THE WATER-SOLUBLE CARBOHYDRATES OF GRASSES. II.*—Grasses Cut at Grazing Height several times during the Growing Season

By R. WAITE and J. BOYD

Four perennial grasses were cut with a motor mower each time they reached a height of about 8–10 in. during the growing seasons of 1951 and 1952. The water-soluble carbohydrates, glucose and fructose (reported together as free hexoses), sucrose and fructosan were determined in each cut. Grasses in which the growing point had recently changed to a floral development (in May or June) contained more carbohydrate than grass grown and cut later in the season. The primary reason for this was that the later grasses were more leafy, and, secondly, that their stems contained much less fructosan than those of the earlier cuts.

In Part I[1] the changes in the amounts of the water-soluble carbohydrates in four perennial grasses during their normal growth-cycle from April to October were described. These grasses passed through the flowering and seed-setting phases, and it was suggested that these physiological changes were probably responsible for the fluctuations in the content of the major carbohydrate fraction, the fructosan, most of which was present in the stem. The content of free hexoses and sucrose varied with the stage of growth, but there was little difference either between the values for the different species or the values in the two successive years. The value of the free hexoses was low at all times of the season, whereas the sucrose content rose appreciably in May–June.

Having thus established the trend of the carbohydrate changes associated with the continuous growth of grasses, we wished to investigate the composition of the soluble carbohydrates in grass such as would be used for grazing dairy cows. The present paper deals with the same four grasses used previously, but in the present experiments they were cut with a motor-mower whenever they had grown to a height of about 8–10 in. It is realized that this did not closely resemble defoliation by animals on the widely used system of continuous grazing, but it would bear a fair resemblance to the removal of herbage by close-folded animals or in well-managed rotational grazing.

Experimental

The grass plots used were part of those described in Part I, and consisted of rye-grass (*Lolium perenne*, strain S23), cocksfoot (*Dactylis glomerata*, strain S143), meadow fescue (*Festuca pratensis*, strain S53) and timothy (*Phleum pratense*, strain S48) in their first and second harvest years (1951 and 1952). The plots received 2 cwt./acre each of potash (60% K_2O) and superphosphate (18% P_2O_5) in February of each year, and a further 1 cwt./acre of each fertilizer in June. ' Nitro-Chalk ' (15·5% N) was applied as follows : 2 cwt./acre in April and 3 cwt./acre after each cut until August, when the amount was reduced to 1 cwt./acre after each cut. The plots had been divided into two ; one half of each grew on without check (as described in the previous experiment[1]), but the other half was cut whenever it reached a height of about 8–10 in. The grass was cut between 9 and 10 a.m. with an Allen motor-scythe. It was considered that the height of the stubble left by this machine, 1–2 in., would more closely resemble that left by the grazing animal than that remaining after cutting by hand with scissors or sheep shears, as in the previous work. The length of the cut grass was therefore about 6–8 in.

The yield of grass was weighed on the plot immediately after cutting, and a sample of 2–3 kg. taken. From this a representative sub-sample of 400–600 g. was dried in the forced-draught oven previously described.[1] It is considered that enzyme action in this sample would be stopped within about 30–45 minutes after cutting. Half of the dried sample was milled at once and the other half separated into leaf and stem portions. These were weighed to provide the ratio of leaf to stem, and milled separately. As before, the length of 100 representative tillers from the original sample was measured and an average length for the cut grass calculated. The stage of development of the primary growing-point, and its length and position in the stem, were again determined under a low-power microscope in samples taken the day before cutting.

The temperature and rainfall during the growing period have already been given in the previous paper.[1]

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Chemical analyses of the dried grass

Moisture content and crude protein (N × 6·25) were determined on all samples. Glucose, fructose, sucrose and fructosan were estimated by the copper-reduction method of van der Plank,[2] according to the scheme of de Man & de Heus,[3] as already described.[1] Glucose and fructose, although determined separately, have again, because of their low values, been reported only in sum as ' total free hexoses '.

Results

Tables I and II give details of the yield of dry matter and the stage of growth reached by the four grasses and the soluble-carbohydrate content at each cut in 1951 and 1952.

Table I

The stage of growth of the grasses and the crude protein and soluble carbohydrate contents (percentage of dry matter) at each cutting in 1951

Grass	Date cut (1951)	Interval between cuts, days	Yield of dry matter, lb./acre	Leaf–stem ratio (dry wt.)	Average height, in.	Development of growing points, % in floral state	Crude protein	Hexoses	Sucrose	Fructosan	Total soluble sugars
Rye-grass	15 June		1920	2·7	11·2	100	8·3	1·9	5·4	16·1	25·5
	12 July	27	675	12	6·2	0	16·9	1·0	3·5	4·6	9·1
	27 July	15	685	15	6·1	0	24·9	1·5	2·8	1·4	6·0
	15 Aug.	19	660	22	5·8	0	26·1	1·9	4·5	0·5	7·2
	30 Aug.	15	549	24	6·6	0	25·3	1·4	4·7	0·7	7·1
	18 Sept.	19	948	21	6·8	0	24·3	1·0	3·9	1·1	5·8
Timothy	18 June		2340	1·2	8·2	100	8·4	1·8	3·9	9·3	16·3
	17 July	29	1010	9·0	6·4	0	16·9	1·0	3·9	1·4	6·7
	20 Aug.	34	946	5·6	7·0	0	14·6	1·7	5·9	5·8	14·3
	17 Sept.	28	895	12	8·2	0	21·9	0·8	3·9	0·6	5·6
Fescue	17 May		703	1·4	7·2	100	15·0	3·0	10·3	11·6	26·3
	8 June	22	1130	0·8	7·9	100	11·8	3·9	7·4	7·7	20·3
	28 June	20	550	7·2	6·4	n.d.*	16·3	1·8	3·7	6·9	11·9
	12 July	14	390	21	6·1	0	25·7	1·1	2·0	1·1	4·3
	27 July	15	830	14	6·0	0	24·4	1·6	2·0	1·1	5·0
	14 Aug.	18	640	21	6·8	0	25·0	1·6	5·6	0·6	8·2
	28 Aug.	14	384	23	6·6	0	26·4	2·2	5·5	0·7	8·8
	2 Oct.	35	822	19	7·9	0	21·6	1·2	3·2	2·3	7·2
Cocksfoot	15 June		2270	3·0	10·0	30	8·0	2·0	4·5	10·7	18·6
	8 July	23	627	8·0	8·1	0	20·2	0·9	3·6	0·8	5·9
	24 July	16	809	8·0	8·9	0	22·9	1·5	5·0	0·9	7·7
	15 Aug.	22	740	14	5·4	0	24·7	1·0	3·4	0·9	5·5
	30 Aug.	15	580	16	7·5	0	29·2	0·7	2·8	0·3	4·0
	17 Sept.	18	643	13	7·9	0	30·6	0·5	3·5	0·2	4·4

* Not determined

In 1951 the first cut of all the grasses except fescue was delayed too long, resulting in high yields, low protein contents and longer grass than was intended. In 1952 the grass was cut at the proper stage of growth but, despite this, the yield of dry matter in both the first and second cuts was much higher than in any subsequent cut. This effect is probably associated partly with the greater ' stemminess ' of the primary tillers and the more vigorous growth of secondary tillers in May and June.

The values for the leaf–stem ratio and the stage of development reached by the growing point clearly show the changing habit of the grass as the growing season progressed. Up to early or mid-June, depending on the species, the grasses would have formed flower heads if grown to maturity, but after that date the growing point was entirely vegetative; even if continued growth had been allowed, no flower heads would have been formed, despite the generous fertilizer-treatment. This behaviour resulted in much higher leaf–stem ratios and higher crude-protein values after the second cuts. From the values for the soluble carbohydrates it can be seen that the previous position was now reversed. It was the earlier cuts, taken up to about mid-June and containing florally developing growing-points, that possessed the greatest amounts of total sugars. (The term total soluble sugars is here taken to mean the sum of reducing sugars after hydrolysis of both the ethanolic and water extracts.) The

Table II

The stage of growth of the grasses and the crude protein and soluble carbohydrate contents (percentage of dry matter) at each cutting in 1952

Grass	Date cut (1952)	Interval between cuts, days	Yield of dry matter, lb./acre	Leaf–stem ratio (dry wt.)	Average height, in.	Development of growing points, % in floral state	Crude protein	Hexoses	Sucrose	Fructosan	Total soluble sugars
Rye-grass	20 May		1560	1·8	7·8	90	14·1	2·9	5·0	9·0	17·9
	25 June	36	1195	7·0	5·9	30	14·4	1·7	5·4	12·7	21·6
	11 July	16	900	10	6·5	0	26·4	1·3	2·3	0·9	4·6
	29 July	18	1050	8·0	7·0	0	23·0	2·6	6·7	2·6	12·4
	4 Sept.	37	1526	16	7·8	0	16·5	1·7	4·7	4·0	11·6
	21 Oct.	47	1000	31	5·2	0	20·3	1·6	6·9	3·0	12·1
Timothy	12 May		1495	4·3	7·1	70	18·1	1·8	4·8	5·7	12·7
	24 May	12	1100	1·9	6·4	n.d.*	22·6	2·5	6·4	0·7	9·8
	19 June	26	910	1·5	5·5	100	19·1	1·4	5·1	3·8	11·0
	12 July	23	1323	3·7	6·7	0	21·8	1·3	3·6	0·5	5·5
	29 July	17	692	6·0	7·1	0	25·2	1·7	5·8	0·3	8·2
	21 Aug.	23	725	26	7·3	0	25·2	1·3	4·4	0·3	6·3
	30 Sept.	40	1070	25	7·1	0	21·1	0·9	6·1	1·0	8·9
Fescue ..	28 Apr.		1150	2·9	7·1	70	24·8	1·9	5·3	2·9	10·3
	20 May	22	1430	0·9	7·7	50	18·1	2·0	3·8	2·4	8·5
	19 June	29	691	23	5·8	0	18·5	1·8	6·2	5·2	14·0
	3 July	14	719	20	5·9	0	26·1	0·9	5·0	0·4	6·6
	22 July	19	1122	20	8·4	0	25·6	0·9	2·3	0·2	3·5
	20 Aug.	29	744	17	7·0	0	23·1	1·1	3·5	0·8	5·8
	29 Sept.	40	773	33	5·8	0	19·2	1·1	5·6	1·9	9·3
Cocksfoot	7 May		1240	2·1	8·4	70	20·9	2·1	4·3	2·4	9·1
	22 May	15	830	4·8	7·4	25	25·6	0·9	3·5	0·5	5·0
	13 June	22	735	6·3	8·0	0	24·4	1·1	3·9	0·4	5·6
	2 July	19	1045	6·5	8·4	0	26·4	0·8	1·6	0·0	2·6
	22 July	20	1010	6·3	10·7	0	24·9	0·9	1·0	0·1	2·0
	20 Aug.	29	670	13	9·3	0	23·0	0·3	2·1	0·1	2·7
	29 Sept.	39	1370	8	8·1	0	18·5	1·1	3·3	1·0	5·8

* Not determined

values in July, August and September fell to about one-half or one-quarter of the amounts present in May and early June. It can be seen that, of the three fractions contributing to the total soluble sugars (free hexoses, sucrose and fructosan), it was the low values of the fructosan that caused the drop in total sugars. This was to be expected, since the later cuts were predominantly leaf, and leaf has been shown[1] to be generally low in fructosan content. Moreover, the figures in Table III, for the carbohydrate contents of the leaf and stem separately, demonstrate that in grass grown and cut after mid-June both the leaf and the stem individually possessed much less fructosan than before mid-June. It may be noted that in the last cut of three of the grasses and the last two cuts of rye-grass the fructosan content had risen to a higher value. This may have been the result of the slower growth in autumn, when the interval before the last cut was 20–50 days.

Discussion

In Part I it was shown that the rise in fructosan content of the grass, particularly in the stem, was stopped about the time of flower initiation and that some of this carbohydrate was then utilized, most probably by the rapidly-developing growing-point. In the first cut in both years, and also in the second cuts in 1952, the growing-point in a high proportion of tillers had changed to the floral state (Tables I and II). The small size of the primordia and its position near the base of the stem indicated that the change had taken place only a short time before the grass was cut. Thus it would be expected that in these grasses the fructosan content of the stem would be relatively high, and this was generally so. Where the growing season started early, as in 1952, these initial fructosan values were considerably lower than in the colder spring of 1951, and it has been suggested[1] that the rapid early growth gave little chance for a carbohydrate reserve to accumulate. An exception to the general association of a young floral growing-point with a high fructosan value occurred in the second cuts of timothy

Table III

The soluble carbohydrate and crude protein contents (percentage of dry matter) of leaf and stem at each cutting (1952)

