl	75 mM-KCl	Activity at 75 mM-KCl expressed as % of activity at 7.5 mM-KCl
37	0	6.78	52.7	6.6	12.3
46	0	6.89	50.4	3.8	7.6
47	0	6.64	39.6	5.4	18.3
39	1.25	6.35	56.6	12.4	22.0
43	2.0	6.42	54	8.8	16.1
41	2.0	6.25	43.6	22.0	50.5
38	3.0	5.70	54.1	45.2	83.6
37	4.0	5.63	48.9	29.6	60.6
42	4.0	5.79	55.8	45.8	82.1
40	6.0	5.79	68.5	51.3	75.0
42	24	n.d	49.9	38.0	76.2
44	24	5.89	58.3	38.4	66.0

[a] Assay conditions: 25 mM-Tris–HCl, pH 7.6, 2.5 mM-Tris–ATP, 2.5 mM-$MgCl_2$, 7.5 or 75 mM-KCl. 0.8 to 1.0 mg of protein/ml. Final volume, 2 ml.

The results obtained using the Mg^{2+}-stimulated ATPase of actomyosin isolated from muscle which had been stored for 1 to 2 h were somewhat variable, some preparations showing the characteristics of the enzyme from fresh muscle, i.e. sensitive to increasing salt concentration, and others those of stored muscle, i.e. less sensitive. Accepting that the onset of rigor in broiler chicken muscle occurs approximately 2 h after death,[14] the observed variations may be related to the degree of rigor of the muscle.

In order to ascertain whether the marked sensitivity of Mg^{2+}-stimulated ATPase activity to increasing KCl concentration noted above, was due to the enzymic system not being fully active, the Mg^{2+}-stimulated ATPase activities of fresh and stored actomyosins were measured in the presence and absence of 0.1 mM-$CaCl_2$. At 10 to 35 mM-KCl, the enzymic activity of fresh actomyosin was 9 to 12% higher in the presence of $CaCl_2$ than in its absence, while in the presence of 75 or 100 mM -KCl there was no effect of added Ca^{2+} on the enzymic activity. The activity of stored actomyosin ATPase was unaltered by the addition of Ca^{2+} to the assay medium.

In the presence of 50 mM-KCl the ATPase activity of actomyosin from both fresh and stored chicken muscle was maximal when the concentration of added $MgCl_2$

equalled that of the ATP present in the assay medium (2.5 mM) and decreased as the Mg concentration was increased beyond that value. $MgCl_2$ has been shown to have a similar effect on the ATPase activity of rabbit actomyosin.[15, 16] The decrease in ATPase activity at 1 mM-Mg shown in the lower curve of Figure 2 was consistently noted when assaying fresh actomyosin and also isolated from muscle stored for short periods of time but never in actomyosin from muscle stored for 1 h or more. A further difference existed between fresh and stored actomyosins in their behaviour in the presence of variable Mg concentration in that while the enzymic activity of fresh actomyosin was only slightly higher (approximately 24% in the experiment illustrated in Figure 2) at the optimum Mg^{2+} concentration than in the absence of added cation the activity of 2 h or 4 h actomyosin was 100 to 200% higher.

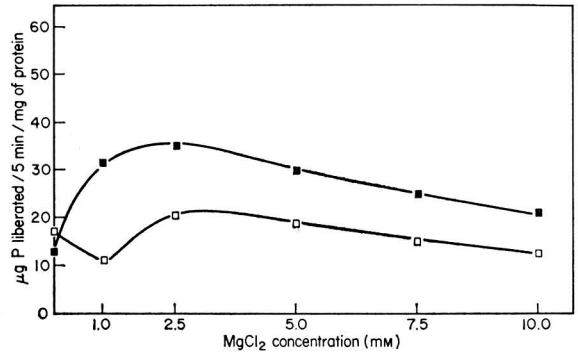

Figure 2. Effect of KCl of variable magnesium chloride concentration on actomyosin Mg^{2+}-stimulated ATPase activity. Assay media contained 25 mM-Tris–HCl, pH 7.6, 2.5 mM-Tris–ATP, 50 mM-KCl, 0.62 mg protein and indicated concentrations of $MgCl_2$.
–□–□–, Fresh actomyosin; –■–■–, 4 h actomyosin.

3.2. Changes in superprecipitation of actomyosin resulting from storage of muscle

Actomyosin from fresh and stored muscle superprecipitated very rapidly in the presence of 2.5 mM-$MgCl_2$ and 25 mM-KCl. As the KCl concentration in the test solution was raised from 25 mM to 75 mM the rate of superprecipitation of actomyosin from fresh muscle decreased. The "clear" phase (Spicer)[17] was noted in the presence of 50 and 75 mM-KCl. The actual time before the onset of superprecipitation varied slightly from preparation to preparation, as might be expected when different birds were used as the source of experimental material. By contrast actomyosin from muscle stored for 2 to 24 h did not exhibit a "clear" phase at 50 mM-KCl and only very occasionally was one noted at 75 mM-KCl. The superprecipitation of stored actomyosin was much more rapid than that of fresh actomyosin (Figure 3), as has recently been shown with rabbit actomyosin from muscle stored for 1 to 8 days at 25 °C.[18]

Actomyosin from muscles stored for 1 to 2 h varied in superprecipitation behaviour, some preparations exhibiting a "clearing" phase before the onset of superprecipitation, while with other preparations the clearing phase was not observed.

The superprecipitation of actomyosin isolated from fresh muscle and that which had

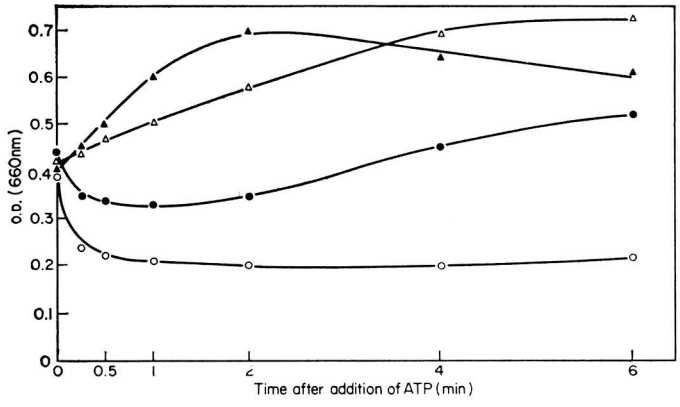

Figure 3. Effect of KCl on superprecipitation of fresh and 4 h actomyosins. Test solutions contained 25 mM-Tris–HCl, pH 7.6, 2.5 mM-Tris–ATP, 2.5 mM-$MgCl_2$, 0.78 to 0.85 mg protein/ml and 50 or 75 mM-KCl.

–●–●–, Fresh actomyosin, 50 mM-KCl; –○–○–, fresh actomyosin, 75 mM-KCl; –▲–▲–, 4 h actomyosin, 50 mM-KCl; –△–△–, 4 h actomyosin, 75 mM-KCl.

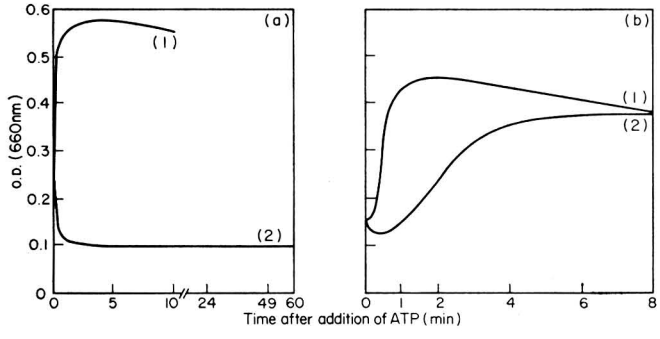

Figure 4. Effect of EGTA on superprecipitation of 3 h actomyosin complex before and after removal of proteins soluble at low ionic strength. Test solution contained 25 mM-Tris–HCl, pH 7.6, 2.5 mM-Tris–ATP, 2.5 mM-$MgCl_2$, 50 mM-KCl, 0.98 mg of protein/ml and 0.1 mM-EGTA where appropriate.

(a) Superprecipitation before treatment with 2 mM-Tris–HCl, pH 7.6. (b) Superprecipitation after treatment with 2 mM-Tris–HCl, pH 7.6. Actomyosin was dialysed against 2 mM-Tris–HCl, pH 7.6 and the insoluble protein resuspended in 50 mM-KCl. The superprecipitation of this protein was measured in the presence and absence of EGTA. (1) Superprecipitation in absence of EGTA. (2) Superprecipitation in presence of EGTA.

been stored at 25 °C for up to 24 h was strongly inhibited in the presence of the chelating agent ethylene glycol bis(aminoethyl)tetra-acetic acid (EGTA) Figure 4.

3.3. Effect on superprecipitation of the removal of proteins soluble at low ionic strength from the actomyosin complex

While polyacrylamide gel electrophoresis[19] performed in the presence of 0.1 % sodium dodecyl sulphate (SDS) and 0.1 % 2-mercaptoethanol revealed that fresh and stored actomyosins were qualitatively similar in composition (Figure 5), the possibility

existed that the increased rate of superprecipitation exhibited by stored actomyosin may have resulted from the alteration of a regulatory protein in the actomyosin complex. Proteins soluble at low ionic strength were therefore removed from the complex by exhaustive dialysis of actomyosin (in 50 mM-KCl) against 2 mM-Tris–HCl, pH 7.6, followed by centrifugation of the suspension at 55 000 **g** for 30 min. The superprecipitation behaviour of the insoluble protein was examined in the presence and absence of

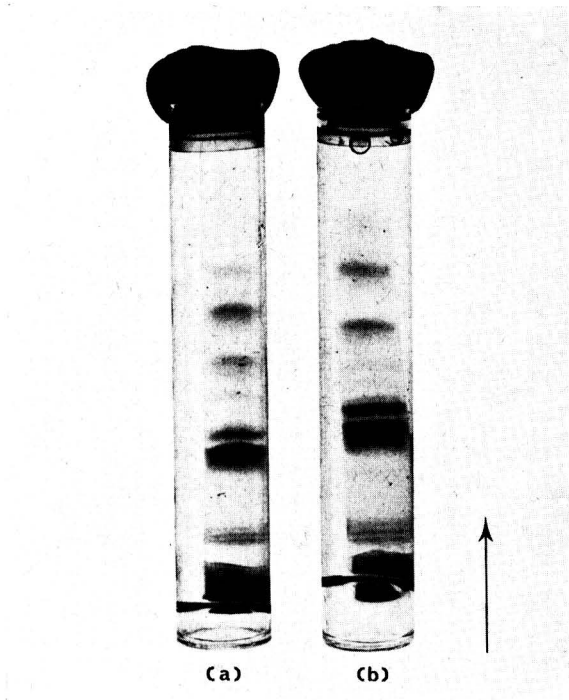

Figure 5. Polyacrylamide gel electrophoresis of fresh and 4 h actomyosins. The protein (5 to 6 mg/ml in 0.1 % SDS–0.1 % mercaptoethanol–10 mM phosphate, pH 7.0) was diluted with 20 % sucrose and 50 μl applied to the top of the gel. Electrophoresis was carried out for 4 h at 8 mA/gel.
(a) Fresh actomyosin; (b) 4 h actomyosin.