Grass	Date cut (1952)	Leaf					Stem				
		Hexoses	Sucrose	Fructosan	Total soluble sugars	Crude protein	Hexoses	Sucrose	Fructosan	Total soluble sugars	Crude protein
Rye-grass	20 May	1·8	3·5	4·8	10·3	16·8	4·4	5·9	15·1	27·4	7·7
	25 June	1·8	5·4	12·1	21·0	15·4	1·1	5·1	16·7	26·1	7·3
	11 July	1·2	2·2	0·4	3·9	27·6	2·4	3·0	5·4	11·6	13·8
	29 July	2·7	6·4	1·7	11·3	24·5	1·7	8·7	9·5	21·4	11·2
	4 Sept.	1·7	4·8	3·2	10·6	17·0	2·0	7·2	16·7	28·2	7·9
	21 Oct.	1·6	6·8	2·4	11·4	20·7	2·4	8·6	20·9	34·6	9·3
Timothy	12 May	1·3	4·4	2·3	8·4	18·5	2·5	2·5	8·7	14·8	11·2
	24 May	2·3	6·7	0·1	9·4	24·5	2·9	3·9	1·3	8·4	16·1
	19 June	1·2	6·3	0·9	8·8	24·0	1·8	3·4	8·0	14·3	11·9
	12 July	1·1	3·6	0	4·8	23·8	2·2	3·4	2·2	8·2	14·6
	29 July	1·6	6·0	0	7·9	26·6	2·6	4·3	2·3	9·7	15·5
	21 Aug.	1·3	4·5	0·2	6·2	25·6	2·4	3·2	1·9	7·9	14·7
	30 Sept.	1·1	6·2	0·6	8·6	21·5	1·4	4·5	10·0	17·2	12·9
Fescue	28 Apr.	1·2	3·8	0·6	5·7	25·2	2·6	3·9	5·1	12·4	15·4
	20 May	1·4	3·1	0·5	5·2	24·1	3·0	3·9	4·0	11·5	12·6
	19 June	1·8	6·2	4·8	13·6	18·9	2·2	5·5	13·9	23·4	9·9
	3 July	0·8	5·0	0·4	6·5	26·7	2·1	4·0	1·5	8·0	15·4
	22 July	0·9	2·3	0·2	3·5	26·0	1·3	1·9	1·0	4·4	16·9
	20 Aug.	1·1	3·5	0·6	5·5	23·7	1·7	3·6	4·0	9·9	13·9
	29 Sept.	1·1	5·6	1·7	9·0	19·2	1·8	5·2	8·5	16·7	12·0
Cocksfoot	7 May	1·3	4·4	0·5	6·5	22·9	4·2	3·3	4·4	12·6	13·8
	22 May	0·5	3·9	0·2	4·6	29·1	1·5	1·6	0·1	3·3	18·5
	13 June	0·8	4·1	0·4	5·5	25·8	2·8	2·7	0·5	6·2	15·9
	2 July	0·6	1·9	0	2·6	28·0	1·8	1·0	0	2·8	15·8
	22 July	0·8	1·0	0·1	2·0	26·4	1·2	0·8	0	2·0	15·5
	20 Aug.	0·1	2·1	0·1	2·5	23·5	2·9	1·8	0·4	5·2	14·8
	29 Sept.	0·9	3·4	0·6	5·2	19·4	2·7	2·8	4·4	10·6	10·6

and cocksfoot in 1952, when the stem fructosan, and hence that of the whole plant, was surprisingly low. The reason for this is not clear, but growth at that time was extremely rapid, the interval between the first and second cuts being 12 and 15 days respectively, which again may have operated against accumulation of fructosan. It is particularly interesting that the cocksfoot, which in general had the greatest weight of stem at each cut, had, after the first cut, the lowest amounts of soluble carbohydrate recorded.

It would therefore appear from the present and the earlier results that grass in which the flower initials have just started to develop will usually contain more soluble carbohydrates than grass in which the growing-point is vegetative, and that the latter condition is to be expected in tillers growing after early June in the latitude (55½° N.) of these plots. The chief factors influencing flower development in perennials are (*a*) exposure to a low temperature during the winter, (*b*) adequate day-length and temperature during the growing period and (*c*) sufficient time to come to the stage of 'ripeness to flower'.[4, 5] Perennial grasses are mainly long-day plants, and, according to Cooper,[4] require a minimum of 11 hours of light per day for flower production. This was available until the end of September; moreover, the temperature was higher in July and August than in May or June. It seems probable, therefore, that after the removal of the early flowering tillers the side buds had insufficient time to become 'ripe to flower', and so only leaves were formed.

Whatever the true explanation is, there seems little doubt that animals grazing young leafy grass in late summer and autumn would take in very different amounts of soluble carbohydrate than from an equal weight of grass of a similar height in the spring. Not only would the actual amount of water-soluble sugars be different, but the ratio of nitrogenous compounds to carbohydrate would be considerably changed. It seems likely that these two differences might affect the performance of cattle subsisting mainly on grazing.

Summary

1. Four perennial grasses—meadow fescue, cocksfoot, timothy and rye-grass—grown in their first and second harvest years (1951 and 1952) were cut whenever a height of 8–10 in.

was reached. This resulted in 4–7 cuts during the season. The yield of dry matter and the stage of growth, as indicated by the leaf–stem ratio and microscopic examination of the growing-point, were recorded at each cut.

2. The grasses were analysed for crude protein and water-soluble carbohydrate, the latter comprising the free hexoses (glucose and fructose), sucrose and fructosan.

3. Grass grown and cut before about mid-June had florally-developing growing-points, a low leaf–stem ratio, and relatively low protein and high soluble-carbohydrate contents. Grass grown and cut after that date was vegetative in character and had a high leaf–stem ratio, high protein and low carbohydrate contents.

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STUDIES ON EGG SHELLS. I.—The Determination of Membrane-, Pore- and Matrix-Protein

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The protein in egg shells consists of the matrix-protein closely associated with the mineral matter, the protein in the pores and a thin cuticle on the outside. In addition, the shell membranes are usually considered under the same heading although, strictly speaking, they are not part of the shell. A method has been devised by which the shell-plus membrane-protein can be divided into three distinct entities, by making use of the different rates at which these entities dissolve on boiling with sodium hydroxide solution. The method is arbitrary, and thus not absolutely accurate, but it appears to give a measure of entities which probably represent (1) membrane- and cuticle- (2) pore- and (3) matrix-protein.

A number of practical problems are associated with egg shells and their properties. Two of the most important are (*a*) the question of microbiological infection *via* the pores, with subsequent rotting of the egg, and (*b*) the question of cracking of egg shells during transport. Much work has already been done on egg shells, but before these problems can be solved further fundamental work is required.

It is clear from the work of Needham[1] that few analytical data for egg shells are available. Those that exist are far from complete and a value for protein is not always given. Further, there is rarely, if ever, any indication whether the analyses refer to shells with or without membranes. This point is important, for Tyler[2] showed that the protein content of the shell plus membranes was exceedingly variable, chiefly owing to variations in the relative amounts of true shell and membrane, whereas the percentage of calcium and carbonate in the nitrogen-free shell material was remarkably constant over a range of hen eggs.

During the course of other work it became apparent to the authors that, not only were figures for total protein required, but if possible some effort should be made to distinguish between membrane-, pore- and matrix-protein. This paper is an account of the attempt to deal with that problem ; hen-egg shells were used for the work.

Briefly, the protein of the shell plus membranes is made up of the membranes themselves on the inside of the shell, the cuticle on the outside of the shell, the plugs in the pores, and the matrix cementing together the mineral matter of the shell.

The authors have tried to determine the membrane-protein, including the small quantity of cuticle-protein, the pore-protein and the matrix-protein. The values have been obtained by multiplying nitrogen values by the factor 6·25, not because this is necessarily correct for all proteins, but because the nature of the proteins in question is not certain and 6·25 represents the generally accepted factor.

Moran & Hale[3] found that the shell membranes consist of mucin and keratin, and Romanoff & Romanoff[4] infer that the pore-protein is similar to membrane-protein, when they state that it stains in the same manner as the shell-membranes. Almquist[5] regards the matrix-protein as a collagen-like substance. It would not seem practicable, at present, to distinguish between membrane-, pore- and matrix-protein by a method dependent on the identification and determination of particular proteins. The authors therefore tried other methods, based on different rates of solution of the three types of protein when exposed to protein solvents.

It is not claimed that the methods to be described determine membrane-, pore- and matrix-protein accurately. Some pore-protein may be included with the membrane-protein, and some matrix-protein with both membrane- and pore-protein. These and other possibilities are bound to make the determinations, to some extent, inaccurate. Nevertheless, the authors feel that the methods employed make it possible to determine certain distinct entities and that the evidence suggests that these entities correspond, though not necessarily accurately, to membrane-, pore- and matrix-protein. Despite these limitations, these terms have been used in the paper instead of others which might be more accurate but at the same time more cumbersome.

Method of analysis

All nitrogen determinations were carried out by means of the Kjeldahl digestion process, followed by distillation on a semi-micro scale.

Shell membrane

The obvious method of removing the membranes is to moisten them with water and then to scrape them off with a knife. This, however, is a very laborious task, and, what is more important, is not reliable. Sometimes, after scraping, particles of membrane are left on the shell, and at other times scraping is too thorough and particles of true shell are removed. The solvent method was therefore tried and after a considerable amount of preliminary work, chiefly with sodium sulphide and with sodium hydroxide, the authors concluded that 2·5% sodium hydroxide solution seemed most efficient, so this was investigated in more detail.

An egg shell with membranes was washed and dried, broken into pieces of approximately 1-sq. cm. area, and then the pieces were shared out at random to give a number of samples, which were weighed. One sample was left untreated, and the others were boiled separately for different lengths of time, up to ten minutes, in 2·5% sodium hydroxide solution. At the end of the appropriate time the solution, along with the shell particles, was tipped into a large volume of cold water to stop the reaction at once. The sample was then washed thoroughly and dried. Nitrogen was determined on all samples, and Table I shows the values obtained from three different eggs, which are typical of many others.

From these data, it would appear that there is a first stage when protein is being destroyed rapidly and a second stage during which there is practically no further loss up to ten minutes. The two stages overlap and this has been shown in the Table by figures in italics. The data in the overlap were disregarded and regression lines and correlation coefficients corresponding to the two stages were calculated; they indicate a highly significant relationship. The equations for the two stages, values of r and values for the point of intersection of the two lines, are given in Table II. It will be observed that in all cases the first stage is represented by a steeply sloping line and the second by an almost horizontal line. The point of intersection is at 2·8–3·7 minutes. The chain of events that is seen to occur while the shell particles are being boiled indicates that removal of the membranes is being achieved and it supports the analytical figures given in Tables I and II. Soon after being placed in the boiling sodium hydroxide solution the membranes on the pieces of shell begin to rise from the shell and form a blister, which enlarges and finally covers the whole area of the shell. The membranes then break away and float in the liquid while the shell sinks. Judged by their appearance when

Table I

The variation in the action of 2·5% sodium hydroxide solution on egg shell plus membrane as influenced by time of boiling

Time, min.	Percentage protein Shell 1	Shell 2	Shell 3
0·0	5·76	5·03	4·77
0·5	5·53	4·89	4·42
1·0	5·23	4·22	4·10
1·5	4·64	3·91	3·69
2·0	3·42	2·38	3·32
2·5	*1·95*	*2·10*	2·82
3·0	1·99	2·09	*1·95*
3·5	*1·87*	*1·83*	*1·70*
4·0	1·79	1·81	1·64
4·5	1·80	1·78	1·60
5·0	1·80	1·72	1·60
6·0	1·78	1·78	1·57
7·0	1·78	1·72	1·53
8·0	1·78	1·71	1·56
9·0	1·74	1·71	1·56
10·0	1·69	1·69	1·51

Table II

Linear regression lines for the two sections of the curve as divided in Table I, with values for their points of intersection

Shell	Steep section: Pairs of readings	Regression line	r	Flatter section: Pairs of readings	Regression line	r	Point of intersection: Time (x), min.	Protein (y), %
1	6	$Y=6{\cdot}28-1{\cdot}48x$	$-0{\cdot}94$***	8	$Y=1{\cdot}87-0{\cdot}015x$	$-0{\cdot}86$**	3·02	1·83
2	6	$Y=5{\cdot}36-1{\cdot}29x$	$-0{\cdot}97$**	8	$Y=1{\cdot}85-0{\cdot}016x$	$-0{\cdot}83$*	2·77	1·81
3	7	$Y=4{\cdot}91-0{\cdot}89x$	$-0{\cdot}98$***	8	$Y=1{\cdot}68-0{\cdot}017x$	$-0{\cdot}87$**	3·70	1·62

* Significant at $P = 0{\cdot}05$ ** Significant at $P = 0{\cdot}01$ *** Significant at $P = 0{\cdot}001$

they break away, much of the membrane must have already dissolved, and further boiling results in complete solution.

Microscopic examination of those shells that had been treated for five minutes or more showed a clean inner surface with no loose protein fibres, such as are found when inadequate scraping has not removed all the membrane. Further, the inner surface of mineral particles was not broken as it frequently is when scraping has been too drastic.

It should be further observed in Table II that for the second stage the coefficient of x is always negative, very small, and in all three cases is very similar. This indicates that the rate of attack on the protein of the true shell, as distinct from the protein of the membrane, is very small between about three minutes and ten minutes. It also shows that the protein of the true shell is, in each case, attacked at about the same rate.

Examination of the values in Table I at 4·5, 5 and 6 minutes' boiling shows that they are very similar for each particular shell, although they represent different samples of that shell and slightly different times of boiling. This strongly suggests that replicate results for a given shell and a fixed time of boiling would be in close agreement.

On the basis of these results, it was decided to use 2·5% sodium hydroxide solution and to boil for 5 minutes to remove the membrane. Shells treated in this manner will be referred to throughout the paper as stripped or true shells.

Pore- and matrix-protein

It is generally believed that the protein of the true shell consists of (*a*) the plugs of protein filling up the pores and (*b*) the matrix-protein cementing the mineral particles together. The fact that the latter must be very intimately mixed with the mineral substance is suggested by the observation made by the authors that pieces of shell kept in warm 20% sodium sulphide solution for six months showed no sign of disintegration. If this is so, then with particles of shell the plugs of protein exposed at their ends might be readily attacked by a protein

solvent and ultimately dissolved completely, while only the matrix-protein on the surface, but not that on the inside of the particles, of the shell would be dissolved. With shell ground to a fine powder, most of the plugs of protein would be quickly attacked by a protein solvent. On the other hand, the total surface area would be large, resulting, not only in a large amount of matrix-protein going into solution, but also perhaps in the further disintegration of the shell, with possible loss of mineral matter. Preliminary experiments with finely ground shell confirmed that the amounts of shell lost on boiling with various strengths of sodium hydroxide solution were far greater than that caused by the removal of the protein, and that ground material was therefore quite unsuitable for the work. This loss occurred in the manipulations between boiling and the determination of nitrogen, and is probably associated with the formation of particles of mineral matter small enough to pass through the filter paper. Since nitrogen is determined in the residue on the filter paper but calculated on the original weight of ground shell taken, any loss of material will lead to error.

It is clear that ground egg shells could not be used, but it was still necessary to consider what size of particle should be used. When shells are broken, lines of fracture will pass through some pore plugs and thus expose their sides as well as their ends to attack by protein solvents. The smaller the particle size, the greater will be the number of pore plugs exposed at their sides as well as at their ends; hence, the quicker will be the effect of the protein solvent. This is desirable because it will simultaneously cut down the length of time that the matrix-protein is exposed to attack. Thus, to avoid finely ground material and yet to ensure reasonably quick attack on the pore-protein, a particle size in the range 1–2 mm. was decided on, after preliminary tests had proved it suitable. In all further experiments material passing a 2-mm. sieve but retained by a 1-mm. sieve was used.

The next step was to ascertain the most suitable strength of sodium hydroxide solution to use and the length of time to boil. Four stripped shells were broken up and sieved to give the correct grade of material; this was well mixed and divided into sub-samples. The sub-samples were each weighed and then treated for a specified time with solution of a specified strength; heating was carried out on a boiling-water bath under reflux (95°). The results are set out in Table III.

Table III

Percentage protein in shell after treatment with sodium hydroxide solutions of different strengths for different lengths of time (original protein, 1·65%)

Strength of NaOH, %	Percentage of protein after heating for (h.)							
	0·5	1·0	2·0	3·0	4·0	5·0	7·0	12·0
5·0	1·45	1·39	1·20	1·05	1·00	0·94	0·92	0·76
7·5	1·38	1·34	1·09	0·91	0·87	0·83	0·80	0·80
10·0	1·20	1·15	0·93	0·89	0·87	0·83	0·79	0·74
12·5	1·20	1·13	0·96	0·90	0·83	0·83	0·81	0·77
15·0	1·18	1·04	0·92	0·84	0·87	0·84	0·73	0·70
20·0	0·98	0·76	0·67	0·60	0·57	0·57	0·48	0·47

Table III indicates that 7·5, 10·0 and 12·5% sodium hydroxide solutions give very similar results, particularly by the time the values for each strength of solution are reaching a fairly constant figure at about 7 hours. The same degree of dissolution is not produced by 5% solution in 7 hours, and the 15% solution seems to go a little beyond the 7·5, 10·0 and 12·5% solutions. The values for the 20% solution are quite anomalous. In this experiment the weight of shell material before and after treatment was known, and it was observed that with 7 hours' boiling 20% sodium hydroxide solution caused a loss of 15% by weight of shell. This is far in excess of the loss due to protein, and explains why the results for this strength of solution are poor. The loss at 7 hours for the 15% solution was 8%, which is still too great, but the losses for solutions of 12·5, 10 and 7·5% were only 4·0, 0·4 and 0·2% respectively. It is evident that these three strengths, particularly 10% and 7·5%, are suitable, because they give values unaffected by losses of shell material.

It was therefore decided to use 10% sodium hydroxide solution for the removal of pore-protein. There was already strong evidence from Table III that 7 hours was sufficient to reach a maximum degree of solution of protein, but one further experiment was carried out to verify this.

Six stripped shells, each broken to the correct grade, were treated separately with 10% sodium hydroxide solution. Two weighed samples were taken from each shell; one portion was treated for 7 hours and the other for 12 hours. The results are shown in Table IV. It

Table IV

Percentage protein in true shell after treatment with 10% sodium hydroxide solution for 7 or 12 hours

Shell	0 h.	7 h.	12 h.
1	1·64	0·73	0·70
2	1·45	0·83	0·82
3	1·42	0·82	0·80
4	1·75	0·78	0·76
5	1·59	0·87	0·86
6	1·52	0·83	0·82

is clear that the removal of the protein soluble in 10% sodium hydroxide solution is virtually complete in 7 hours, since only a very small decrease in protein content occurs in the next 5 hours. Thus it appears that with particles of a size 1–2 mm. there is some form of protein that is readily removed in about 7 hours by boiling with 10% sodium hydroxide solution, and that further protein is not apparently removed up to 12 hours. The authors consider that the protein removed therefore represents an entity and is probably the pore-protein, and that that remaining behind is the matrix-protein.

Suggested procedure

A standard procedure can now be suggested.

(*a*) *Membrane-protein.*—An egg shell with membranes intact is washed and dried. A weighed sample of this is then taken and the percentage of total protein (N × 6·25) is determined. A further weighed sample, consisting of pieces each about 1 sq. cm. in area, is then dropped into boiling 2·5 sodium hydroxide solution and kept there for five minutes. Suitable quantities are 0·5 g. of shell and 30 ml. of solution. At the end of the time the boiling solution and shell particles are tipped into a large volume of cold water to stop the reaction. The pieces of shell are then removed, washed thoroughly and dried. The percentage of total protein in this stripped (true) shell is then determined. From these two values it is possible to calculate the amount of membrane as a percentage of either the original shell plus membrane or as a percentage of the true shell. Although it is perhaps less correct, the authors have calculated it as a percentage of the true shell, so that values can be compared directly with the other data on pore- and matrix-protein that are correctly expressed as a percentage of true shell.

(*b*) *Matrix-protein.*—A weighed sample of the stripped shell, broken to pass a 2-mm. sieve but to be retained on a 1-mm. sieve, is heated on a boiling-water bath under reflux (95°) with 10% sodium hydroxide solution for 7 hours, then removed, washed thoroughly and dried. Suitable quantities are 0·5 g. of shell and 50 ml. of solution. The amount of protein in this sample is then determined and expressed as a percentage of the true shell. This protein represents the matrix-protein.

(*c*) *Pore-protein.*—The difference between the values for percentage of total protein in the stripped shell determined in (*a*) and the percentage of matrix-protein in the stripped shell will give the percentage of pore-protein in the stripped shell.

Some typical values

Table V gives a set of typical values for hen-egg shells, with membranes, obtained by the procedure outlined above. It will be seen that the greater part of the protein is membrane-protein, that the matrix-protein and the pore-protein are much smaller, and on the average about equal in amount and fairly constant, but that the pore-protein is more variable than the matrix-protein.

Discussion

It is recognized that the procedure is somewhat arbitrary, but the authors believe that, until some better method can be found, it will provide useful comparative data.

With this method the authors hope to obtain information about egg shells from a number of different species of birds. Further, it should be possible to study the question of the possible relationship between the three forms of shell-protein, porosity coefficients as measured by Tyler's method,[6] and pore counts.

With shells of other birds it might be necessary to alter the times of boiling, both for membrane- and pore-protein removal, but this does not affect the underlying principle.

Table V

Typical values for membrane-, pore- and matrix-protein in normal hen-egg shells (plus membranes), expressed as percentages of true shell

Egg	Protein		
	Membrane	Pore	Matrix
1	7·25	0·83	0·76
2	6·44	0·79	0·85
3	6·00	0·83	0·81
4	5·94	0·74	0·78
5	5·51	0·78	0·80
6	5·38	0·77	0·71
7	5·29	0·99	0·73
8	5·04	0·65	0·89
9	4·97	0·61	0·81
10	4·41	0·84	0·77
11	4·11	0·72	0·78
12	3·86	0·75	0·76
Mean	5·35	0·78	0·79
Coeff. of variation	18·2	12·6	6·3

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STUDIES ON EGG SHELLS. II.*—A Method for Marking and Counting Pores

By C. TYLER

A method has been developed for marking the pores in egg shells by immersing them in concentrated nitric acid for very short periods of time. This makes the pores easily visible under the microscope, and counting can then be done by a statistical method. Not only is the total number of pores determined, but also a frequency distribution is obtained which indicates that the pores are not distributed at random.

Numerous workers, e.g.,[1–3] have described methods for marking pores in egg shells and estimates have been made of the number of pores in an egg shell. In every case the method has depended upon a staining technique, followed by a direct count or a qualitative assessment. Bryant & Sharp,[4] however, created a vacuum over water in which the egg was immersed. From the shell rose bubbles, whose sources were regarded as pores. Very low values, up to 50 pores per egg, were obtained, but these authors admitted that probably many other smaller pores existed. As far as the present author is aware the problem of counting the pores has never been approached statistically; neither has any method, other than staining, been used for marking the pores, with the one exception mentioned. It is the purpose of this paper to describe a new method of marking the pores and a statistical approach to the problem of counting them.

* Part I: *J. Sci. Fd Agric.*, 1953, **4**, 261

Method of counting

It is essential first to obtain a reliable method of counting. To attempt to count all the pores in a shell is almost impossible, and therefore a random sample of shell must be used. The method finally evolved which logically meets this requirement was as follows: An ordinary low-power microscope (eye-piece × 10; objective × 10) had the tube-length so adjusted that the observed field of view of an object placed on a slide on the stage was exactly 1 sq. mm. in area. With such an arrangement the object was not necessarily in perfect focus, but this is not essential when the problem is merely one of counting very obvious marks.

A piece of shell, about 10–15 sq. mm. in area, with pores suitably stained or otherwise marked, was taken at random, placed on a slide on the microscope stage and the pores in the 1-sq. mm. field counted. The only precaution taken was to ensure that the whole field was occupied by shell, otherwise any sq. mm. area on the shell was viewed at random. The process was repeated for x pieces of shell and the sum of all the counts gave the number of pores in x sq. mm., from which the number of pores per unit area and per egg could easily be calculated. This method has a further advantage in that it gives additional information. Counts of this type were found to give values from none to about 6 pores per sq. mm. with 1, 2 or 3 pores usually occurring most frequently. Counting of such numbers, where the chance of occurrence is small, has analogies with the Poisson distribution. It should therefore be possible, not only to compare the frequency distributions for individual shells, but also to determine whether or not they are Poisson distributions. This point is discussed below.

Before this method of counting was finally decided on, it was necessary to determine how many pieces of shell should be used to give a reliable count. Obviously, with only 10 or 20 pieces of shell, i.e. 10 or 20 separate sq. mm.-areas counted, the error would be great. Six samples of 50 pieces each from the same shell were therefore counted and these were used to calculate the mean count, its standard deviation and the coefficient of variation. Successive pairs of samples of 50 pieces were then combined, giving three samples each of 100 pieces, and similar statistics were calculated for these. The results are set out in Table I; it is evident that the coefficient of variation varies greatly with different shells, but that the counts of 100 pieces, in every case, show a smaller coefficient of variation than the counts of 50 pieces. Further, with one exception the counts of 100 pieces have a coefficient of variation of less than 5%. Many biological measurements tend to give coefficients of variation of at least this value, and often greater, so that it was thought that counts of 100 pieces would be suitable. The 100 pieces will also give the count for 1 sq. cm. directly.

Table I

Values for the coefficient of variation as influenced by different shells and by size of sample in the same shell

Shell	Size of sample, pieces	No. of samples	Mean count	Standard deviation	Coeff. of variation
1	50	6	105·2	3·66	3·48
	100	3	210·4	4·04	1·92
2	50	6	74·5	6·38	8·56
	100	3	149·0	10·15	6·81
3	50	6	79·0	8·17	10·34
	100	3	158·0	7·81	4·94
4	50	6	79·8	0·98	1·23
	100	3	159·6	0·58	0·36
5	50	6	80·5	3·94	4·89
	100	3	161·0	7·21	4·47

Marking the pores

Other work on egg shells is being done by the author, and counting of pores has therefore to be done on egg shells that have been dried with the membranes on. The application of aqueous solutions of dyes to the membrane side of these shells, even for long periods, results in few, if any, stains on the outer surface. It is therefore necessary to remove the membrane in order that the dye placed on the inner surface can penetrate through the pores and show as spots or stains on the outer surface. The membrane was removed by the method of Tyler & Geake,[5] and it was then found that the dye penetrated almost instantaneously through the

treated shells. Dyes such as methylene blue and crystal violet in 1% aqueous solution gave discrete spots on the outer surface, but Congo red gave spots which quickly spread and often merged into each other. Alcoholic solutions of dye went through very rapidly but the spots quickly spread like those of Congo red.

The counting method described above makes it essential that there should be no doubt as to whether the mark of a pore is in or out of the microscope field. Even with spots made by methylene blue or crystal violet this is not always easy, because under the microscope some of the spots are very indistinct in outline and shade off imperceptibly into the surrounding unstained shell.

The author decided, therefore, to try an entirely different approach. It was thought that if the shell was dipped in acid for a short time, the acid might perhaps attack the pores more readily and enlarge them, until they were visible under the microscope, long before the shell was decreased in thickness sufficiently to break up. Tests showed that dilute acids acted slowly, over the whole shell. Concentrated hydrochloric acid acted quickly at first, but then a scum of shell protein on the shell slowed down the reaction. Finally concentrated nitric acid was tried and it worked very well. Immersion times of 20–25 sec. for hen-egg shells from which the membranes had previously been removed resulted in the appearance of very clearly defined, almost circular, holes in the shell. Under the microscope, with transmitted light these could be seen as full white circles on a dark background. There could be no error in deciding whether or not a hole was there, and even where two holes had coalesced it was still obvious that there had been two holes originally, because the ' double ' hole had the shape of a solid figure of eight. Further, the definite holes made it possible to judge whether a hole should be counted in the field or not. Thus there was no doubt that the holes produced in this way were ideally suited for the counting technique suggested and were much better for this purpose than spots of dye.

To speed up the immersion process a wooden holder was made. Two grooves were cut in the surface of a block of wood 4 in. × 2 in. × ½ in., each groove being ¼ in. deep and ¼ in. wide. The grooves were parallel to the long side and were 1 in. apart. Between these grooves a vertical handle, 3 in. long, was fixed. Paraffin wax, heated until it was soft, was inserted in the grooves, about 20 pieces of shell, each 1–2 sq. cm. in area, were pressed into the soft wax so that they stood up vertically, and the wax was then allowed to set. The holder was next taken by the handle and held for exactly 25 sec. in a bath of concentrated nitric acid, care being taken to see that the pieces of shell were completely immersed. It was then removed, plunged immediately into cold water and finally washed under the tap. Afterwards the pieces of shell were broken off about 1 mm. from the surface of the wax, dried and broken up to give a large number of smaller pieces for counting.