0.1 mM-EGTA. Comparison of Figure 4(a) and 4(b) shows that in the case of 3 h actomyosin, treatment with 2 mM-Tris–HCl, pH 7.6, removed some components of the EGTA-sensitising factor, but did not substantially modify the rate of superprecipitation in the absence of EGTA.

3.4. Analytical ultracentrifugation of actomyosin preparations

A feature of all the actomyosin preparations examined (protein concentration 2.9 to 4.5 mg/ml in 0.6 M-KCl, pH 6.0) was the presence of material which sedimented rapidly as the centrifuge was taken up to speed without giving a discrete peak. This material probably corresponded to the gel component observed by Johnson and Rowe[20] in their

study of rabbit actomyosin. Stored chicken actomyosin preparations contained more gel component than did those of fresh actomyosin and after 24 h storage the preparations consisted almost exclusively of this component. Sedimentation diagrams (Figure 6) revealed further differences between fresh and stored actomyosins. Fresh actomyosin contained two main components together with some slow-moving diffuse material. The faster of the main components had an uncorrected sedimentation coefficient at 20 °C of 36 to 38 *s*, while the slower component had a sedimentation coefficient of 32 to 36 *s*. 2 h actomyosin also contained two main components, the faster of which (sedimentation coefficient 47 to 53 *s*) was more clearly resolved than the corresponding component in fresh actomyosin. The sedimentation coefficient of the slower component was 42 to 44 *s*. The faster main component was either absent or much reduced in quantity in 4 h actomyosin, while the slower component (34.5 to 37 *s*) was apparently homogeneous.

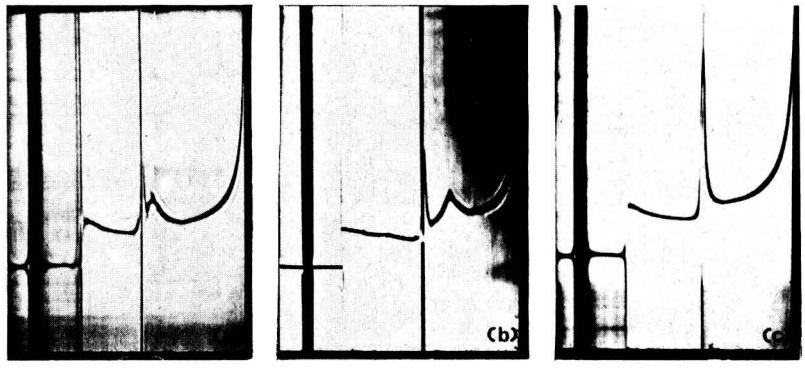

Figure 6. Analytical ultracentrifugation of fresh and stored actomyosins.
(a) Fresh actomyosin B. 3.1 mg/ml, 29 min; (b) 2 h actomyosin 3.5 mg/ml, 23 min; (c) 4 h actomyosin 3.3 mg/ml, 37 min.
Solvent: 0.6 M KCl, speed: 30 000 rev./min. Time in min is period after reaching full speed.

3.5. Modification of actomyosin

Since the initial pH value of the Weber–Edsall extract of fresh muscle was normally 7.8 to 8.0, while that of the extract of muscle stored for 4 h or more was 6.5 to 6.7, it was possible that differences in pH during the extraction may alone have been responsible for the observed differences in sedimentation behaviour and enzymic properties of fresh and stored actomyosin. In order to investigate this possibility, samples of fresh muscle extract were acidified to approximately pH 6.5 by the addition of 1 M-HCl at 0 °C and the pH of stored muscle extract raised to approximately 8.0 by the addition of 0.6 M-KCl–0.2 M-$NaHCO_3$ or 1 M-KOH immediately prior to the 20-h extraction period. Actomyosin was isolated from the modified extracts in the usual manner and the sedimentation behaviour and Mg^{2+}-stimulated ATPase activity compared with those of the corresponding complex isolated from unmodified extracts. Ultracentrifugal analysis revealed that the faster-moving component of fresh actomyosin was either absent or much reduced in quantity in the preparations isolated from modified extracts

while this component was more prominent in modified than in unmodified 4 h actomyosin. The sedimentation diagram of acid-modified 2 h actomyosin also differed from that of the unmodified complex (Figure 7). The main component present in the preparation of modified fresh actomyosin had a sedimentation coefficient of 29 to 35 *s* while sedimentation coefficients of 27 and 30 *s* were obtained for the main components of modified 4 h actomyosin.

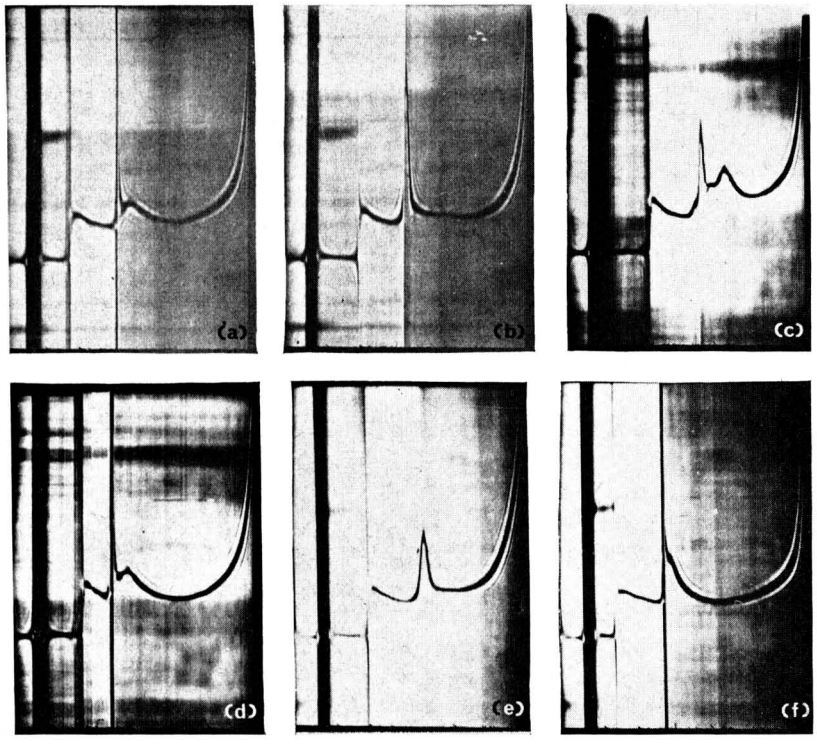

Figure 7. Modification of sedimentation pattern of actomyosin by pH adjustment of initial muscle extract. Immediately prior to the 20 h extraction period, the pH value of the Weber–Edsall solution containing the muscle mince was adjusted with acid or alkali. The sedimentation pattern of actomyosin isolated from the modified extract was compared with that of actomyosin isolated from unmodified extract.

(a) fresh actomyosin (3.5 mg/ml). Initial pH of extract: 8.0, 27 min; (b) actomyosin (3.3 mg/ml) isolated from modified extract (pH 6.42) of fresh muscle, 23 min; (c) 2 h actomyosin (3.7 mg/ml). Initial pH of extract: 7.42, 14 min; (d) actomyosin (3.5 mg/ml) isolated from modified extract (pH 6.5) of 2 h muscle, 16 min; (e) 4 h actomyosin (4.3 mg/ml). Initial pH of extract: 6.65, 20 min; (f) actomyosin (3.9 mg/ml) isolated from modified extract (pH 7.9) of 4 h muscle, 27 min.

Solvent: 0.6 M-KCl, pH 6.0, speed: 30 000 rev./min. Time in min is period after reaching full speed.

While acid or alkali treatment of the appropriate extract consistently altered the sedimentation patterns of the actomyosins isolated from the modified extracts, measurement of the Mg^{2+}-stimulated ATPase activities of the modified actomyosins indicated that the properties of the various enzymes could not be reversed by merely altering the

pH of the extract from which they were isolated. On the whole, acid treatment of fresh muscle extract resulted in the production of actomyosin preparations whose Mg^{2+}-stimulated ATPases were less sensitive to KCl concentration than untreated samples, while alkali-treatment of stored muscle extract gave actomyosins with varying properties, some preparations becoming more sensitive to salt than those from untreated extracts while others did not exhibit this phenomenon (Table 2).

TABLE 2. Modification of actomyosin by pH adjustment of Weber–Edsall extract

Prep. no.	Storage period (h)	pH value of extract Initial	Adjusted	Mg^{2+}-ATPase activity[a] 7.5 mM-KCl	75 mM-KCl	Activity at 75 mM-KCl expressed as % of activity 7.5 mM-KCl
46	0	8.0		56.0	4.2	8.0
			6.42[b]	57.0	7.0	12.0
48	0	7.4		44.0	6.0	14.0
			6.5[b]	44.8	14.7	33.0
45	4	6.65		50.6	28.8	57.0
			7.9[c]	46.8	12.1	25.0
48	4	6.72		65.0	47.0	72.0
			7.98[c]	58.0	45.0	76.0

[a] Assay conditions as for Table 1.
[b] pH adjusted by the addition of acid immediately prior to 20 h extraction period.
[c] pH adjusted by the addition of alkali immediately prior to 20 h extraction period.

4. Discussion

Fujimaki and coworkers[3,4] found that the Mg^{2+}- and Ca^{2+}-stimulated ATPase activities of actomyosin isolated from rabbit skeletal muscle which had been stored for 2 days *post-mortem* at 4 °C were 15 to 25% higher than the activities of actomyosin isolated from at-death muscle. Actomyosin isolated from bovine muscle stored for 12 to 24 h *post-mortem* at 2 or 16 °C also had higher Mg^{2+}- and Ca^{2+}-ATPase activities than at-death actomyosin.[5] The present work shows that at potassium chloride concentrations (35 to 40 mM) similar to those used in the earlier studies, actomyosin isolated from chicken skeletal muscle stored *post-mortem* for 4 h to 24 h at 25 °C has a higher Mg^{2+}-stimulated ATPase activity than actomyosin isolated from at-death (fresh) muscle. Chicken actomyosin isolated from *post-mortem* muscle differed, however, from the corresponding rabbit and bovine proteins in that its Ca^{2+}-stimulated ATPase activity was lower than at-death actomyosin. Actomyosin from *post-mortem* rabbit muscle has been shown to contain more actin, which is enzymically inactive, than at-death muscle[4] and thus the decreased Ca^{2+}-stimulated ATPase activity of stored chicken actomyosin may indicate the presence of more enzymically inactive protein in these preparations than in fresh actomyosin. In view of this, the observed higher Mg^{2+}-stimulated ATPase activity of actomyosin from stored chicken muscle is surprising and suggests that a modification of the myosin–actin interaction occurs during storage. Such a modification, the exact nature of which is unknown, may also be responsible for the rapid onset of superprecipitation observed with actomyosin from stored muscle.

The change in biological properties of actomyosin occurring during storage of chicken muscle may, on the other hand, be due to factors other than an alteration in the binding of myosin to actin, such as changes in the properties of one or more of the minor components of the myofibril. The rate of superprecipitation of stored actomyosin was not substantially decreased by the removal of proteins soluble at low ionic strength from the complex, suggesting that alterations in the properties of these proteins were not responsible for the observed differences between fresh and stored actomyosins.

Polyacrylamide gel electrophoresis of stored actomyosin in the presence of SDS showed that the complex was identical with fresh actomyosin, thus supporting the conclusion of Penny[21] that degradation or loss of individual myofibrillar proteins did not occur during the storage of muscle.

The increased rate of sedimentation of the two main components of 2 h actomyosin, together with the disappearance of the faster component of 4 h actomyosin indicates that fundamental changes in the physico-chemical properties of actomyosin occurred during storage of chicken breast muscle at 25 °C. While these changes apparently resulted from the decrease in pH value of the muscle during storage, investigations during which the pH value of the medium used for the extraction of actomyosin was altered, indicated that the decrease in pH value was not responsible for the differing enzymic properties and superprecipitation behaviour of fresh and stored actomyosin.

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Technology of Biscuit Manufacture: Investigation of the Role of Fermentation in the Manufacture of Cream Crackers

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It has been shown that satisfactory cream crackers can be made, both in a pilot bakery and on commercial cracker plants, from doughs which have been mechanically developed to about 5 Wh/lb[a] and subsequently fermented for 30 min at 100 °F (37.8 °C). Evidence is presented to support the suggestion that the amount of separation of the laminations during the baking of cream cracker doughs is related to the rate at which carbon dioxide is being produced in the dough at the end of the fermentation process. Taste panels were unable to distinguish between crackers made from doughs prepared by long and short fermentation procedures.

1. Introduction

Cream crackers are manufactured from a yeast-fermented wheat-flour dough. The conditions of fermentation used by different firms vary from about 4 h for a straight dough process to 24 h for a "sponge and dough" process. During fermentation the consistency of the dough changes from a short, tight mass to a smooth extensible, but elastic, material. This change is brought about by the conversion of the hydrated flour proteins into gluten. The products of fermentation are also believed to contribute to the flavour of the finished cracker. The commercial success of the Chorleywood Bread Process (CBP),[1] in which a period of bulk fermentation of bread dough lasting about 3 h is replaced by a short period of intense mechanical work under closely controlled conditions, prompted the present investigation to determine if a similar process might be developed for use in the manufacture of cream crackers.

2. Development of a pilot scale test baking procedure

A general account of the methods used in the commercial manufacture of cream crackers has been given elsewhere.[2] For the purpose of the investigation a pilot scale test baking procedure was developed based on a 4-h straight-dough fermentation procedure.[3]

[a] Throughout this paper 1 lb = 0.454 kg.

2.1. Recipe

The recipe used is given in Table 1(a). The flour used was an untreated bakers' flour of protein content 10 to 12%. Several samples of flour were used during the course of the investigation. The level of dough water required by each flour was determined experimentally as that amount giving a dough of optimum handling characteristics in the post-fermentation stages of the test baking procedure, as judged by an experienced biscuit baker.

TABLE 1. Recipes used in the manufacture of cream crackers

(a) Recipe used for cream cracker doughs		
Flour	840	g
Vegetable shortening	120	g
Diastatic malt flour	30	g
Bakers compressed yeast	12	g
Sodium bicarbonate	0.75	g
Sugar (pulverised)	12	g
Salt	10	g
Water (approximate)	270	ml
(b) Preparation of cracker "dust"		
Flour	1680	g
Vegetable shortening	540	g
Salt	30	g
These ingredients are lightly mixed together to form a freely flowing powder.		

The ingredients, other than the yeast, were stored at ambient temperature (65 to 70 °F) (18 to 21 °C). The yeast, a fresh sample of which was obtained each week, was stored at about 40 °F (4 °C).

2.2. Mixing and fermentation of the dough

Doughs were mixed in a Morton Duplex laboratory mixer, model 0. Equipment connected to the mixer motor enabled the mechanical energy expended on the dough to be measured electrically; a correction was automatically applied for the energy required to drive the empty mixer.[1] The dry ingredients other than the salt and yeast were mixed together on slow speed for 2 min. The salt, dissolved in 150 ml of the dough water, was added and mixed in for 5 s. The yeast, suspended in the remaining dough water was added and mixing continued on slow speed for a further 3 min. The work input during this final stage of mixing was about 1 Wh/lb of dough. The temperature of the mixer jacket was adjusted to give a dough temperature of 80 ± 2 deg. F (26.7 ± 1 deg. C) at the end of mixing.

The dough was transferred from the mixer to a preheated enamelled container with a loose fitting lid and held in a constant temperature cabinet at 80 °F (26.7 °C) for 4 h.

2.3. Lamination and formation of the dough sheet

After fermentation the dough was laminated on a reversing pastry brake (A "Rondo" model S6 was used during most of the investigation). Because of the viscoelastic properties of cracker doughs strict control of the laminating process was necessary. The laminating procedure is illustrated in Figure 1. A total of 12 layers was produced using a "three fold" [Figure 1 (b)] followed by a "four fold" [Figure 1 (d)] folding procedure.

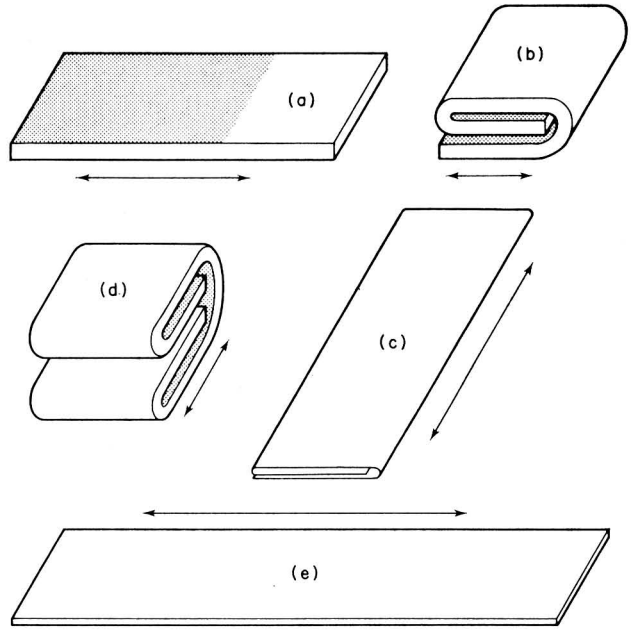

Figure 1. Diagram (not to scale) illustrating the folding procedure used to laminate cream cracker dough on the pastry brake in the pilot bakery. (The arrows indicate the direction of the isotropic tension in the dough sheet at each stage of the lamination process.) (a) Initial dough sheet with cracker dust spread over two-thirds of its surface. (b) Initial dough sheet folded in three. (c) Three fold sheet after its thickness has been reduced on the brake. (d) Three fold sheet after a further folding into four layers. (e) Final dough sheet made up of 12 laminations from which the cracker shaped dough pieces are cut.

215 g of cracker dust (see Table 1(b)) was incorporated in the dough at the first folding. The dough was turned on the brake table through 90° after each folding to distribute more evenly the tensions built up in the dough by repeated rolling. The laminated dough was finally passed between two power driven gauge rolls (21.6 cm diameter) with an effective gap of about 1.10 mm. The actual gauge roll gap used was varied slightly with different flour samples in an attempt to maintain the weight of the finished crackers reasonably constant. However, the gap was maintained constant throughout each set of experiments carried out on a given flour. The cream cracker shaped dough pieces, bearing suitable lettering and docker holes, were mechanically cut from the final dough sheet and mechanically transferred to wire mesh baking trays.

2.4. Baking and cooling

The crackers were baked on wire mesh trays in a pilot scale travelling oven of the forced convection type manufactured by the Spooner Food Machinery Engineering Co. Ltd. A brief description of this oven has been given elsewhere.[4] The oven settings used for baking cream crackers are shown in Table 2; the baking time was 4¼ min. These conditions produced acceptable crackers having moisture contents of about 3 to 4%.

After baking, the crackers, still on the baking trays, were cooled in a rack for 20 min before examination.

TABLE 2. Oven settings used for baking cream crackers in the pilot scale travelling oven

Oven first section	
Temperature (°F)	330 (166 °C)
Top heat damper settings	low, low
Bottom heat damper settings	low, low
Rate of water injection	0
Dew point of circulating air[a]	below ambient
Oven second section	
Temperature (°F)	530 (277 °C)
Top heat damper settings	½, low
Bottom heat damper settings	½, ¼
Dew point of circulating air[a]	below ambient

[a] Measured before dough pieces entered the baking chamber. See reference 4.

2.5. Examination of the crackers

Each sample consisted of five trays each carrying twelve crackers in the order deposited by the cutting machine. The crackers from trays two to four were taken for subsequent examination.

The following measurements were recorded on each sample of crackers.