Examination of the pieces of shell left in the wax showed clearly that the nitric acid attacked them as far as the surface of the wax, so that, by breaking off about 1 mm. above this surface, there was no danger of including in the sample areas of shell that had not been fully attacked.

Before proceeding further, it was necessary to show that the holes developed by nitric acid coincided with the spots made by the dye, i.e. that they represent pores and are not artifacts. To do this, a piece of shell without membrane and about 1 sq. cm. in area was taken. This was treated on the inner surface with crystal violet, and the usual spots appeared on the outside. A photograph of the outer surface of this piece of shell was taken by reflected light. The piece of shell was then immersed in concentrated nitric acid for 5 seconds, washed, dried and photographed again in exactly the same position in relation to the camera as before, but with transmitted light. The process was repeated by dipping for a further 5 seconds, making 10 seconds in all, and so on up to a total of 35 seconds in 5-second intervals, with a photograph at each stage. All the photographs were then enlarged to exactly the same extent and from each a tracing was made. These tracings were then transferred to a single sheet of paper, all of them being accurately superimposed one upon the other; Fig. 1 shows the result. In Fig. 1 a portion of the shell has been marked off in order to avoid the edges. The reason for this is that some erosion takes place at the edges and, whereas a stain might show very close to the original edge, no hole shows after 25 seconds, not because there was no hole corresponding to that stain, but because that part of the edge containing the hole has itself been dissolved. Considering then the area enclosed in the rectangle ABCD it will be seen that most of the holes developed by nitric acid correspond exactly with the stains made by the dye, that some single stains correspond to two closely adjacent holes, which in some cases have run together but form the typical figure of eight instead of a circle, and that a few holes have appeared, usually after about 25 seconds, where there was no dye stain.

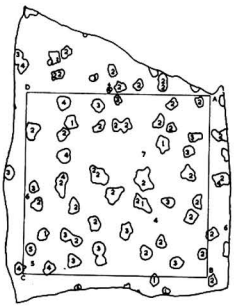
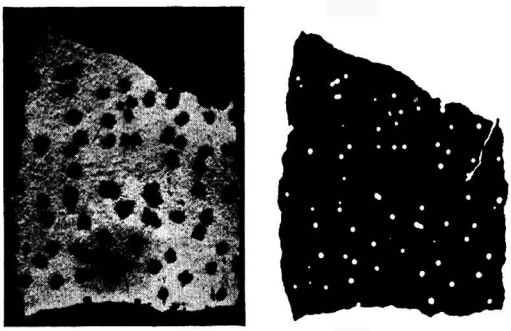


FIG. 1.—*Diagram of a piece of shell showing areas which were stained with dye. Within these areas the numbers represent times at which holes appeared when the shell was immersed in nitric acid* (1 = 5, 2 = 10, 3 = 15 *sec., and so on for other figures*)



(*a*) (*b*)

FIG. 2.—*Photograph of a piece of shell* (*original size about* 1 *cm. each way*)

(*a*) *Showing pores stained with crystal violet* (*reflected light*)

(*b*) *After* 25 *seconds' immersion in concentrated nitric acid* (*transmitted light*)

There seems to be no doubt that, in general, the stains made by the dye and the holes made by nitric acid are both manifestations of the same thing, namely pores. The nitric acid method gives the higher and presumably the more correct count, because it clearly distinguishes between two closely adjacent pores which may show as only one stain when a dye is used. The only difficulty is that the nitric acid reveals holes which are not shown up with a dye. This may be because some pores are blocked somewhere along their length, thus preventing dye moving along, but not preventing the action of nitric acid. Thus it must be recognized that the nitric acid method probably gives the maximum pore count. Fig. 2 shows photographs of the stained piece of shell and the same piece after 25 seconds' dipping in nitric acid.

The work was then continued quantitatively by counting 100 pieces of shell stained with dye and comparing with 100 pieces of the same shell treated with nitric acid for 25 seconds. Table II gives the results for five different shells, and it will be seen that the nitric acid gave a somewhat higher count. The difference between any pairs of counts will include errors of sampling, occasional counting of two pores with acid against only one with dye, and the counting of ' blocked ' pores which appear only in the acid treatment. Thus since the differences are small there can be little variation caused by counting ' blocked ' pores. It would thus appear that the nitric acid method is a very reliable method of making pores visible for counting.

Table II

Pore counts in 1 *sq. cm. of shell as given by the staining and nitric acid method on the same shell*

Shell	Staining	Nitric acid
1	142	143
2	151	161
3	133	138
4	138	144
5	161	172

Another point to be settled was the time of immersion, and some guidance was obtained from the photographs mentioned previously. The number of holes appearing in the rectangle ABCD at each 5-second interval has been set out in Table III. From this it is evident

Table III

Number of pores appearing in a given area of shell after different times of immersion in concentrated nitric acid

Time of immersion, sec.	Pores appearing at 5-sec. intervals	Cumulative count
5	4	4
10	23	27
15	12	39
20	5	44
25	3	47
30	1	48
35	1	49

Staining gave 40 pores in this area of shell

that after about 20–25 seconds the maximum has almost been reached. This is fortunate, for after 30–35 seconds the shell disintegrates because of its extreme thinness. A further set of data obtained in rather a different manner, but supporting this time of 20–25 seconds, is given in Table IV. In this case five samples were taken from the same shell and each sample given a different time of immersion, followed by counting in the usual way. The maximum was reached much earlier, but 20–25 seconds gave a reliable figure ; also, although at 25 seconds the shell was exceedingly thin, yet not even then did holes appear as artifacts, since the count at 25 seconds is very little greater than at 20 seconds.

Table IV

Pore counts in 1 sq. cm. of shell after different times of immersion in concentrated nitric acid

Time, sec.	Pore counts	Remarks
5	128	Extremely small holes
10	144	Still many small holes
15	152	Holes all circular, but some still small
20	152	All holes circular and easily seen
25	156	Large holes, very thin shell
30	—	Shell broke up

Finally, it was decided to test whether the removal of protein plugs from the pores would have any effect on this method. The method of Tyler & Geake[5] was used to remove the protein plugs, but heating with the 10% sodium hydroxide solution was continued for 12 hours and samples of shell were withdrawn at 2-hourly intervals. Each of these samples were then immersed for 25 seconds in nitric acid and counts made. Table V shows the results, and it is evident that whether the pores are plugged with protein or whether this protein has been partially or wholly removed the method gives the same pore-count, within the limits of experimental error.

Table V

Pore counts in 1 sq. cm. of shell as influenced by boiling the shell with 10% sodium hydroxide solution for different periods of time

Time of boiling, h.	Pore counts
0	83
2	85
4	85
6	84
8	81
10	81
12	81
Mean	82·9
Standard deviation	1·865
Coeff. of variation	2·25

Suggested procedure

A standard procedure can now be suggested.

The membranes are first removed from a random sample of the shell by the method of Tyler & Geake.[5] About 20 pieces of this true shell, each about 1–2 sq. cm. in area, are then taken at random and fitted into the holder already described. These pieces are immersed in concentrated nitric acid for exactly 25 seconds, then washed in water and finally broken off about 1 mm. above the surface of the wax. The pieces of shell are then dried and broken to give about 150–200 smaller pieces of various sizes. One hundred of these are then taken at random.

Each one is, in turn, placed on the platform of a microscope set to view 1 sq. mm. of the surface of the object, and a random view is taken of 1 sq. mm. of the piece of shell.

The number of pores is recorded 0, 1, 2 . . . or n. The total number of pores in 100 pieces examined represents the number per sq. cm.; in addition the frequency distribution is obtained.

To calculate the number of pores per egg the number per sq. cm. is multiplied by the surface area, which can be obtained from the formula of Mueller & Scott:[6] $S = 4{\cdot}67W^{2/3}$, where S represents the surface area of the egg, in sq. cm., and W the fresh weight of the egg, in g.

Some typical results

In Table VI the results from some normal egg shells are given. It will be observed that there is considerable variation in pore counts per sq. cm. and also per shell. Counts per shell

Table VI

Some typical pore counts in hen-egg shells

Shell	Count/sq. cm.	Count/shell
1	101	7161
2	112	8221
3	166	12,450
4	193	13,047
5	221	15,072
6	237	17,088

vary from 7000 to 17,000, but according to Romanoff & Romanoff,[7] who quote two other workers beside themselves, the value is about 7500; at the same time they admit that, because the pores in an egg shell are numerous, and counting is laborious, there is little information concerning the numbers present. The explanation of the much larger numbers obtained by the author is probably twofold. First, it has been shown clearly that the nitric acid method gives higher but more accurate counts than the staining method. Secondly, inspection of Table II shows that even the counts made after staining are much greater than 7500. If it is assumed that the shells in this test had a surface area of about 70 sq. cm. then the pore counts per shell vary from 9310 to 11,270 even with staining, and it may be that the removal of the membrane before staining has something to do with it, particularly since the dried membrane on a shell allowed no aqueous dye to pass. All other workers who used a staining technique left the membrane on. Rizzo[1] and Stewart[3] obtained results of about 7000, and in each case the aqueous dye was allowed to come outwards. Almquist & Holst[2] obtained lower results, but they allowed an alcoholic solution of dye to move inwards.

Mention has been made above of the fact that this method of counting enables the frequency distribution of counts per sq. mm. to be obtained. Table VII gives two examples of counts, one of 320 pieces and one of 640 pieces, on different shells. The full frequency distribution is given and has been compared with the Poisson distribution. From this comparison, made by means of the χ^2 test, it is clear that neither shell shows the Poisson distribution, and, since this would occur if the pores were distributed at random, it follows that in these two shells the pores were not distributed at random. Further examination shows that the frequency of the counts directly on each side of the mean count tend to be greater and other frequencies to be less than with the Poisson distribution. No further comment on these results will be made now, but counts are being made on many more shells, after which further discussion may be possible.

Table VII

Frequency distributions of pore counts per sq. mm. for two different shells and a comparison with the Poisson distribution

Count	Actual frequency	Theoretical frequency, m (Poisson)	Difference, d	d^2/m
Example 1				
0	11	39·1	− 28·1	20·19
1	70	82·2	− 12·2	1·81
2	140	86·5	+ 53·3	33·09
3	74	60·6	+ 13·4	2·96
4 and over	25	51·8	− 26·8	13·87
	320	320·2		71·92 = χ^2
Mean count = 2·10				
Example 2				
0	80	136·5	− 56·5	23·39
1	255	210·9	+ 44·1	9·22
2	209	162·9	+ 46·1	13·05
3	72	83·9	− 11·9	1·69
4 and over	24	45·1	− 21·1	9·87
	640	639·3		57·22 = χ^2
Mean count = 1·55				

$P = 0{\cdot}001$; $n = 3$; $\chi^2 = 24{\cdot}32$

Conclusions

By the method of marking and counting pores described above it will be possible to compare counts and frequency distributions for different shells and to compare the distribution of pores in different parts of the shell. It will also be possible to compare pore-counts with such other values as pore-protein and porosity.

It is intended to use this method with the shells of eggs from other birds, but it is clear that 25 seconds' dipping in concentrated nitric acid will be too long for very thin shells, and the time may be too short for very thick shells. It will therefore be necessary to carry out tests to discover the appropriate immersion times for each type of shell, but this in no way detracts from the general principle of the process.

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THE SEED FAT OF THE KERGUELEN CABBAGE *(PRINGLEA ANTISCORBUTICA)*

By H. H. HATT and A. Z. SZUMER

The seed fat of the Kerguelen cabbage, an isolated member of the Cruciferae, has been analysed. The fatty acids have been found to include erucic acid as a major component (13·5%). *cis*-11-Eicosenoic acid is also present in approximately equal amount (13·8%), together with linolenic acid (30·3%) and linoleic acid (20·1%). Other acids are present in smaller amounts.

Analysis of the fat has been made by ester distillation and also by amplified distillation of the methyl esters of the saturated acids and of the hydrogenated acids. The merits of the two methods are compared. The oxidation of the monoenoic acids to dihydroxy-acids with performic acid is a convenient method for their characterization.

The Kerguelen cabbage (*Pringlea antiscorbutica* Hook, fl.) is a member of the Cruciferae family which is restricted in its range to the isolated islands of the Kerguelen–Heard and Prince Edward–Crozet groups in the Southern Ocean.[1, 2] On these treeless islands it is the largest and most striking of the few flowering plants, for it can attain a height of three to four feet.[3] It seeds prolifically and the small heart-shaped seeds are said to be the chief food of the local teal.[4] It seemed of interest to discover whether the seed fat of this isolated species of the Cruciferae would contain the erucic glycerides usually present in the fats of this family, and also whether those of eicosenoic acid were present, for these have recently been found in appreciable amount in the seed fats of some Cruciferae.[5–8] A preliminary investigation[9] had already shown that the saponification and iodine values of this fat were unusually high for a fat of the Cruciferae.

The seeds used were kindly collected for us at various times by members of the Australian National Antarctic Research Expedition and of the Mission Française à l'Archipel de Kerguelen. The yields of fat from the four samples and some physical and chemical constants are compared in Table I. All samples have high saponification and iodine values, and it is noteworthy that the two from Heard Island have iodine values (150) considerably above the iodine values (145) of the two from Kerguelen Island. This conforms with the generalization that the iodine value of a seed fat increases with the latitude of its source.