1. Stack height of 20 crackers (cm).
2. Total length of 10 crackers placed edge to edge in the direction of travel of the oven conveyor (cm).
3. Total width of 10 crackers placed edge to edge in the direction across the oven conveyor (cm).
4. Weight of 10 crackers (g).
5. Texture meter reading[5] of the crackers (s).
6. Moisture content (%) (loss in weight of 5 g ground cracker during 1.5 h at 130 °C).
7. Measurement of the percentage reflectance of incident light (Baker Perkins Ltd, "Biscuit Colour Comparator").

In addition to the above measurements each sample was examined by two experienced technicians. Particular attention was paid to the internal structure of the crackers and, in many cases, a photographic record was made of the structure of representative samples.

2.6. Comparison of cream crackers made in the pilot bakery with commercial crackers

Cream crackers produced by the test baking procedure described above, although of acceptable appearance and eating quality, had a much more open structure than commercial cream crackers [Figure 2 (a) and (b)]. This difference was shown to be associated with the difference in conditions used to laminate the doughs. The majority of commercial cracker doughs are laminated in continuous sheets by a machine known as a laminator.[2] The pastry brake from the pilot bakery was set up alongside a commercial laminator. Samples of the commercial cracker doughs were laminated on the brake and the resultant sheet passed through the commercial cutting machine and oven. The crackers obtained were similar in structure to those shown in Figure 2(b). No

TABLE 3. Comparison of some properties of typical cream crackers made in the pilot scale bakery with a range of commercial crackers. (Cracker weights have been compared on a basis of unit area to compensate for variations in size of commercial crackers from different manufacturers)

	Stack height 20 crackers (cm)	Wt/unit area (g/cm^2)	Texture meter reading (s)	Reflectance (%)
Commercial crackers, 6 samples from 4 manufacturers				
Mean	14.2	0.166	14	52
Range	12.4–15.1	0.160–0.178	10–20	47–54
Crackers from pilot bakery (8 replicates) (flour 1)				
Mean	17.1	0.179	11	53
Range	16.4–18.6	0.171–0.186	10–13	49–56
Crackers from pilot bakery (5 replicates) (flour 2)				
Mean	17.3	0.175	10	49
Range	17.1–17.6	0.171–0.183	10–11	49–51

modification to the brake procedure was found which materially reduced the difference in structure between the crackers made by the test baking procedure and the commercial product.

A comparison of some physical properties of commercial cream crackers with those of crackers made by the test baking procedure is shown in Table 3. In spite of every effort to maintain the operating conditions in the test baking procedure constant, the batch to batch reproducibility of some of the physical properties of the cream crackers was poor. The effect of this variation was reduced by replicating each group of experiments (usually three times) and averaging the values obtained.

3. Results

3.1. Effect of replacing the fermentation period by mechanical development of the dough to various levels of work input

Series of doughs were prepared, using the same recipe and ingredients as used for the fermented doughs, but mixing to levels of work input of 1, 5 and 15 Wh/lb of dough. The temperatures of the dough water and the water jacket on the mixer were adjusted

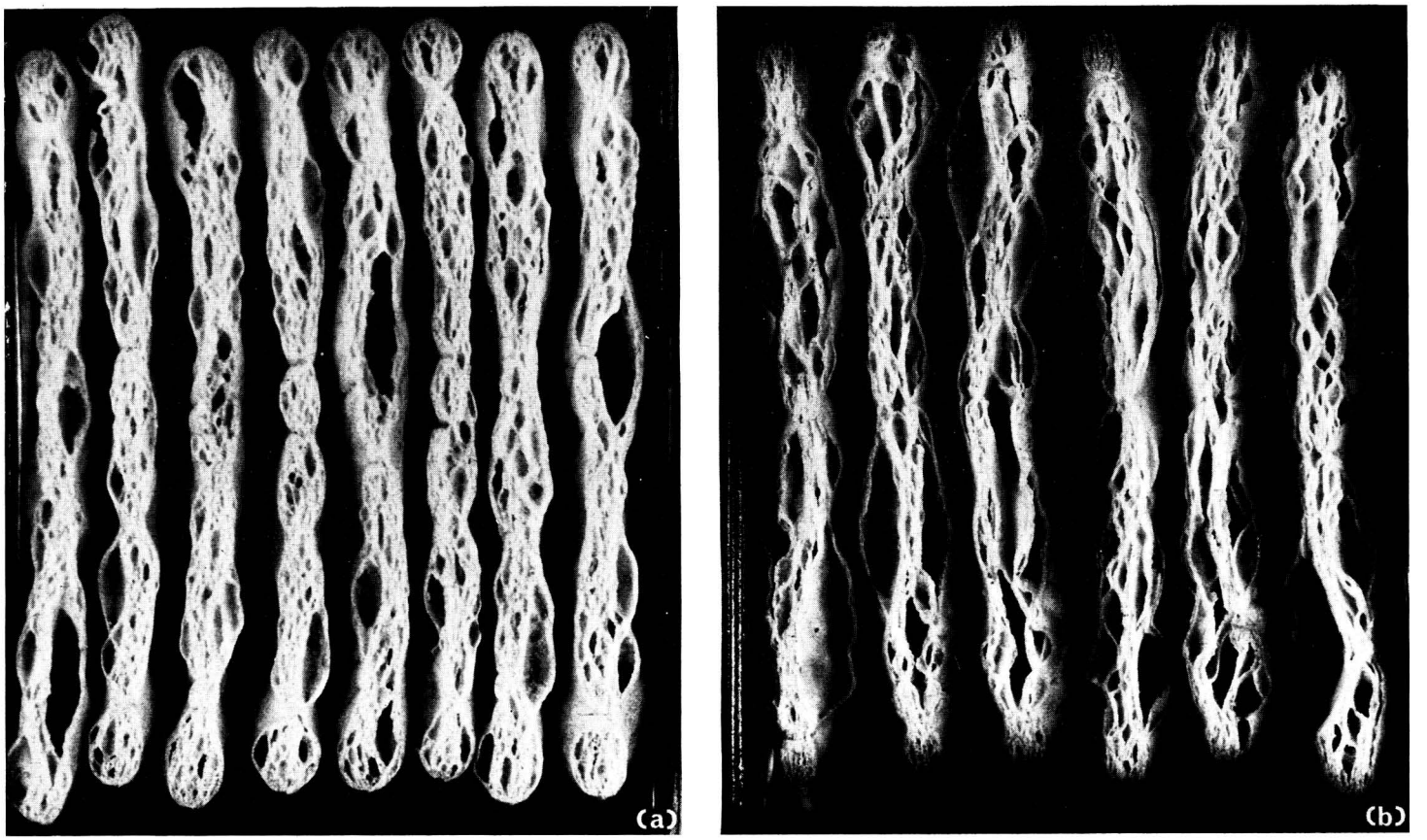

Figure 2. Comparisons of the internal structure of some cream crackers. (a) Commercial crackers. (b) Crackers made in the pilot bakery from doughs fermented for 4 h at 80 °F (26.7 °C).

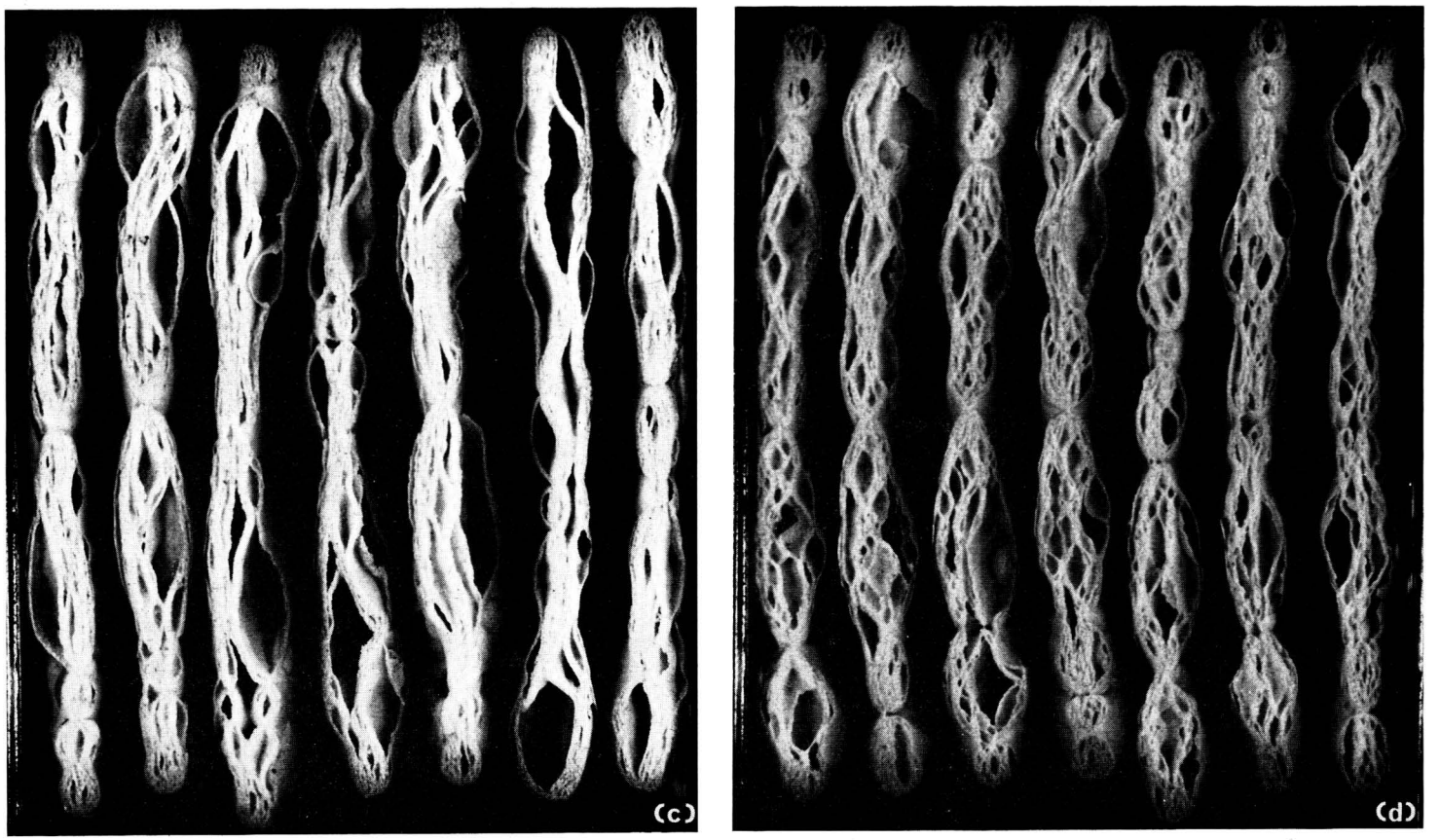

Figure 2. (c) Crackers made in the pilot bakery from dough prepared by the short fermentation process at 80 °F (26.7 °C). (d) Crackers made in the pilot bakery from dough prepared by the short fermentation process at 100 °F (37.8 °C).

so as to maintain the final dough temperature as near 80 °F (26.7 °C) as possible. A fermented dough was prepared 4 h before each group of mechanically developed doughs to serve as a control. The consistency of each dough was assessed subjectively. At 1 Wh/lb the doughs were short and tight, being closely similar to doughs intended for fermentation. The doughs mixed to 5 Wh/lb, the optimum level of work input for the CBP, were softer and slightly extensible but much tighter than the fermented controls. The doughs mixed to 15 Wh/lb were smooth and extensible, similar in consistency to the fermented controls.