Table I

Physical and chemical constants for samples of seed fat

Sample	1	2	3	4
Place of collection	Kerguelen I.	Kerguelen I.	Heard I.	Heard I.
Date of collection	March 1949	March 1951	May 1949	July 1951
Wt. of 1000 seeds, g.	3·70	2·97	1·92	1·40
Fat content, %	13	22·1	25·1	18·5
Moisture in seeds, %	16·1	—	9·5	8·3
Refractive index of fat (n_D^{25})	1·4755	1·474	1·475	1·475
Acid value	—	—	9·6	8·3
Saponification value	184	180·5	187	186·2
Iodine value (Wijs)	144·8	144·8	149·6	149·9
Conjugated dienoic glyceride (as linolein)	—	1·37	1·40	0·58
Conjugated trienoic glyceride (as linolenin)	—	0	0	0
Diallylic glyceride (as linolein) by isomerization	—	19·5	20·1	18·2
Triallylic glyceride (as linolenin) by isomerization	—	31·9	30·3	33·0

Composition of the fat

Sample 3 was the only large one (1580 g.) and the fuller chemical examination was made on the fat from it. Further properties of this fat are given in the Experimental section. The small percentage of non-saponifiable material present (1·7%) and the yield of glycerol (9·1%) show it to be a true fat; the low optical activity and low Reichert–Meissl and Polenske values show that glycerides of hydroxy-acids are probably absent, and that there can be little of those of lower fatty acids present.

The mixture of fatty acids was analysed by two methods. In the first the conjugated dienoic and trienoic glycerides were determined in the fat spectroscopically, and then diallylic and triallylic glycerides* were determined spectroscopically after isomerization. The methyl

* It seems desirable to assign some general name to the unsaturated fatty acids that can be determined spectroscopically after isomerization with alkali. Since they contain two or more adjacent allylic units ($-CH_2-CH=CH-$), it is suggested that they be called the polyallylic acids

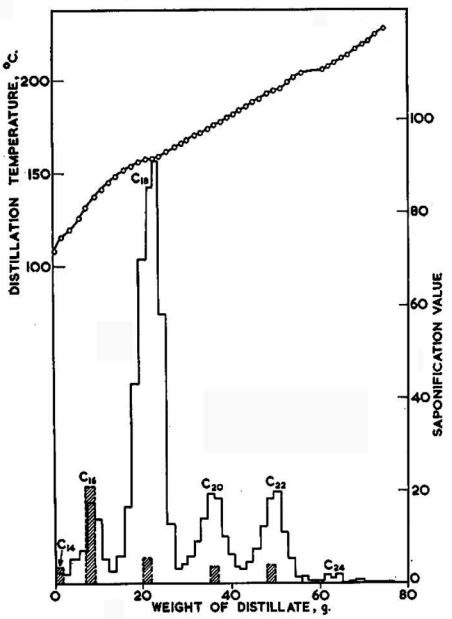

FIG. 1.—*Amplified distillation of the hydrogenated methyl esters*

esters of the mixed fatty acids were then prepared, purified by rapid distillation under reduced pressure, hydrogenated, and a small quantity (5·1 g.) was analysed by the method of amplified distillation.[10, 11] The record of the distillation is given graphically in Fig. 1 and the calculated composition in column I of Table II. The saturated acids in the fat itself were then determined and isolated by the method of Bertram. Two determinations, each on 5 g., gave the values 7·42, 7·67%. The saturated acids obtained were esterified quantitatively with diazomethane and the esters were analysed by amplified distillation. The results are given in column II of Table II and are shown as proportionate areas beneath the graph for the analysis of the hydrogenated esters (Fig. 1). Combination of these three sets of data gives the composition of the fat recorded in column III of Table II. Less than 20 g. of fat was needed for this analysis.

The second method used the more conventional ester fractionation. The mixed fatty acids were resolved into three groups by crystallization from acetone at − 30 and − 60° and the amounts of di- and triallylic acids in each fraction were determined spectroscopically. Table III shows the results.

Subsequent analysis was by distillation of the derived methyl esters, combined with determinations of saponification and iodine values on the fractions made. For the plateau fractions from distillations II and III these were

Table II

Composition of the fat obtained from amplified distillation of the hydrogenated esters and saturated (Bertram) esters

Acid	Composition of hydrogenated esters, %	Composition of saturated esters, %	Composition of the fat, %
Myristic	0·5	10	0·5
Palmitic	8·0	50	3·7
Stearic	60·6	15	1·1
Arachidic	14·8	10	0·8
Behenic	14·8	15	1·1
Tetracosanoic	1·3	0	0
Hexadecenoic			4·1
Oleic			7·9
11-Eicosenoic			13·8
Erucic			13·5
Tetracosenoic			1·3
Linoleic			20·1
Linolenic			30·3
Non-saponifiable ..			1·7

Table III

Crystallization of fatty acids from acetone

	Fraction I (crystallized at − 30°)	Fraction II (crystallized at − 60°)	Fraction III (soluble at − 60°)
Yield, %	15·1	26·8	58·1
Equiv. wt.	307·7	298·9	289·4
Iodine value	46·9	87·2	204·4
Diallylic acids, %	0·42	4·9	34·0
Triallylic acids, %	0·11	3·2	48·9

supplemented by spectroscopic estimations of the polyallylic acids. The distillation curves are shown in Fig. 2. From this analysis the percentage composition of the whole fat was calculated, and it is shown in Table IV; in addition to the glycerides listed, spectroscopic examination of the fat indicates that a variable small amount of conjugated dienoic acids may also be present.

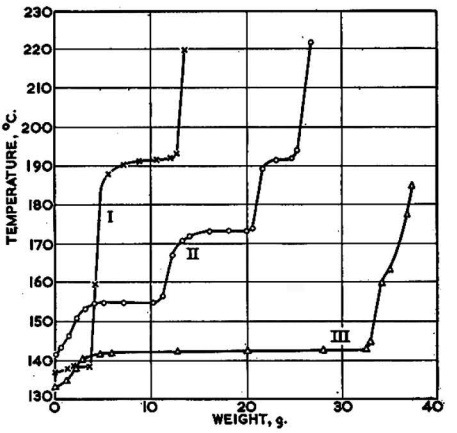

FIG. 2.—*Distillation of methyl esters of fractions I, II and III from low-temperature crystallization of the fatty acids*

I. From acids crystallizing at − 30° (distilled at 1 mm. mercury)
II. " " " " − 60° " " " "
III. " " " " " " " 0·5 " "

Discussion

The two methods of analysis give results which are in essential agreement except for the content of oleic glyceride. There are smaller but significant differences for the linolenic, eicosenoic and erucic glycerides. Compared with amplified distillation, ester fractionation gives a value 6·5% high for oleic glycerides and 2% low for two of the others; the value for linolenic glyceride is also low. These differences we attribute largely to the weaknesses in ester fractionation cited by Hilditch,[12a] namely, that with highly unsaturated fats there is invariably some polymerization and oxidation during the processes of crystallization and distillation, with resulting slight decreases in iodine value and saponification equivalent. Here these have led to the allocation of too great proportions to oleic and stearic glycerides. Moreover, the use of a good fractionating column does not give complete separation into families and, where one or two unsaturated fatty acids are present in each family, saponification and iodine values, though supplemented by spectroscopic data, do not always permit a single solution of the composition. Simplifying assumptions must be used[12b] with accompanying uncertainty. On the other hand, the information obtainable from the amplified distillation of hydrogenated and saturated esters, though more limited, is unequivocal, and where it applies we consider it the more accurate. It has the advantage of using very little material. Ester fractionation is needed to provide information this method cannot give. It shows the distribution of unsaturation between the carbon families and permits the isolation and determination of the exact structures of the unsaturated acids. Spectroscopic examination of the plateau fractions of distillation II showed that the C_{20} fraction contained 1·7% diallylic and 0·4% triallylic esters; the C_{22} fraction contained 2·7% diallylic and 0·6% triallylic esters. In this fat there is therefore definite evidence of the presence of C_{22} diallylic glycerides (as Hilditch & Baliga[6] found also of the related rape-seed fat) and of the C_{20} diallylic glycerides.

As noted previously,[13] ester distillation in a good spinning-band column gives fractions very rich in one material. The second plateau fraction in distillation I contained 95·8% of methyl erucate; the first, second and third plateau fractions of distillation II contained respectively, 70% methyl oleate (10% linoleate), 96·4% methyl 11-eicosenoate and 81·6% methyl erucate. These fractions were used to identify the particular acids.

The finding that *cis*-11-eicosenoic acid and *cis*-13-docosenoic (erucic) acid are major component acids of Kerguelen cabbage-seed fat conforms with the view that erucic acid is a

Table IV

Composition of whole fat

Glyceride	%	Glyceride	%
Palmitic and lower	4·5	Linoleic	20·4
Stearic	2·6	Eicosadienoic	0·1
Arachidic	0·7	Docosadienoic	0·1
Behenic	0·5	Linolenic	28·6
Hexadecenoic	3·7	Non-saponifiable	1·7
Oleic	14·4	Eicosatrienoic } Docosatrienoic }	Traces
11-Eicosenoic	11·2		
Erucic	11·5		

characteristic component of seed fats of the Cruciferae and with the recent observations[5, 14, 15] that this acid may not be the major component, and that eicosenoic acid may be present in comparable amount. Similar high contents of linolenic acid have been reported for the seed fats of one or two Cruciferae.[14, 16]

Experimental

Extraction and properties of the fat

The seeds of sample 3 (1·75 kg.) were collected in May–June, 1949, at Atlas Cove Head, Heard Island, dried at 20° and stored dry until the return to Australia in May, 1950. The average dimensions of the seed (1580 g.) obtained after cleaning were: length, 3·5 mm.; width, 2·0 mm.; thickness, 1·0 mm. The seed was ground in a cooled Christie & Norris mill and extracted with a purified light petroleum (b.p. 50–65°) under nitrogen and the solvent removed, finally, under reduced pressure. The orange-yellow oil (Lovibond (1 cm.): N.T., 2·0; R, 3·6; Y, 20·0) possessed the typical odour of mustard-seed oil. In addition to the properties already recorded, the following were measured: d_4^{25}, 0·9173; η^{25}, 49·6 centipoises; $[\alpha]_D^{25} + 0{\cdot}1°$; dispersion, 13·5; glycerol yield (Zeisel–Fanto[17]), 9·1%; Reichert–Meissl number, 0·08; Polenske number, 0·1. The yield of fatty acids was 93·9; 92·7%.

Determinations of the thiocyanogen number made on two different samples of fat (Kerguelen Island, Heard Island) at different times gave values in close agreement: 108·4, 109·5. The fat was carefully dried, but during the determination a considerable yellow precipitate always formed; the figures have not been used for analytical purposes. The reaction mixtures remained clear in thiocyanogen determinations made simultaneously on other fats. The fatty acids gave considerable quantities (20%) of precipitate in the hexabromide determination, but the yields were very variable. The precipitate was 9 : 10 : 12 : 13 : 15 : 16-hexabromostearic acid. After crystallization from xylene its melting point and its mixed melting point with an authentic specimen was 183·5–184°(corr.).

The oil-free seeds contained: N, 6·6% (equivalent to 41·2% of protein), starch, 9·2%, and crude fibre, 15·6%. They yielded 9·2% of ash.

Determination of saturated acids

The oxidation conditions recommended by Pelikan & von Mikusch[18] proved unsuitable for this fat. Large quantities of insoluble dibasic acids, mainly suberic, azelaic and sebacic acids, but also some of higher molecular weight, were formed and they made extraction of the saturated monobasic acids extremely difficult. The iodine value also remained too high (3 to 5). When the oxidation conditions recommended by Hilditch & Priestman[19] were used there was considerably less insoluble acid material, and extraction of the saturated acids was possible. The iodine value of the saturated acids was also lower (usually about 1). These conditions were therefore used.

Formation and fractionation of the methyl esters

The fatty acids esterified readily with methanol in the presence of sulphuric acid with yields of esters from 94 to 95%. Catalyst poisons in these esters prevented direct hydrogenation using Adams' catalyst. The esters were purified by rapid distillation from a Claisen flask at 0·2 mm. pressure, which gave 95% recovery without noticeable change in iodine value (146·8). Hydrogenation then proceeded readily at room temperature and the product had an iodine value of less than 0·1.

The small amount of saturated acids (0·67 g.) available for amplified distillation was esterified in ether in the presence of excess of diazomethane, the ether was removed, and the methyl esters were mixed with 60 g. of amplifying oil and distilled at 1·00 mm. pressure.

Amplified distillations were made in the manner already reported.[11] For normal fractional distillation of the esters (Fig. 2) a pressure of 1 mm. and a take-off rate of 3 g. per hour was used, except for distillation III, where the pressure was reduced to 0·5 mm. and the take-off rate increased to 7 g. per hour. A mineral-oil 'chaser' was employed to drive over the last fractions.

Low-temperature crystallizations

Except that acetone was used as a solvent and that crystallizations were made at − 30 and − 60°, the method used was that described previously;[13] the crystalline fractions obtained at − 30 and at − 60° were each recrystallized. Esterification with methanol and sulphuric acid gave the esters in 95–99% yields.

Spectroscopic determinations of polyallylic acids

Brice & Swain's procedure[20] was used and measurements were made with a Unicam S.P. 500 spectrophotometer.

Identification of the acids

The methyl esters of monoenoic acids were identified in the appropriate plateau fraction. Determinations of saponification equivalent and iodine value showed that the main plateau fraction of distillation I contained 95·8% of methyl erucate and that the third plateau fraction of distillation II contained 81·6%. The erucic acid from the first of these melted at 32–33°, and after crystallization from alcohol it formed needles of m.p. 33–33·5°. The brassidic acid obtained by elaidinization formed plates of m.p. 59·4–60·0°. The first plateau fraction of distillation II was estimated to contain 70% of methyl oleate, which was identified by conversion to the high- and low-melting 9 : 10-dihydroxystearic acids. The second plateau fraction of distillation II was estimated to contain 96·4% of methyl 11-eicosenoate. It was identified as the *cis*-form by oxidation with alkaline permanganate to the high-melting 11 : 12-dihydroxyeicosanoic acid, and by oxidation with hydrogen peroxide in formic acid to the low-melting 11 : 12-dihydroxyeicosanoic acid. The position of the double bond was confirmed by oxidation of the high-melting form with periodic acid and isolation of nonaldehyde as its 2 : 4-dinitrophenylhydrazone, m.p. 105·5–106·5°. The six dihydroxy-acids prepared were purified by crystallizing the high-melting forms three times from alcohol, and by crystallizing the low-melting forms three times from ethyl acetate. Their melting points were determined in small resistance-glass capillaries, and the corrected values and the mixed melting points are shown in Table V.

Table V

Acids	$C_{18}H_{36}O_4$	$C_{20}H_{40}O_4$	$C_{22}H_{44}O_4$	$C_{18}H_{36}O_4$ + $C_{20}H_{40}O_4$	$C_{20}H_{40}O_4$ + $C_{22}H_{44}O_4$	$C_{22}H_{44}O_4$ + $C_{18}H_{36}O_4$
High-melting dihydroxy-acids						
M.p.	129·5–130°	128·2–128·8°	129·6–130·3°	115–117°	119–122°	118–121°
Low-melting dihydroxy-acids						
M.p. (obs.)	93·4–94·0°	96·8–97·4°	99·6–100·4°	84–91°	88–93°	85–89°
M.p. (literature)	94° (*a*)	90–92° (*c*)	101° (*e*)			
	95° (*b*)	94–95·5° (*d*)	98–99° (*d*)			

(*a*) Swern *et al.*[21]
(*b*) Hilditch[22]
(*c*) Hilditch & Boliga[6]
(*d*) Hopkins[5]
(*e*) Dorée & Pepper[23]

The melting points obtained for the C_{18} and C_{22} high-melting forms are about 2° lower than those usually cited in the older literature, but more recent values agree better with ours. The melting points previously recorded for low-melting 11 : 12-dihydroxyeicosanoic acid appear to be several degrees low. Preparation of the high-melting hydroxy-acids is somewhat lengthy, and their melting points differ only slightly. They are therefore less suitable derivatives for the purpose of identification than the low-melting forms, which can be prepared quickly and on a small scale by the method of Swern *et al.*,[21] and the melting points of which differ significantly and rise with increasing molecular weight.

The saturated acids were identified by their melting points and by those of their methyl esters. The first plateau of distillation I contained 82% methyl palmitate and the pure ester was obtained by crystallization. A small amount of methyl behenate was isolated from the last portions of distillation I. Stearic and arachidic acids were obtained from the Bertram acids after amplified distillation.

The crystalline tetrabromide of linoleic acid could not be obtained from the residues left after the preparation of hexabromostearic acid. The presence of linoleic acid in the fat was established by oxidation of the plateau fractions of distillation III with 1% permanganate at 0°. The crude hydroxy-acids were freed of dihydroxy-acids by extraction with ether, and the hexahydroxy-acids were then extracted with hot water. The remainder, m.p. 163–176°, on extraction with alcohol gave crystals of the high-melting tetrahydroxystearic acid (m.p. 172–173°) (Found: O, 27·4, 27·7. $C_{18}H_{36}O_6$ requires O, 27·55%). Subsequent extraction with boiling acetic acid and cooling gave crystals, which when recrystallized from acetic acid possessed the melting point of the eutectic mixture of the two tetrahydroxystearic acids (155–157°).

Of the component acids present to the extent of more than 1%, only hexadecenoic acid has not been identified. Its ester was calculated to form 30–40% of the small early fractions of distillation II, the remainder being methyl oleate and methyl palmitate.

Acknowledgment

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A NEW METHOD FOR ASSAY OF THE 'PYRETHRINS'

By Wm. MITCHELL

A new method for assay of 'pyrethrins' is described. It is suggested that its successful operation requires less experience than do the existing methods. Comparative results on pyrethrum flowers and extracts by the new method and the A.O.A.C. (Association of Official Agricultural Chemists) method are given. A comment is made on the standard of accuracy to be expected from pyrethrum analyses in general.

No method of determining the individual active constituents of pyrethrum as such has yet been devised. The difficulty of doing so is increased now that it is known that the terms 'pyrethrin I' and 'pyrethrin II' refer not to single substances, but to groups of esters of varying molecular size and insecticidal potency.[1, 2] The two groups respectively comprise esters of the keto-alcohols pyrethrolone and cinerolone with (*a*) chrysanthemic acid (chrysanthemummonocarboxylic acid) and (*b*) pyrethric acid (the monomethyl ester of chrysanthemumdicarboxylic acid). The Seil method[9] and the A.O.A.C. (Association of Official Agricultural Chemists) (mercury-reduction) method,[10, 11] currently in use, both depend on determination of the chrysanthemum-mono- and -di-carboxylic acids obtained on hydrolysis of the total esters. The results were formerly calculated as 'pyrethrins' on the basis that the acids were in fact derived entirely from pyrethrins. This method of calculation has latterly been continued, as a matter of expediency, since there is no means of determining what proportions of the acids

are derived from cinerins, polymerized (and hence non-insecticidal) pyrethrins and cinerins, other (probably inert) esters, or initially free acids.

A systematic study of these methods has already been made by Mitchell *et al.*[3] The methods employ essentially the same procedure for extraction of the flowers, hydrolysis of the esters and isolation of the chrysanthemumdicarboxylic acid. The actual determination of the latter is by titration with alkali, and hence is non-specific. In fact, a significant proportion of the acidic material titrated consists of uncrystallizable, resinous, acidic matter, from which the true crystalline chrysanthemumdicarboxylic acid can be separated by extraction with hot water. This inherent error in the 'pyrethrin II' figures gives considerable point to the suggestion made by Tattersfield *et al.*,[4] and repeated by Mitchell & Tresadern,[5] that pyrethrum should be standardized only on its content of 'pyrethrin I'. However, it is common experience that very old, or badly stored, pyrethrum flowers or extracts often show more 'pyrethrin II' than 'pyrethrin I'. Such a result renders the sample suspect, and its insecticidal potency should then also be tested biologically before use. Hence, even if the suggestion were adopted, it would still be wise to retain the 'pyrethrin II' determination, since its ratio to the 'pyrethrin I' figure affords such a valuable indication of quality.

It is in the isolation of the chrysanthemic acid that the methods differ. The Seil method depends on the fact that this acid is steam-volatile, whereas the dicarboxylic acid is not. Unfortunately there is a considerable and variable loss during this procedure. It was suggested by Mitchell *et al.*[3] that this was due to hydration, and it was confirmed by Harper *et al.*,[6] who isolated the hydroxy-acid formed. The loss may be partly offset by the presence of other volatile acids,[7] since any such acids could well be included as chrysanthemic acid, the determination by titration with alkali being non-specific.

These shortcomings have probably contributed to the recent wider use of the mercury-reduction method, especially since it appears to be relatively specific for chrysanthemic acid. In this method, the acid is selectively extracted with light petroleum, in which solvent the dicarboxylic acid is only very sparingly soluble. Unfortunately, as has been shown by Bray *et al.*,[8] and confirmed by Mitchell *et al.*,[3] the strictest adherence to the experimental details of the (imperfectly understood) mercury-reduction reaction is necessary; and even then the reproducibility of results between laboratories is not of a high order.

More recently, the Colonial Products Advisory Bureau's Consultative Committee on Insecticide Materials of Vegetable Origin has published the Report[9] of its standing Sub-Committee on Methods of Analysis of Vegetable Insecticides dealing with a World-Wide Collaborative Analysis of Pyrethrum Flowers. This report shows that the results were disappointing. In particular, inter-laboratory errors were high and of approximately the same order either by the Seil or mercury-reduction procedures described. In fact, it was concluded that a difference of 0·3% between the results of two different laboratories on a sample of flowers containing between 1 and 2% of total pyrethrins should not be regarded as significant!

From these considerations, the author was led to the following conclusions:

(*a*) that the apparent advantage of a specific determination of chrysanthemic acid by the mercury-reduction method is outweighed by the empirical nature of the procedure, whereby gross errors can readily be caused by small deviations in technique.

(*b*) that it should be possible to determine the chrysanthemic acid by simple alkalimetry, after separating it by extraction with light petroleum (as in the mercury-reduction method), provided means could be found of removing the extraneous acids known[3] to be co-extracted.

Preliminary experiments on the lines suggested under (*b*) suggested that extraneous acids could be largely removed by repeated washing of the light-petroleum extracts with water. Control experiments, using pure chrysanthemic acid under similar conditions, indicated that a fairly constant loss of about 3% of the acid occurred, owing to its solubility in water. Although this was considerable, it was thought that, provided it was a consistent loss, the method was worth further study. For this purpose, a series of pyrethrum extracts was subjected to the procedure described in the Experimental section. The results are given in Table I, along with the corresponding results by the A.O.A.C. method (7th Edition,[10] modified,[11] using hydrochloric acid).

A limited number of analyses of pyrethrum flowers was also made by this method. The flowers were extracted by continuous percolation with cold light petroleum (b.p. 40–60°) in order to minimize the extraction of polymerized pyrethrins.[6] For this purpose a simple continuous extractor was devised; it is described in an Addendum to this paper. The results of these analyses are given in Table II, in comparison with results by the A.O.A.C. method.[11] In addition, comparative results by the A.O.A.C. method, but using hot extraction, are given in parentheses; they confirm the desirability of cold extraction.

Table I

Comparative analyses of pyrethrum extracts by the proposed method and the A.O.A.C. method

Sample No.	Proposed method (pyrethrins, %)			A.O.A.C. method (pyrethrins, %)		
	I	II	Total	I	II	Total
1	18·5	12·8	31·3	17·8	12·8	30·6
2	15·4	10·5	25·9	15·0	10·5	25·5
3	12·4	9·0	21·4	12·0	9·1	21·1
4	16·2	11·0	27·2	15·9	11·1	27·0
5	12·8	8·8	21·6	12·3	8·8	21·1
6	18·1	12·8	30·9	17·5	12·9	30·4
7	12·6	Not determined		12·2	Not determined	
8	13·4	,,	,,	13·0	,,	,,
9	18·5	,,	,,	17·9	,,	,,

All results are means of at least two analyses in close agreement

Table II

Comparative analyses of pyrethrum flowers by the proposed method and the A.O.A.C. method (figures in parentheses refer to extraction with warm solvent)

Sample No.	Proposed method (pyrethrins, %)			A.O.A.C. method (pyrethrins, %)		
	I	II	Total	I	II	Total
1	0·49	0·37	0·86	0·47 (0·48)	0·36 (0·42)	0·83 (0·90)
2	0·80	0·60	1·40	0·78 (0·79)	0·62 (0·66)	1·40 (1·45)
3	0·32	0·37	0·69	0·29 (0·31)	0·38 (0·42)	0·67 (0·73)
4	0·44	0·29	0·73	0·42 (0·43)	0·28 (0·30)	0·70 (0·73)
5	0·68	0·54	1·22	0·66 (0·69)	0·53 (0·57)	1·19 (1·26)

All results are means of at least two analyses in close agreement

From these results it seems clear that the loss of chrysanthemic acid in the water washings is more than offset by other extraneous acidic matter that is not removed by water-washing. Thus the figures for 'pyrethrin I' by the present method are consistently some 3–4% higher than those obtained by the A.O.A.C. method. The method involves simpler manipulative procedure than that of either the Seil or A.O.A.C. methods. In these Laboratories the method has given equally good results in the hands of those with or without experience in pyrethrum analysis. Thus, although it is not claimed that the method gives absolute figures for chrysanthemic and chrysanthemumdicarboxylic acids, it is suggested that it may provide a simpler means, less liable to errors due to inattention to minute detail, and hence capable of being performed by less skilled analysts, for the assay of pyrethrum flowers and preparations. It is hoped that other Laboratories will experiment with and comment on it. Whether it could be effective in reducing the very large inter-laboratory errors encountered in the World-Wide Collaborative Trial[9] is doubtful. The writer is of the opinion that, probably owing to the high value and relatively large quantity of pyrethrum flowers used and hence the large sums of money involved, there has been a tendency to expect an unattainably high degree of accuracy in its analysis. Apart from any errors inherent in such a method itself, there are personal errors, and especially volumetric-reagent errors, to be added. One needs only to consult Pharmacopoeial and other official analytical standards to observe that for other drugs limits of error of $\pm 5\%$ or more are the rule, presumably to allow for these collective errors. It seems not unlikely that a collaborative trial, on the scale of that conducted for pyrethrum flowers, of drugs such as opium or hyoscyamus might disclose inter-laboratory errors no less than those found in pyrethrum analysis.

Experimental

Method for analysis of pyrethrum extract

Weigh accurately a quantity of the extract expected to contain about 0·125 g. of total pyrethrins. Add 0·5N-potassium hydroxide in ethylene glycol monoethyl ether (20 ml.) and boil the mixture under reflux for 30 minutes. Dilute the cooled mixture with water (about 170 ml.), transfer to a 250-ml. graduated flask, add Filter-Cel (1 g.) and 10% (w/v) aqueous

barium chloride (10 ml.), allow the precipitate to settle and test the supernatant liquid for completeness of precipitation. If necessary, add more (up to 5 ml.) barium chloride solution. Make up to volume with water, mix and allow to stand for at least 30 minutes. Filter off 200 ml. through a 15-cm.-diameter fluted filter paper. (To avoid subsequent separation difficulties, this filtrate must be obtained bright, if necessary by refiltering through the same paper.)

Acidify the filtrate (Congo red) with hydrochloric acid and extract it by vigorous shaking with light petroleum (b.p. 40–60°; 50, 50, 25 ml.). Reserve the extracted acid liquid (A). Unite the light-petroleum solutions and extract them by vigorous shaking with 0·1N-aqueous sodium hydroxide (20 ml.) and then with water (three portions, each of 10 ml.); discard the extracted light petroleum.