The doughs mixed to 1 Wh/lb were rather difficult to handle during the early stages of the laminating process, but the consistency steadily improved during processing. In the later stages of laminating no differences in handling characteristics were noted between the fermented doughs and any of the mechanically developed doughs. So marked was the change in consistency during lamination of the doughs mixed to 1 Wh/lb that measurements were made to see if extra work was being expended on

TABLE 4. Work input during lamination of cream cracker doughs prepared in various ways

Method of preparation of dough	Work input during lamination (Wh/lb)
Fermentation [4 h at 80 °F (26.7 °C)]	0.25
Mechanically developed to 1 Wh/lb at 80 °F (26.7 °C)	0.33
Mechanically developed to 5 Wh/lb at 80 °F (26.7 °C)	0.30
Mechanically developed to 15 Wh/lb at 80 °F (26.7 °C)	0.18

these doughs by the brake during lamination. (These measurements were made electrically in a similar manner to those made on the mixer.) The work expended on the dough during lamination was much smaller than the amount expended on the doughs during mixing and was difficult to measure accurately. However, the results obtained (Table 4) suggested that the work expended during lamination decreased as the work expended during mixing increased.

The cream crackers obtained from the mechanically developed doughs differed from the control crackers principally in being thinner and lighter in weight (Table 5) and in having a much poorer separation of the inner layers [Figure 2(c)].

Under commercial operating conditions the mixing of a batch of dough would be completed at least 15 min before the batch was required for use on the plant. In addition, the time taken to process a normal batch of dough (about 1000 lb) through the laminator would be 20 to 30 min. During these periods some fermentation of the mechanically developed doughs would occur. The above experiments were therefore repeated but at each level of work input doughs were allowed to stand, under the same conditions used for the fermented doughs, for periods of up to 1 h before lamination. Changes in the subjectively assessed consistencies of the doughs during this standing period were

noted. The doughs mixed to 1 Wh/lb softened considerably and at the end of 1 h were similar in consistency to the fermented controls. The doughs mixed to 5 Wh/lb also softened and were of similar consistency to the controls after only 30 min. The doughs mixed to 15 Wh/lb softened only slightly during the first few minutes standing. After the initial softening of the doughs mixed to 5 and 15 Wh/lb had taken place no further change in consistency was observed when the doughs were allowed to continue standing for up to one hour. The changes in the consistencies of the various doughs produced no systematic changes in the physical measurements or structures of the finished crackers.

In subsequent experiments attention was concentrated on doughs mixed to a work input of 5 Wh/lb and subsequently fermented for 30 min under controlled conditions to allow the consistency of the doughs to stabilise. Doughs prepared under these conditions are referred to as "short time doughs".

TABLE 5. Effect of replacing fermentation by various levels of work input during mixing on some physical properties of cream crackers

Method of preparation of dough	Stack height 20 crackers (cm)	Total length 10 crackers (cm)	Total width 10 crackers (cm)	Weight of 10 crackers (g)	Texture meter reading (s)	Reflectance (%)
Fermentation [4 h at 80 °F (26.7 °C)]	17.1	64.1	70.5	78.5	11	47
Mechanically developed to 1 Wh/lb at 80 °F (26.7 °C)	15.7	65.4	70.7	78.2	12	53
Mechanically developed to 5 Wh/lb at 80 °F (26.7 °C)	15.7	65.5	70.7	75.8	12	52
Mechanically developed to 15 Wh/lb at 80 °F (26.7 °C)	15.7	67.0	70.5	74.8	11	48

3.2. Effect of addition of oxidising agents to short time doughs

An important feature of the CBP is the addition to the dough of controlled amounts of suitable oxidising agents. Two of the most commonly used agents are potassium bromate and ascorbic acid. Experiments were therefore carried out in which these reagents were added in increasing amounts to short time doughs prepared at 80 °F (26.7 °C). The maximum levels of addition used were 200 parts/million of ascorbic acid and 40 parts/million of potassium bromate, both figures expressed on flour weight basis. (These levels of addition are some three times higher than the usual levels used in bread.) No significant effect on the properties of dough or finished crackers were found at any level of addition.

A further feature of the CBP is the requirement for a level of addition of fat such that the dough will contain the equivalent of 8 oz (227 g) of solid fat per 280 lb flour at a temperature of 100 °F (37.8 °C). This requirement was more than adequately met in the case of cream crackers by the large amount of fat already present in the recipe. Dilatation measurements on this fat showed it to contribute about 2 lb of solid fat per 280 lb of flour at a temperature of 100 °F (37.8 °C).

3.3. Determination of the carbon dioxide content of cream cracker doughs

It appeared possible that the difference in structure between cream crackers made from fermented doughs and those made from short time doughs might be associated with the presence of a higher level of carbon dioxide in the former than in the latter. Measurements were therefore made of the carbon dioxide content of fermented doughs [4 h at 80 °F (26.7 °C)] and of short time doughs prepared at 80 °F (26.7 °C). The carbon dioxide content of the doughs was also determined on the cut dough pieces after lamination of these doughs. The measurements were made using a semi micro procedure based on the Chittick method.[6] The results obtained, Table 6, showed that before lamination the fermented dough contained about three times as much carbon dioxide as the short time dough, but during lamination most of the excess gas was removed from the fermented dough. It appeared unlikely that the difference in carbon dioxide content of the doughs remaining after lamination was sufficient to account for the marked difference in the structure of the crackers made from the two types of dough.

TABLE 6. Carbon dioxide content of fermented and short time cracker doughs [both at 80 °F (26.7 °C)] before and after laminating. Each figure is the average of determinations on five separate doughs

	Fermented dough	Short time dough
	CO_2 (ml/17 g of dough)	
Before lamination	11.6	3.8
After lamination	4.3	3.3

3.4. Measurements of the activity of yeast in cream cracker doughs at different temperatures

Measurements were next made of the rate at which carbon dioxide was produced by cream cracker doughs at succeeding stages during fermentation at different temperatures. Four 60-g portions of each dough were placed in small flasks held in a water bath which was thermostatically controlled at the required temperature. The carbon dioxide evolved from each portion of dough was collected in a calibrated tube over 25% w/v salt solution which had previously been saturated with carbon dioxide. The volumes of carbon dioxide evolved were noted at frequent intervals during a period of 5 h and the readings used to calculate the rate of gas production at successive stages of fermentation. Measurements were made on duplicate doughs mixed to a work input of 1 Wh/lb and temperatures of 70, 80, 90 and 100 °F (21.1, 26.7, 32.2 and 37.8 °C). Additional measurements were made on duplicate doughs mixed to a work input of 5 Wh/lb at a temperature of 80 °F (26.7 °C) to determine if the amount of work input during mixing affected the rate of gas production. The results obtained are summarised in Figure 3. From these results it is seen that the rate of carbon dioxide production during the early stages of fermentation is, as expected, markedly increased by increasing dough temperature but does not appear to be affected by work input during mixing of the dough over the range 1 to 5 Wh/lb.

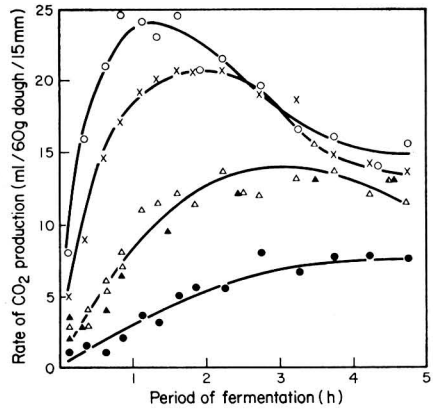

Figure 3. Rate of gas production by cracker doughs at various stages during fermentation at four temperatures.

Work input during mixing of dough (Wh/lb)	Fermentation temperature (°F)	(°C)	Symbol
1	70	(21.1)	●
1	80	(26.7)	△
5	80	(26.7)	▲
1	90	(32.2)	×
1	100	(37.8)	o

3.5. Effect of temperature of short time doughs on the properties of the finished cream crackers

The difference in the amount of separation of the laminations of cream crackers made from doughs prepared under different conditions can be explained on the basis of the results shown in Figure 3 if it is assumed that the degree of separation of the laminations is related to the rate of carbon dioxide production by the dough at the end of the fermentation period. The crackers having good separation of the inner laminations were obtained from doughs fermented for 4 h at 80 °F (26.7 °C) which would have been producing carbon dioxide at the rate of 13 ml/60 g dough/15 min. The crackers showing less separation of the inner laminations were obtained from doughs fermented for 0 to 60 min at 80 °F (26.7 °C), which would have been producing carbon dioxide at rates of 0 to 8 ml/60 g dough/15 min. On this hypothesis, the curves shown in Figure 3 suggest that short time doughs mixed to progressively higher temperatures should yield crackers of progressively better structure. A series of such doughs was therefore prepared at temperatures of 70, 80, 90 and 100 °F (21.1, 26.7, 32.2 and 37.8 °C). The consistencies of the doughs changed considerably with increasing temperature, the dough mixed to 100 °F (37.8 °C) being quite soft, but no difficulty was experienced in lamination of the doughs. The crackers obtained were once again thinner and lighter in weight than the control crackers made from doughs fermented for 4 h at 80 °F (26.7 °C)

(Table 7). However, the inner structure of the crackers showed a marked improvement with increasing temperature of the doughs over the range 70 to 90 °F (21.1 to 32.2 °C). The crackers produced from the doughs mixed to 90 and 100 °F (32.2 and 37.8 °C) were closely similar in structure to the control crackers made from fully fermented dough [Figure 2(d)]. This latter observation indicates that the maximum separation of the laminations is obtained from doughs producing carbon dioxide at the rate of about 13 ml/60 g dough/15 min, no further improvement occurring at higher rates of gas production.

TABLE 7. Effect of dough temperature on the properties of crackers made from short time doughs

Method of preparation of dough	Stack height 20 crackers (cm)	Total length 10 crackers (cm)	Total width 10 crackers (cm)	Weight of 10 crackers (g)	Texture meter reading (s)	Reflectance (%)
Fermentation [4 h at 80 °F (26.7 °C)]	18.3	64.3	70.7	82.8	13	50
Short time doughs prepared at:						
70 °F (21.1 °C)	17.5	65.7	70.7	81.0	14	51
80 °F (26.7 °C)	17.6	64.7	71.1	77.9	11	48
90 °F (32.2 °C)	17.4	64.6	70.7	78.4	10	47
100 °F (37.8 °C)	17.2	65.0	70.4	76.9	11	47

3.6. Experiments carried out on commercial cream cracker plants

Experiments were carried out on a number of commercial cream cracker plants. The results obtained confirmed that cracker doughs of satisfactory consistency were obtained by mechanical development to about 5 Wh/lb (observed range 3 to 7 Wh/lb). Crackers of satisfactory appearance and structure were obtained from doughs mixed to these levels of work input and held at 95 to 100 °F (35.0 to 37.8 °C) for 30 min before

TABLE 8. Comparison of properties of cream crackers made by long and short fermentation procedures on two different commercial cracker plants. At plant C the normal fermentation time was 16 h and at plant D 24 h

	Stack height 20 crackers (cm)	Total length 10 crackers (cm)	Total width 10 crackers (cm)	Weight of 10 crackers (g)	Texture meter reading (s)	Reflectance (%)
Results obtained on Plant C						
Long fermentation	14.7	66.1	72.0	76.0	14	51
Short fermentation	14.2	66.1	72.2	76.6	14	51
Results obtained on Plant D						
Long fermentation	14.2	69.5	65.7	81.1	12	—
Short fermentation	14.2	69.8	66.1	78.9	12	—

lamination, provided that the doughs contained not less than the level of yeast used in the pilot bakery recipe (4 lb/280 lb flour). Where a very low level of yeast was employed in a long, sponge and dough, fermentation procedure it was necessary to increase the amount of yeast added to at least this level. The differences in stack height between commercial crackers made from fully fermented doughs and those made from the short time doughs at 100 °F (37.8 °C) were much less than the differences observed in the pilot bakery (Table 8).

3.7. Organoleptic comparison of crackers made by long and short fermentation procedures

At each stage of the investigation, both in the pilot bakery and in the various commercial plants, crackers made by long and short fermentation procedures were submitted to taste panels 24 hours after preparation of the crackers. The panels used at Chorleywood consisted of about 40 people drawn from members of the staff and were not specially selected. In industry the panels were usually smaller, consisting of about 15 members of staff regularly concerned with cracker production and quality control. A triangular test was used, the crackers being presented in subdued red light to minimise any small differences in appearance. Eighteen panels were held. In each case the number of correct selections was close to that which would have been made on a purely random basis.

4. Discussion

The relatively poor reproducibility of the properties of cream crackers, both in the pilot bakery and under industrial conditions, is widely recognised. Many of the difficulties arise at the lamination stage of the process and are associated with the complex viscoelastic properties of the dough. However, the observations on which the main conclusions of the present investigation have been based were sufficiently consistent to be considered reliable.

Cream cracker dough, like hard sweet biscuit dough, owes its rheological properties to the presence of a three dimensional gluten network derived from the flour proteins. The effect of changes in work input at constant dough temperature on the consistency of cream cracker doughs was somewhat greater than the effect reported in the case of hard sweet biscuit doughs.[4] This difference in behaviour between the two types of biscuit dough is probably associated with the differences in composition. The cracker dough contains much less sugar and more water than the hard sweet dough, which may allow a greater amount of gluten development in the former.[7,8] The large effect on the consistency of some cream cracker doughs produced by the relatively small amounts of work done during lamination suggests that this small amount of work is used more efficiently than is the much larger amount of work expended on the dough during mixing. The effect of dough temperature at the end of mixing on the consistency of cream cracker doughs was similar to the effect observed on hard sweet biscuit doughs.[4] As in the case of hard sweet biscuit doughs, changes in the mixing conditions of cracker doughs prepared by the short fermentation procedure in the pilot bakery caused some variations in the measured properties of the finished crackers. However, the results show no evidence for a critical level of work input.

The above observations suggest that cracker doughs intended for a short fermentation process should be mixed to a constant final dough temperature. Consideration of the effect of temperature on the activity of the yeast indicates that this temperature should be in the range 90 to 100 °F (32.2 to 37.8 °C). Moderate variations in the level of work input are of no importance provided that the general level is such as to give a dough of satisfactory consistency after fermentation. An appropriate level would appear to be within the range 3 to 7 Wh/lb.

The results obtained in the present investigation indicate that the amount of separation of the laminations of cream cracker doughs during baking is related to the rate of gas production by the dough at the end of the fermentation period. However, the stack height of the crackers did not increase as the separation of the laminations increased, indicating that this property is controlled by other factors. The lower stack heights of the crackers made from short time doughs in the pilot bakery compared with the stack heights of crackers made from fully fermented doughs may be partly associated with the somewhat lower weights of the former crackers when compared with the latter (see Tables 5 and 7). In the experiments on commercial cracker plants the gauge roll gap was adjusted to obtain crackers of similar weight from both types of dough and crackers of similar stack height were also obtained (Table 7).

Under industrial conditions, the changeover from the production of cream crackers from fully fermented dough to dough produced by the short fermentation process was accompanied by an almost complete disappearance of the aroma of baked crackers from the vicinity of the plant. However, taste panel comparison 24 hours later failed to show any difference in the flavour of the products. A similar finding has been reported in the case of bread.[9]

For some years now a short fermentation procedure based on the results reported above has been used commercially for the manufacture of cream crackers with very satisfactory results. This procedure is the subject of a British Patent.[10]

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Estimation of Available Copper, Iron, Manganese and Zinc in Soils using Cation-exchange Resin

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Studies on the extractability of available Cu, Fe, Mn and Zn in soils using cation-exchange resin were carried out. A procedure involving the shaking of 5 g soil with 5 g of resin in 200 ml deionised water for 2 h at 25 ± 1 °C followed by colorimetric determination of the micronutrient elements in solution was adopted. This was compared with known chemical extraction methods in the prediction of availability to *Phaseolus mungo* as test plant. Correlation analysis showed that the method successfully indicated the availability of Cu, Mn and Zn but was not sensitive enough for the prediction of the availability of Fe. The proposed method is simple, convenient and compares favourably with the chemical extractants in the prediction of availability of Cu, Mn and Zn.

1. Introduction

Any soil test method for assessing nutrient availability must be able to (1) extract a measurable amount of the nutrient from the soil that is related to the uptake by plants and (2) it should be cheap, reproducible and capable of being incorporated into a laboratory routine.[1] Although considerable progress has been made one of the most important analytical problems facing soil testing as an aid in the diagnosis of production difficulties related to soil fertility is the development of suitable tests for micronutrients.[2]

Various different chemical extractants have been used to assess the availability of Cu, Fe, Mn and Zn in soils. For copper: 0.1 N-NH_4NO_3 and NH_4OAc;[3] 1 N-HCl and NH_4 citrate–Na_2EDTA[4] have been tested as indices of availability. For iron: reducing agents; iron-complexing agents; dilute acids or buffer solutions of low pH including NaOAc at pH 4.8;[5] N-NH_4OAc at pH 4.8[6] have been used. For manganese: extractants have ranged from water through electrolytes of salts including N-NH_4OAc at pH 7.0, 0.5 M-$Mg(NO_3)_2$, $Ca(NO_3)_2$, $NH_4H_2PO_4$, Na_2EDTA, to reducing agents such as hydroquinone, hydroxylamine hydrochloride and hydrosulphite, to mineral acids such as H_3PO_4.[7] For zinc: various chemical methods have been proposed but only two of these—1 N-NH_4OAc–dithizone[8] and 0.1 N-HCl[9]—have been sufficiently calibrated. It would be simpler, cheaper and more convenient if one good extractant could be found for several micronutrient elements. This would provide greater economy in soil testing. In an attempt in this direction, Dolar, Keeney and Walsh[10] tested extractions

with N-$Mg(NO_3)_2$ at pH 5.9, 0.1 N-HCl, 0.01 M-EDTA–N-NH_4OAc at pH 7 and 0.005 M-DPTA (diethylenetriaminepenta-acetate) in 0.01 M-$CaCl_2$–0.1 M-triethanolamine at pH 10.9 and found that none of these alone successfully predicted uptake of all the three micronutrient cations Cu, Mn and Zn.

Cation-exchange resins have been used successfully by several workers in extracting the fraction of non-exchangeable potassium that is taken up by plants during the cropping period.[11] It appears that cation-exchange resin could be conveniently used in extracting other cationic plant nutrients including the micronutrient elements. The following investigation was therefore conducted with the aim to finding out whether a satisfactory procedure for the estimation of available Cu, Fe, Mn and Zn could be developed using cation-exchange resin as extractant.

2. Experimental

2.1. Soils used

Samples of 14 top soils (0 to 22 cm) were used in these studies. Some properties of the soils are given in Table 1. The soils were crushed and then sieved and subsamples of the <2 mm fraction were ground in an agate mortar to pass a 70-mesh sieve. Care was taken at all stages of soil preparation to avoid contamination by using polythene containers and sieves.

2.2. Analytical methods

Exchange capacity of the soils was determined using N-NH_4OAc at pH 7.0. Organic carbon was determined by wet-combustion with dichromate and mechanical composition by the Bouyoucos hydrometer method.[12] Total analysis for Cu, Fe, Mn and Zn was carried out by fusion with sodium carbonate[13] followed by colorimetric estimation using biscyclohexanone,[14] *o*-phenanthroline,[15] periodate[16] and dithizone[17] methods, respectively.