Neutralize the united alkaline aqueous liquids (phenolphthalein) with concentrated hydrochloric acid, add 1 ml. in excess, and extract by vigorous shaking with light petroleum (b.p. 40–60°; 50, 25, 25 ml.). (This re-extraction with light petroleum has already been shown[8] to eliminate traces of chrysanthemumdicarboxylic acid present in the first extract.) Wash the combined light-petroleum extracts with water (five portions, each of 20 ml.; the last extract should be practically neutral to phenolphthalein; if it is not, one or more further washings should be given, as necessary). Each aqueous washing is in turn washed with the same portion (20 ml.) of light petroleum (b.p. 40–60°). Add the first two washings and the extracted aqueous acid liquor to the liquor (A) reserved for the determination of 'pyrethrin II'; discard the later washings.

Transfer the light-petroleum solutions quantitatively (using 10 ml. of the same solvent to wash the separators) to a 200-ml. glass-stoppered bottle containing 15 ml. of freshly boiled and cooled water neutralized (phenolphthalein) with 0·02N-sodium hydroxide solution. Titrate, as in the Seil method, with 0·02N-sodium hydroxide solution (phenolphthalein) until the aqueous layer is just pink and remains so for at least one minute. Each 1 ml. of 0·02N-sodium hydroxide solution is equivalent to 0·0066 g. of 'pyrethrin I'. [In conducting the titration, care should be taken to minimize entry of carbon dioxide by adhering to the size of bottle stated, and reducing the number (and time) of individual additions of alkali to the minimum; the first addition should amount to 80% of the expected total.]

Determine the content of 'pyrethrin II' in the reserved acid aqueous liquors (A) by the A.O.A.C. method[10] as recently modified.[11]

Correct the figures for 'pyrethrin I' and 'pyrethrin II' by deduction of the corresponding values obtained in a parallel 'blank' assay from which the pyrethrum extract is omitted.

Method for analysis of pyrethrum flowers

Weigh accurately 12·5 g. of pyrethrum flowers (30-mesh powder) and extract in a suitable continuous-extraction apparatus with cold light petroleum (b.p. 40–60°) for four hours. Recover the solvent from a water bath, avoiding over-heating of the residual extract. Add 20 ml. of 0·5N-potassium hydroxide in ethylene glycol monoethyl ether and proceed as described above for pyrethrum extracts.

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Addendum

A CONTINUOUS COLD-EXTRACTION APPARATUS (by P. F. Barker)

The continuous extractor described below was designed and constructed in this Laboratory primarily for the extraction of pyrethrum flowers with light petroleum at room temperature; its use for this purpose has been described above.

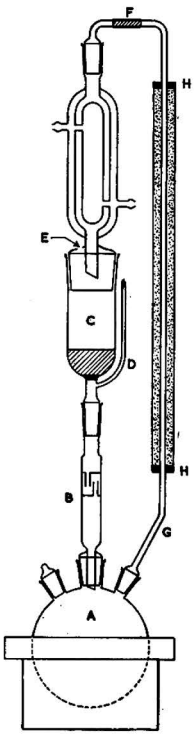

FIG. 1

The apparatus is compact, being based on an electric heating-mantle and requiring the support of only one retort-stand. The component parts are of interchangeable-joint standard glassware with, in addition, some special pieces made in the laboratory. These pieces are simple and require no special glass-blowing skill.

Details of the assembly are shown in Fig. 1. The centre neck of a 250-ml. three-necked flask A carries the distillate return tube, one side-neck supports the vapour tube G, and the other can be used for solvent addition. The distillate return tube B has a simple liquid-seal vapour trap. A B.14 joint connects the trap to the outlet of the drip extractor C. This component carries a vent-tube D, lightly plugged with cotton wool. A vent is also blown (at E) on the B.34 joint of the condenser. These two vents ensure that there is no pressure difference on either side of the plug of material being extracted. The solvent vapour is conducted to the top of the condenser by a lagged tube G. This is insulated by means of cork dust packed in the annular space bounded by a concentric, wider-bore glass tube supported on corks H (the inner tube was finally bent to shape after the outer tube and lagging were applied). The necessary flexibility in the apparatus is provided by the right-angle tube F incorporating a short rubber connexion arranged so that the glass ends almost touch.

The apparatus has been used for extractions with light petroleum, over periods of four hours or more, without appreciable loss of solvent. The extraction rate was found to be considerably higher than that of an ordinary Soxhlet extractor. The present apparatus is considered to be simpler and more compact than that recently described by Waters.[1]

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Reference

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THE DEVELOPMENT OF A NUMERICAL SCORING SYSTEM FOR THE SENSORY ASSESSMENT OF THE SPOILAGE OF WET WHITE FISH STORED IN ICE

By J. M. SHEWAN, RUTH G. MACINTOSH, C. G. TUCKER and A. S. C. EHRENBERG

An attempt is made to classify the sensorily perceptible quality-factors of cod fish, stored in ice, throughout the spoilage chain from absolute freshness to putridity. The classification is given numerical form to ease the handling and interpretation of the resulting data. A panel has been trained to agree in assessing any one sample, and its internal consistency is discussed on the basis of some experimental data.

The importance of the method would seem to be that those factors are classified which would give rise to opinions of preference in the ordinary consumer, were the food presented to him. In practice the accuracy obtained by the panel was such that samples could be clearly differentiated, in terms of periods of storage in ice under certain standard conditions, to within a day or two.

Introduction

At the end of the war, particularly during the years 1946–47, concern was expressed in many quarters about the poor quality of most of the ' white ' fish reaching the consumer. As a result, an informal interdepartmental Committee consisting of members from the Ministry of Food, Ministry of Agriculture and Fisheries, Scottish Home Department, Health Departments, Ministry of Transport and British Railways, Department of Scientific and Industrial Research and other bodies, was set up in November, 1947, to consider what measures could be adopted to improve the quality of fish. Evidence was collected from a variety of sources, both trade and Departmental, and a working party was constituted to initiate and carry out research on several immediate problems, the solution of which would do much, it was considered, to improve quality. It soon became obvious, however, that all the problems, whether of a short-term or long-term nature, involved some method of quality assessment. Thus, if any alterations in the normal techniques of handling or stowage at sea were to be attempted, the effect of such alterations would have to be judged ultimately by the quality of the fish on landing. Some means of assessing quality that permitted more quantitative interpretation than hitherto, and of determined validity in use, had to be devised. Although quality judgments are made daily at the markets, shop, or at home, few people recognize the many factors involved.

Quality, as applied to foodstuffs, is a complex term having many aspects—physiological, psychological, hygienic etc.—so that no one analytical method can suffice to determine it in its entirety. It is not surprising, therefore, that the concept of quality may have quite different meanings for different people. In the present paper it is proposed to consider only the effect of spoilage on the complex of factors such as appearance, odour, flavour and texture, which may affect the sensory judgments of ' quality ' by the purchaser and consumer, and which for convenience may here be called the ' factors ' of ' eating quality '. The changes occurring in the individual quality factors during the deterioration of fish from absolute freshness to putridity have been identified and described. They are set out in order of occurrence during spoilage, and given numerical form to ease handling of the resultant data. Such a classification, it was thought, should make it possible to place in order, according to their sensorily perceptible quality factors, any samples of fish that might ordinarily be encountered, whatever their storage history. The internal consistency of a panel, trained to agree in assessing samples by this technique, is also described on the basis of some experimental data.

Previous work at Torry Research Station (Department of Scientific and Industrial Research) in quality assessment

In most of the research work on the storage and preservation of both fresh and processed fish carried out at the Torry Research Station over the past two decades, quality has been assessed by a combination of two kinds of techniques. One technique consisted in performing bacterial counts or determining the chemical and physical properties of samples of the foodstuff usually regarded as being ' objective ', i.e. largely independent of the observer, and although few of these objective data were closely correlated with ' eating quality ', they often supplied valuable supplementary data which greatly assisted the interpretation of results. The other technique consisted of presenting samples to a ' panel ', normally of from two to six individuals directly concerned or interested in the experiment, who were asked to comment in any way they pleased on the appearance, odour and texture of the raw product and on the appearance, odour,

flavour and texture after cooking. This primarily depended on the sensory reaction of the observer to the foodstuff and varied from individual to individual. A considerable amount of empirical knowledge concerning the quality of a variety of fish and fish products, both fresh and processed (salted, smoked, dried etc.), has been built up in this way at Torry, and several members of the staff have become expert judges in their own particular fields. It was always recognized, however, that these subjective panel judgments were obtained in a rather haphazard manner, and probably would not stand up to rigorous analysis.

As a result of the Interdepartmental Committee's deliberations, considerable impetus was given both by the Ministry of Food and the Department of Scientific and Industrial Research to a study of the problem of subjective assessment of ' eating quality ', and of the suitability and validity of one or two of the more promising chemical methods.

Methods for assessing factors of quality

As just indicated, there are in general two types of methods available for assessing quality. The first entails the instrumental determination of some physical, chemical or biological property which alters with fish, for example, as they deteriorate from absolute freshness to putridity [an absolutely fresh fish is defined here as one which is in or just coming out of *rigor mortis*—with iced cod fish usually 24–40 hours after death[1]] ; the second involves the sensory perceptions on whose basis such concepts as ' freshness ', ' putridity ' and the intermediate stages of eating quality might be defined.

For fish, various physicochemical analytical procedures have been suggested.[2] It is not intended here to review the methods proposed or used ; suffice it to say that few have been found successful in practice in specifying stages of ' eating quality ', although at least four—the chemical estimation of total volatile bases, of di- and of tri-methylamine and of volatile acids—appear to be promising.

Some of the sensorily perceptible factors affecting quality, e.g. colour and size, can of course be also assessed physically ; but the evidence is that with the less thoroughly studied factors, such as flavour and odour, ultimate reference has to be made to direct sensory perceptions, the assessment of which therefore becomes of paramount importance.

Such assessments, based on the psychological evaluations of physiological sensations, are at present the subject of discussion in many fields, not least in that of food science. Previous views about the validity of subjective judgments have ranged from those of complete scepticism[3] to almost the other extreme where it is claimed that such techniques have the ' power of a new laboratory tool as refined as a micro-analytical balance and as sensitive as a spectroscope '.[4] Crocker[5] is probably nearer the truth when he states that ' a considerable degree of reproducibility may be obtained in organoleptic testing ', and ' even persons of ordinary discrimination may become good flavour discriminators if adequately trained '. Quite recently at a Symposium on Subjective Judgments grave doubts were expressed, particularly by some of the academic psychologists, concerning many of the subjective measurements now being attempted in various applied fields.[6]

It may be true that much of the reported work entailing subjective judgments, at least in the field of food science, is carried out without a clear conception of what is involved in the experimental methods employed or in the analysis of the data obtained ; but in many fields where sensory judgments are used it seems to be accepted that, provided the problem is properly stated, techniques are available from which significant conclusions may be drawn.

Among methods of sensory assessment, two types may here be distinguished : (1) methods based on consumer reaction, such as the so-called ' acceptance ' or ' preference ' tests (' preference ' tests are used, for example, to determine what a population prefers when presented with two or more ' different ' kinds of product, and hence the information sought concerns the reaction of the consumer to the given samples—such techniques will not be considered here) ; (2) methods whose aim is to obtain an exact description of the various characteristics of the product, such as toughness, odour, flavour, without any direct reference to the observer's preferences. This method is analytical in nature, and the discipline should be analogous to the process of observation in the ' exact sciences '. Indeed, such methods have been called ' objective sensory tests '.[7]

Assessments based on the characterization and differentiation of quality factors may be made either by a single individual or, more often, by a small panel. The use of a panel, particularly at the early stages of an investigation, permits the immediate estimation, in some degree at least, of the confidence to be placed on the assessments. It is recognized that the results may be subject to more variability than is acceptable as mere ' error of measurement ', as is, of course, not unlikely for any system of observation that has not been standardized over a long period of time.

Techniques used for expressing differences in sensory reactions are numerous, and vary from ' paired comparisons ', ' triangular tests ', or the use of ranks, to descriptive classifications and scoring systems. This aspect of the subject has recently been reviewed by Boggs & Hanson.[7] It is suggested here that the initial recognition of all the quality factors of a foodstuff and their definition and abstraction by training a test panel to recognize them in subsequent tests is a technique which yields maximum information and hence is likely to be of most use in research work. What follows describes the evolution of the process of recognizing various quality factors in white fish (mainly cod) stored in ice, and their formulation in a comprehensive scoring system, together with an analysis of data obtained when the score sheet was used by a trained panel.

The establishment of a technique

Training of the panel and evolution of a system of descriptive terms

A panel consisting of six men and three women, most of whom had no previous experience in judging fish quality, were first subjected to a test for taste acuity, using solutions of pure chemicals, e.g. sucrose, salt and citric acid ; they were able to detect and arrange the solutions in the correct manner. Such preliminary testing for acuity of taste perception, however, is probably unnecessary, as the criterion of selection of a panel member need be nothing more than his or her ascertained performance in using an agreed scale. Panel members who had little or no previous experience with fish were given a short course dealing with the microbiological, chemical and organoleptic changes occurring in fish during spoilage, along with demonstrations of cod fish, both in the raw and the cooked state, at various stages in the degradation from absolute freshness to putridity. After this formal instruction, the panel as a whole began its training period. This consisted in examining daily 3–12 fish that had been stored in ice under the best conditions in the laboratory for periods up to 21 days. At some sessions, all the fish tested had been stored in ice for the same periods ; at others, they had been stored for differing periods.

Previous experience, based mainly on the classic work of Anderson[8] but also on that of other workers, including those at Torry,[2, 9–12] has indicated that seven quality factors are the most important and reliable in the organoleptic examination of fish. The following has been found to be the most practicable sequence of observations.

I. The general appearance. The appearance of the eyes, gills, outer surfaces.
II. The appearance of the flesh, particularly at the cut surface along the backbone and at the ' belly flaps '.
III. The texture of the raw fish, under manual pressure, including the reaction to touch of the outer surface of the fish.
IV. The odour of the raw fish, particularly at the gills and body cavity.
V. The odour of the cooked fish.
VI. The flavour of the cooked fish.
VII. The texture of the cooked fish.