2.3. Extraction procedure

The final method selected for testing with plant uptake was as follows: 5 g of soil ground to pass a 70-mesh sieve were placed in a 250-ml polythene bottle and 5 g of air-dry Zeo-Karb 215 (a strongly acidic polyfunctional phenol–formaldehyde cation-exchange resin in the hydrogen form) were added followed by 200 ml of deionised water. The resin, with particle size between 50 and 70-mesh had an exchange capacity of 3.5 mequiv./g of oven dry material. The soil–resin mixture was shaken in a reciprocating shaker for 2 h at a temperature of 25 ± 1 °C. After shaking the resin was separated from the soil by rinsing with deionised water through a 70-mesh polythene sieve. The soil passed through the mesh and the clean resin was retained on the sieve. The resin was then transferred to a leaching tube similar to that employed by Hislop and Cooke[18] except that flow rate was controlled by a clip instead of a hypodermic needle. The extracted nutrient cations were then eluted from the resin with 2 N-H_2SO_4 until 100 ml were collected in 1 h. Aliquots of the eluate were used for the colorimetric determination of Cu, Fe, Mn and Zn as indicated above. All the extractions were carried out in duplicate or triplicate and the results shown are therefore means. In preliminary studies it was

found that elution with 2 N-H_2SO_4 gave better results, particularly for Mn, than did N-H_2SO_4; but 200 ml of 2 N-H_2SO_4 did not yield better results than when 100 ml was used. In all cases the results of the analyses were reproducible within $\pm$ 5%.

2.4. Preparation and regeneration of the resin

Before use the analytical grade resin was ground in water in an agate mortar and then screened by washing, decanting and sieving to obtain the 50 to 70-mesh size. After use the resin was regenerated in batches of about 200 g by soaking overnight in 1 l of 4 N-H_2SO_4, then transferring into a large cylindrical separatory funnel and leaching with several bed volumes of 2 N-H_2SO_4 followed by deionised water until the effluent was free of sulphate ions. The wet resin was then dried at 60 °C and stored at normal laboratory temperature and humidity.

2.5. Comparison with other extraction methods and with plant uptake

The proposed resin extraction method was compared with other extraction methods for copper,[3,4,19] manganese[7,20] and zinc[9,19,22] by testing against uptake by *Phaseolus mungo* in a pot culture in the greenhouse. Subsamples (200 g) of soil <2 mm were mixed with 200 g acid-washed sand and placed in 500 ml polythene pots. The soil–sand mixture in each pot was treated with 20 mg P in solution, allowed to dry and then thoroughly mixed. Five seeds of *Phaseolus mungo* were then sown in each pot. A solution to supply the following macronutrient elements was then applied to each pot: 25 mg of N as NH_4NO_3; 25 mg of K as K_2SO_4; and 5 mg of Mg as $MgSO_4.7H_2O$. The moisture content of the soil–sand mixture was then adjusted to 25% water holding capacity (w.h.c.).

Six days after germination, micronutrient solutions containing in parts/million 1 Mo, 50 Fe, 50 Mn, 5 Cu and 5 Zn, were applied; pots in which availability of Cu, Fe, Mn and Zn was evaluated received no Cu, Fe, Mn and Zn, respectively. There were three replicates of each treatment and these were arranged as three blocks with the treatments completely randomised within each block. Watering was continued by adjusting the water content daily to 50% w.h.c. by weighing. The plants were harvested at 38 days after planting, washed, dried, weighed and then ground. Sub-samples of the ground material were wet digested with a ternary mixture of HNO_3, H_2SO_4 and $HClO_4$ according to Jackson[13] and the Cu, Fe, Mn and Zn concentrations determined colorimetrically as indicated earlier.

The aim of the experiment was to estimate availability and was therefore not designed as a factorial to test responses to the application of Cu, Fe, Mn or Zn in the 14 soils. Statistical analysis was carried out on the data using Student's t test and calculating the significant difference ($P = 0.05$) from the mean. The relative usefulness of the extraction methods was tested by correlation analysis using the concentration in parts/million of the plant material or the total uptake of the micronutrient element in μg as the dependent variate and the amount extracted by the method as the independent variate. Further, in order to improve the prediction of uptake by correcting for possible diminishing returns between uptake and amounts extracted, the former values were also correlated with the logarithmic form of the latter data.

3. Results and discussion

The total Cu, Fe, Mn and Zn contents and other properties of the soils are given in Table 1. The ranges of the total contents of these microcations in the soils are similar to values reported on other tropical soils e.g. Burridge and Ahn,[23] Sharma and Shinde,[24] Sharma and Motiramani[25] and Murthy and Mehta.[26]

TABLE 1. Some properties of soils used

Soil no.	Great soil group and parent material	Organic carbon (%)	pH 0.1 M ($CaCl_2$)	Clay content (%)	CEC mequiv. (%)	Total content (parts/million) Cu	Fe	Mn	Zn
1	Forest oxysol on biotite granite schists	1.0	4.0	28	11.9	37	15 250	325	116
5	Forest ochrosol on granite	0.4	5.3	6	3.7	21	2687	100	103
6	Tropical black clay on hornblende gneiss	1.3	7.4	33	36.4	77	45 930	2400	94
9	Forest oxysol on biotite granite	0.6	4.7	22	6.1	21	5625	150	33
16	Forest oxysol on phyllite	1.0	4.2	41	11.5	26	1250	850	92
19	Savannah ochrosol on alluvium	1.3	5.3	10	8.5	30	8200	750	87
22	Savannah gleisol on alluvium	0.6	4.2	33	19.0	46	18 750	1900	72
27	Forest ochrosol on phyllite	0.8	5.2	8	8.9	35	7200	425	45
28	Groundwater laterite on shale	0.5	4.1	8	5.9	26	5065	100	42
30	Savannah acid gleisol on granite	0.9	3.9	25	18.9	35	16 000	500	53
31	Forest acid gleisol on phyllite	1.1	4.1	25	11.8	30	8000	550	34
38	Savannah ochrosol on quartzite/ sand stone	0.6	5.1	8	6.2	30	6750	450	41
42	Forest oxysol on tertiary sand	0.8	4.8	8	6.2	30	6625	150	41
45	Savannah acid gleisol on shale	0.6	4.9	6	4.2	26	2600	150	19

3.1. Development of the method

As it has been shown that temperature can affect the adsorption of cations from solutions[22] and also from soils (e.g. potassium)[11] by cation-exchange resin, all the extractions were performed at 25 ± 1 °C in order to avoid any possible influence of temperature variations. Also sulphuric acid was selected as an eluant in preference to hydrochloric acid because chloride ions interfere in the colorimetric analysis for manganese using the periodate method. Adoption of nitric acid (3 N) as eluant was discarded because preliminary tests with nitric acid resulted in erratic values particularly for Cu and Fe. Samuelson[27] has pointed out that nitric acid can attack cation exchangers containing phenolsulphonic acid groups.

The quantities of Cu, Mn and Zn extracted from the soils increased with increasing quantity of resin used for the extraction, but the increases were generally only significant between extractions with 1 g and 5, 10 and 20 g of resin whereas the differences were not significant between extractions with 5, 10 and 20 g of resin (Table 2). Use of 5 g of resin was selected for convenience. It has been shown by other workers[11,21] that the release of cations to the resin is dependent on the exchange capacity of the resin in the soil–resin mixed system. As can be seen in Table 1, in all cases the exchange capacity of the resin in the mixture was in excess of the exchange capacity of the soil so that

TABLE 2. Effect of weight of resin on copper, manganese and zinc extractable from 5 g of soils 6, 16, 22 and 38 during 2 h shaking. (Mean values in parts/million)

Wt of resin[a] (g)	Cu	Mn	Zn	Cu	Mn	Zn
	Soil 6			Soil 16		
1	2.5	200	1.0	1.3	9	1.0
5	8.5	310	2.5	1.6	19	1.8
10	10.6	420	2.3	2.6	26	2.0
20	10.7	480	2.7	2.6	37	2.1
Mean	8.1	352.5	2.1	2.0	22.7	1.7
Significant difference ($P = 0.05$)	3.7	93.3	0.6	0.5	8.9	0.4
	Soil 22			Soil 38		
1	1.8	360	1.3	1.8	220	1.6
5	3.6	440	1.9	4.0	285	2.7
10	4.3	480	2.6	4.7	330	3.0
20	4.7	500	2.9	5.2	330	3.7
Mean	3.6	445.0	2.2	3.9	291.2	2.7
Significant difference ($P = 0.05$)	2.7	46.7	0.5	1.1	39.3	0.6

[a] Exchange capacity was approximately 3.5 mequiv./g of oven dry resin.

TABLE 3. Effect of period of shaking on copper, manganese and zinc extractable by 5 g resin from 5 g of soils 6, 16, 22 and 38. (Mean values in parts/million)

Period (h)	Cu	Mn	Zn	Cu	Mn	Zn
	Soil 6			Soil 16		
0.5	7.2	160	2.3	1.3	19	1.7
1	7.9	240	2.3	1.4	19	1.8
2	8.6	310	2.5	1.7	19	1.8
3	9.7	360	2.5	2.0	19	2.5
4	8.7	380	3.5	2.1	22	2.3
Mean	8.4	290.0	2.6	1.7	19.6	2.0
Significant difference ($P = 0.05$)	0.6	60.5	0.3	0.2	0.9	0.2
	Soil 22			Soil 38		
0.5	3.3	320	1.9	3.0	200	1.5
1	3.4	380	1.8	3.9	200	1.9
2	3.6	440	1.9	3.9	225	1.7
3	4.8	480	1.9	4.3	240	1.7
4	4.4	500	1.8	3.5	240	1.7
Mean	3.9	424.0	1.9	3.7	221.0	1.7
Significant difference ($P = 0.05$)	0.4	49.7	0.05	0.6	13.8	0.09

adsorption of the microcations was not likely to have been limited by complete saturation of the resin with extraneous exchangeable cations.

The data in Table 3 show that the extractable Cu, Mn and Zn sometimes increased with increase in shaking time. However, the increases were small and in most cases insignificant particularly when shaking was continued for more than 2 h. This trend may have been due to complete extraction of the immediately available Cu, Mn and Zn within 2 h of shaking or to the exchange capacity available to maintain adsorption by the resin becoming reduced by the adsorption of other non-exchangeable cations from the soil as the shaking time was prolonged.

As shown in Table 4 there were no significant differences between the Cu, Fe, Mn and Zn extracted with the addition of 50, 100 or 200 ml of deionised water to the soil–resin mixture. The choice of 200 ml deionised water was made because it facilitated the transfer of the mixture onto the polythene sieve (approximately 125 ml volume) for the separation process.

TABLE 4. Effect of volume of water used on copper, iron, manganese and zinc extractable by 5 g of resin from 5 g of soils 16 and 38. (Mean values in parts/million)

Soil no.	Vol. (ml)	Cu	Fe	Mn	Zn
16	50	0.9	288	26	1.4
	100	1.2	212	22	1.6
	200	1.7	340	19	1.6
Mean		1.3	280.0	22.3	1.5
Significant difference ($P = 0.05$)		0.4	57.3	2.7	0.1
38	50	2.0	58	200	1.4
	100	2.1	40	198	1.6
	200	3.9	70	225	1.7
Mean		2.7	56.0	207.7	1.6
Significant difference ($P = 0.05$)		2.6	13.4	14.7	0.2

3.2. Extractable micronutrients and plant uptake

As shown in Table 5 the total uptake and concentration of Fe and Mn in *Phaseolus mungo* were relatively high compared with those of Cu and Zn. Since plant species differ in their requirements for nutrients,[1] it is not strictly justifiable to compare the uptake values in this work with values obtained on other plants. However, it is reasonable to note here that the tissue concentrations agree well with those quoted as intermediate ranges by various workers for copper[28] and zinc.[29] In the case of Fe and Mn most of the plants had comparatively normal levels and a few very high levels.[30,31]

The amounts of Cu extracted by the extractants investigated were in the order: proposed resin method > citrate–EDTA > 2.5% HOAc > dilute HCl > acid NH_4OAc. The amounts extracted by 0.1 N-HCl and citrate–EDTA were lower than amounts quoted for some American soils.[4] Also, contrary to the finding of Cheng and Bray[4] that Cu extracted from soils by citrate–EDTA are usually about the same as those extracted by 0.1 N-HCl, in this study the former extracted slightly higher amounts. However, the quantities extracted by citrate–EDTA fell within the range of 0.6 to 12 parts/million reported as sufficient for normal growth.[32] The Cu extracted by 2.5% HOAc was higher than the range of 0.05 to 1.0 reported for Scottish soils.[33] On the other hand, the NH_4OAc extract Cu was far lower than the range of 0.2 to 5 parts/million suggested for normal growth.[3]

Soil analysis for estimating Fe availability has not been generally useful. Although amounts extracted from three soils by DPTA (diethylenetriaminepenta-acetic acid) have been found to correlate with amounts taken up by plants,[34] this was not tested in the present study. However, the uptake of iron, as indicated earlier, was generally adequate as compared with values reported by Wallihan.[30]

TABLE 5. Ranges and means of copper, iron, manganese and zinc extractable by different chemical methods and uptake by *Phaseolus mungo* from the 14 soils investigated

Method	Reference	Range	Mean
I. Copper			
Soil extraction (parts/million)			
1. Dilute HCl (0.1 N)	Cheng and Bray[4]	0.3–3.4	1.4
2. Acid NH_4OAc (1 N)	Fiskell and Westgate[3]	0.05–0.24	0.12
3. Citrate–EDTA	Cheng and Bray[4]	0.9–3.8	2.4
4. 2.5% HOAc	Tucker and Kurtz[19]	0.7–3.6	1.5
5. Resin	Proposed	1.2–8.5	3.5
Plant uptake:			
total uptake (μg/pot)		8.7–22.2	14.7
concentration (parts/million)		6.6–12.3	9.2
II. Iron			
Soil extraction (parts/million)			
1. Resin	Proposed	9.6–112.0	54.5
Plant uptake:			
total uptake (μg/pot)		88.0–191.8	123.6
concentration (parts/million)		140.0–450.0	247.6
III. Manganese			
Soil extraction (parts/million)			
1. Exchangeable	Sherman *et al.*[20]	0.3–154.2	30.4
2. Easily reducible	Sherman *et al.*[20]	1.2–780.0	132.5
3. Active	Sherman *et al.*[20]	1.5–785.3	157.7
4. H_3PO_4	Browman *et al.*[7]	1.3–195.0	38.3
5. $NH_4H_2PO_4$	Browman *et al.*[7]	2.0–216.0	66.7
6. EDTA	Browman *et al.*[7]	0.4–222.0	45.3
7. Resin	Proposed	15.0–440.0	131.6
Plant uptake			
total uptake (μg/pot)		105.0–1710.0	862.1
concentration (parts/million)		75.0–950.0	458.1
IV. Zinc			
Soil extraction (parts/million)			
1. Dilute HCl	Nelson *et al.*[9]	1.4–11.4	5.2
2. 2.5% HOAc	Tucker and Kurtz[19]	1.0–2.2	1.7
3. NH_4OAc–dithizone	Brown and Krantz[22]	1.0–4.9	2.9
4. Resin	Proposed	1.8–3.8	2.5
Plant uptake:			
total uptake (μg/pot)		22.0–66.6	35.1
concentration (parts/million)		13.1–28.1	20.2

The amounts of Mn extracted generally followed the order: active > easily reducible > proposed resin method > $NH_4H_2PO_4$ > EDTA > H_3PO_4 > exchangeable. The amounts extracted were similar to those reported by Browman, Chesters and Pionke.[35] However, considering the critical limit of deficiency at 3 parts/million exchangeable Mn and 100 parts/million easily reducible manganese,[36] a few of the soils can be considered deficient. This is confirmed by Hammes and Berger's[37] results that Mn deficiency could be expected when soil Mn extracted with EDTA was <50 parts/million, that extracted with hydroquinone was <65 parts/million and that extracted with either H_3PO_4 or $NH_4H_2PO_4$ was <20 parts/million. Surprisingly, the plant uptake was in all cases higher than the minimum required for normal growth.[31]

The amounts of Zn extracted by the extractants varied as follows: dilute HCl > NH_4OAc–dithizone > proposed resin method > 2.5% HOAc. The amounts of Zn extracted by the dilute HCl method appear to be normal; Wear and Sommer[38] reported a range of 1.2 to 4.7 parts/million, and Viets[39] a range of 1.3 to 1.8 parts/million of 0.1 N-HCl extractable Zn for normal plant growth. Similarly, the amounts extracted by NH_4OAc–dithizone were well above the critical level of 0.5 parts/million below which Zn response might be expected in zinc-sensitive crops.[22]

TABLE 6. Comparison of total correlation coefficients between copper, iron, manganese and zinc extractable by the proposed resin method and certain other chemical methods and the nutrient element removed by *Phaseolus mungo* expressed as (a) μg/pot and (b) parts/million

Method[a]	*r* value (a) μg/pot	(b) parts/million
I. Copper		
1. Dilute HCl	0.45	0.66**
2. Acid NH_4OAc	−0.45	−0.51
3. Citrate–EDTA	0.54*	0.50
4. 2.5% HOAc	−0.04	0.18
5. Resin	0.57*	0.61*
II. Iron		
1. Resin	0.15	0.27
III. Manganese		
1. Exchangeable	0.80***	0.85***
2. Easily reducible	0.73**	0.77**
3. Active	0.76**	0.80***
4. H_3PO_4	0.76**	0.81***
5. $NH_4H_2PO_4$	0.87***	0.88***
6. EDTA	0.78***	0.83***
7. Resin	0.81***	0.84***
IV. Zinc		
1. Dilute HCl	−0.39	−0.23
2. 2.5% HOAc	0.27	0.31
3. Dithizone	0.62*	0.67**
4. Resin	0.57*	0.56*

*, ** and *** denote significance at 5, 1 and 0.1% level, respectively.

[a] See Table 5 for references to the methods used.

3.3. Predictability of availability

The total correlation coefficients between the extractable micronutrients and uptake by *Phaseolus mungo* are shown in Table 6. The relationships between uptake and extractable amounts of the micronutrients were not improved by transforming the original data into logarithmic form. It is clear that the proposed method compared favourably with the other known successful chemical extractants in the case of Cu, Mn and Zn. The method was not sensitive for prediction of Fe availability; the high average plant contents of Fe tended to suggest that a considerable amount of Fe not extractable by the resin method was potentially available to plants during cropping. It also suggests that

not all the Fe might have been removed from the resin during elution as Djurfeldt and Samuelson[40] have pointed out that "as far as Fe^{3+} is concerned a maximum displacement is obtained at medium acid concentration, e.g. 5 N-HCl". They have also observed in their studies that elution of the elements under present study can be performed effectively with a minimum volume of eluant if an optimum concentration of 3 to 5 N-HCl is chosen. It is likely that not only is the H_2SO_4 used not as suitable as HCl but also that the concentration was much too low to be very effective for all the micronutrients studied.

The highly significant correlation between the resin-extractable Mn and uptake is not surprising as the former was also highly significantly correlated with exchangeable, easily reducible-, active-, H_3PO_4-extractable and $NH_4H_2PO_4$-extractable Mn ($r =$ 0.96, 0.96, 0.98, 0.95, 0.96 and 0.98, respectively; all significant at $P = 0.001$).

The correlations between the resin-extractable Cu and Zn and uptake, although significant, were not very high. It is possible that because of the low amounts of these elements extracted from the soils the colorimetric methods may have been subjected to some errors but such errors were never greater than $\pm 5\%$. This source of error can be overcome by using a larger amount of soil with corresponding increase in resin and also by the use of more sensitive analytical methods e.g. atomic absorption spectroscopy.

The proposed resin method accounted for only 32.5 and 37.2% of the variation observed in Cu, 65.6 and 70.6% in Mn and 32.5 and 31% in Zn uptake and concentration, respectively, by *Phaseolus mungo*. Admittedly, these variances are low but as a single predictor of uptake of Cu, Mn and Zn, the results obtained are encouraging enough to warrant further studies with field responses for possible adoption for routine use. As shown by several workers[1,7,10] for other chemical extractants, inclusion of one or more soil physical or chemical properties, (including temperature, moisture content, pH, aeration, clay and organic matter contents, available phosphorus and availability of other micronutrients) may increase the predictability. Furthermore, it has the advantages of being simple and economic as it enables the four micronutrients to be determined on one extract; also with adequate regeneration the resin can be reused several times. With the time consuming colorimetric procedures used in these studies it was possible for one laboratory technician to analyse a minimum of 24 soil samples within 2 working days for Cu, Fe, Mn and Zn. In laboratories where dependable atomic absorption equipment is available there is little doubt that several folds of this minimum number can be analysed in a day by adopting in addition time-saving modifications such as measuring the soil and resin volumetrically with a scoop instead of weighing.

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