During the training period, the panel as a group were allowed to examine the fish and to comment as they pleased on each quality factor of each individual sample, so as to arrive at an agreed descriptive judgment. In this way it was possible to build up a collection of descriptive terms, more especially those relating to odours and flavours, for fish stored in ice for any period, a selection of which is given in Appendix I. It was considered essential that each descriptive term or its equivalents should be recognized by all the panel members. Thus, even though some members could never recognize an odour as being ' mousey ' or ' acetamide-like ', it was soon obvious that the terms ' mousey ' and ' musty ' used by different members were largely equivalent ; and similarly with the terms ' oily ' and ' grassy ', and so on (see Appendix I). It was of great assistance to the panel that, at several stages in the spoilage chain, the odours perceived both in the raw and cooked states corresponded without ambiguity to those of several well recognized chemical compounds known to be present in the muscle. Indeed the ideal to be sought for is the replacement of ordinary descriptive terms, many of which may have irrelevant associations, with suitable chemical analogues. All terms implying judgments of merit or preferences, such as ' excellent ' and ' good ', have been categorically avoided ; and imprecise comparative terms such as ' slight ' and ' strong ' are used as little as possible. In this way, ambiguity of definition of the terms has been minimized, facilitating consistent interpretation by the panel and, it is hoped, permitting others to comprehend and reproduce the details of the technique.

At this stage, preliminary studies were made of technical aspects such as cooking methods. Baking and frying were ruled out, the former because it caused too great a loss of the volatiles

owing to the high temperatures used, the latter because it allowed the flavours to be almost completely masked by those of the hot fat. The procedure finally adopted was to steam a 6–8 oz. (170–230-g.) middle cut in a glass casserole of 7 in. (17·7 cm.) diameter, with the lid on, but with no water or condiment added, for 30–35 minutes in a boiling-water bath. In the preliminary tests, samples were kept on a boiling-water bath during testing, but subsequent experience showed that better results were obtained by carrying out the testing on a bath which maintained the fish temperature at about 60°. This temperature suited most palates; moreover, no serious loss of volatiles occurred in the interval between the first and last taster.

By the formulation and recognition of a standardized descriptive terminology for the majority of quality factors in fish of varying degrees of freshness one of the most difficult tasks presented to the panel had been accomplished.

Formulation of a numerical scoring system

The various descriptive terms for each of the seven quality factors as discerned by the panel were arranged in the sequence of their occurrence during the spoilage of iced cod-fish from absolute freshness to putridity. The ordered stages might then have been labelled by any ordered set of symbols such as A B C D, but numerical scores were assigned, as shown in Appendix I, to facilitate statistical analysis; this was particularly necessary, as data of this kind are known to be subject to considerable variability.

In order to place these stages on a scoring scale, the principle that was finally adopted was to attempt to make adjacent unit steps equally perceptible. Since the quality factors which are being assessed are perceived by the senses, it seems reasonable to graduate them in terms of equally noticeable differences of sensation. In general appearance, for instance, the attributes of scores 5 and 3 could easily be defined; however, another stage, symbolized by the score 4, could be recognized but could not be described in precise verbal terms. Such intermediate and even fractional scores are frequently used. It was not thought necessary to try to describe an equal number of distinct stages during spoilage for each of the seven quality factors. Eleven stages were defined in the odours of the raw and cooked fish, nine in flavour and four in all the others. In practice, not all the criteria for any one score are present and considerable overlapping may occur, particularly in odour and flavour, and only experience can guide the panel members on the compromise score finally to be given.

It can be argued that errors of scoring on such a scale will be independent of the level of scoring, permitting simple interpretations of average scores. It should be mentioned that the actual choice of units for any system of measurement does not seem to be fundamental to the interpretation of the resulting data; the meaning of scoring units may be compared with that of degrees of temperature, for example, especially for temperature measurements outside classical physics, where the effects of 'equal' degree differences are by no means equal.

It should be noted that no attempt is made to deduce a formula for the 'general quality' of any one sample as some function, such as for instance the total, of all scores given for the quality factors, general appearance, odour, flavour. All that is done is to derive a technique giving data on which decisions about 'quality' could be made. Thus the various ways of combining scores to obtain an index of quality which have been suggested[13, 14] have not had to be considered here.

The scoring system may be interpreted in terms of the number of days in ice under the best storage conditions required to produce unit fall, or in terms of consumer reaction. In the latter case precise data are lacking, but it is presumed as a working hypothesis that the higher the score, the better the eating quality, and that flavours of 5 or less become increasingly unacceptable.

Some early experiments with the scoring system

The panel, having agreed on descriptive terms and a scoring scheme, were set to examine, under code, a series of cod samples, again of qualities ranging from absolute freshness to putridity. Unfortunately not all the nine panel members were usually available owing to a variety of circumstances; moreover, two of them had to be delegated to prepare, code and cook the samples for each scoring session. As in the training period, all the fish were handled and stowed in ice in the laboratory under the best conditions and were all of known history. Usually six fish stored in ice for two different periods, i.e. two groups of three, were examined in the raw and cooked states each day. On five occasions, however, 18 fish stowed 7, 7, 11, 15 and 21 days respectively, were examined on the same day. These periods of storage were chosen as corresponding to the beginning and end of phase II (7 and 12 days), the end of phase III (15 days), and well into phase IV (21 days) of the spoilage chain.[2] The samples were coded and served in random order, and each panel member was required to assess and record the appropriate

score for appearance, odour, texture and flavour of the raw and cooked fish. In all, about 200 fish were examined over a period of a month.

As the main purpose of these tests was to investigate the scoring technique, it was necessary to enforce a much stricter panel discipline than during the training period. The raw and the cooked samples were placed in two rooms and each panel member was required to examine and score them with only one other person, not a panel member, in the room to record the scores. Members were provided with as favourable an external environment as possible and with the opportunity to concentrate without being influenced, intentionally or otherwise, by the other panel personnel. A full discussion always took place immediately after each session. This was found to be particularly helpful by the less experienced members of the panel and did much to raise quickly the efficiency of the team as a whole. In addition to the subjective tests, a sample representative of the muscle from each fish was taken for the determination of total volatile bases and trimethylamine,[15, 16] with the primary object of investigating the degree of correlation between these chemical methods and the sensory one.

The analysis of the experimental results

The data obtained in these early experiments are here discussed with regard to: (1) the reliability of the panel members, (2) the relationships between the quality-factors, and (3) the validity of the scoring techniques; the three quality-factors of raw odour, cooked odour and cooked flavour are considered in greatest detail. Some of the more purely statistical matters are given in Appendix II.

The analysis and interpretation of the data have been complicated by the day-to-day variation in the composition of the panel, which has already been referred to. Moreover, two panel members (Nos. 8 and 9) arranged the samples for each session, and their scores have had to be considered separately from those of the other panel members. For convenience in analysis, the bulk of the data have been arranged in 10 batches of 18 fish each, so as to give the same combination of panel members throughout each batch and to make the batches as homogeneous in age-in-ice as possible; thus the five batches of 18 fish of one age, each scored on a given day, and five more batches, called 1 to 5, made up of six sets of three fish of one age scored together as follows (cf. also Table II):

1st	batch:	6, 7, 8, 10, 14 and 16	days old
2nd	,,	5, 9, 11, 11, 12 and 13	,, ,,
3rd	,,	19, 19, 20, 20, 21 and 21	,, ,,
4th	,,	3, 6, 11, 13, 13 and 17	,, ,,
5th	,,	9, 10, 14, 15, 16 and 18	,, ,,

The remaining data consist of nine more sets of three fish of one age, ranging from 2 to 21 days.

The reliability of each panel member

Before the reliability of panel members is discussed, it should perhaps be noted that there is no suggestion in the data that the panel members could effectively guess the design of any session, i.e. whether the six samples were all of one age-in-ice (part of a batch of 18 fish) or of two ages (two sets of three fish).

The scores given to any one sample by the different panel members will not necessarily all be the same. Discrepancies between scores given to the same sample will be referred to generally as 'error'. We shall here attempt to distinguish between two kinds or components of error, *biases* and *residual errors*.

It may appear from the examination of the scores that one panel member has tended to score certain samples higher, or lower, than the other panel members. A consistent discrepancy of this kind will be called a *bias*, assuming that it is large enough to be statistically significant. (The term 'personal equation' is sometimes used in a similar connexion, e.g. by Pearson.[17]) Biases may depend upon the quality of the samples, for example the panel member may score good quality higher and poor quality lower than the rest of the panel. A panel member may have a *constant* bias, or a *variable* one which changes from occasion to occasion. In the latter case one would wish to be able to show that each bias depends on factors such as the time of day, fatigue, the number or similarity of the samples tested at once, and so on. A constant bias may be regarded as due to a different interpretation of the score sheet, and this could, in principle, be remedied. It would be difficult to allow for a variable bias in an analysis.

If the bias of a panel member is subtracted from the score that he has given to a sample, the remaining discrepancy with the panel's mean score can be considered a measure of his *residual error*. It may appear that one person has, on the whole, smaller residual errors than

others. He is then more consistent and more discriminating, in that he tends to give similar scores to similar samples and different scores to samples which differ according to the classification of the score sheet. Just like bias, residual errors may depend on the quality of the samples; their size may vary from occasion to occasion, and depend on such factors as are enumerated above in connexion with variable bias.

These two criteria and methods for their statistical estimation are further discussed in Appendix II. The conclusions obtained from the experimental data are summarized here.

Table I

Averaged bias estimates for 10 batches of 18 fish

Panel member	1	2	3	4	5	6	7
No. of samples tested	180	72	126	162	180	144	72
Raw odour	0·23*	− 0·04	− 0·03	0·18*	− 0·04	− 0·31*	− 0·18
Cooked odour	0·01	0·29*	0·23*	0·09*	− 0·10	− 0·01	− 0·34*
Cooked flavour	0·05	0·21	0·36*	0·03	0·06	− 0·17	− 0·35*

* Significant at 5% probability level

Biases.—In the first place, the ten batches of 18 fish mentioned previously have been considered. A panel member's bias has been estimated as the difference between his and the panel's mean scores for a given set of fish. Such an estimate of bias is not independent of the other members present, and, as the composition of the panel varied, the rigorous comparison of biases for different sets of fish is difficult. However, one can form some idea of constant biases by merely averaging each panel member's values (Table I). It is found that panel members 1, 4 and 6 are significantly biased for raw odour and that 2, 3 and 7 have biases for cooked odour and flavour. Numerically these biases are less than half a unit in size. For batches 1 to 5 and the nine additional sets of three fish mentioned above, a more rigorous examination for bias has been made, allowing for the effect of different combinations of persons in the data examined (Table III). The numerical results of these two calculations are in general agreement and are further discussed in Appendix II.

There is also some variable bias, i.e. the biases of some panel members vary significantly from occasion to occasion; it has not been possible to identify from the data causes for this variation.

Residual errors.—As defined above, residual errors are distributed with zero mean; and the variance (the square of the standard error or standard deviation) of the residual errors gives a useful measure of their distribution. An unbiased estimator of a panel member's error variance is derived in Appendix II, and the numerical values obtained are set out in Table II. ('Unbiased' here has no connexion with a panel member's bias; it denotes that the estimate is such that in the long run the average value would be equal to the 'true' value.) To assist in interpreting the significance of a difference between two panel members' variances for the same group of samples, a formula for the sampling variance of such a difference is also given in Appendix II.

There is considerable variation in the values of the variance estimates (Table II). Causes for this variation can to some extent be identified. Thus the scores for 'cooked' quality-factors of the second batch of 7-day-old fish are quite unusual; not only are the panel's error variances consistently greater than average, but the estimated variation of the fish themselves in the batch is given by a variance value of 1·4, as against the average value of 0·3 of all other batches (see below). The values for this batch may therefore on the whole be regarded as exceptional. Again, the 18 fish called '1st batch' were all tested in the first days of the experiment, and this may be the cause of the generally higher errors occurring. The other very erratic groups consist of very stale fish, the 3rd batch (19-, 20-, 21-day-old) and the 15- and 21-day-old batches, and here a distinct source of error is apparent from the individual scores: Occasionally a single panel member scored a sample zero when the rest of the panel gave higher scores (2–4 say). At least one panel member was aware of this tendency to give a zero score for rather stale fish, when with his usual care he could have arrived at a higher value. It seems that this misuse of the scoring system could be remedied; very stale fish will not, in any case, have to be considered frequently by the panel. Here these 'spurious' zero-scores do account for most of the larger variances. Hopkins[18] has suggested that according to his data—obtained without a trained panel—the assessment of the quality of poor samples might be more erratic. But where in the present set of experiments the error variances for stale fish are larger it is due mainly to the particular sources of errors discussed, and the most accurate panel members, 4 and 5, do not show this effect.

Table II

Variance estimates for panel members 1–7 *: raw odour* (R), *cooked odour* (O) *and cooked flavour* (F)
All estimates including ' spurious zero ' scores are marked *. These values, and the two batches marked † which are also discussed in the text, identify most of the larger estimates

Panel members ..	1			2			3			4		
	R	O	F	R	O	F	R	O	F	R	O	F
1st batch† ..	0·71	0·98	1·51	0·59	0·60	0·01	0·47	1·53	3·00	0·32	0·65	0·29
2nd ,, ..	0·02	0·75	0·44				0·51	0·54	0·63	0·32	0·17	0·43
3rd ,, ..	1·54	0·87	3·04*	0·84	0·26	0·32	1·98	0·80	−0·02	0·14	0·27	0·48
4th ,, ..	0·13	0·89	0·70				0·96	1·29	1·79	1·09	0·88	0·25
5th ,, ..	0·25	0·83	0·44	0·55	0·92	0·20	0·59	0·77	0·40	0·45	0·04	0·13
7 days batch ..	0·43	0·35	0·48							0·41	0·30	0·07
†7 ,, ,, ..	0·43	2·46	1·29	0·56	1·10	3·78						
11 ,, ,, ..	0·76	0·09	0·33							0·21	0·01	0·13
15 ,, ,, ..	1·60	0·89	4·35*				0·52	1·65	1·42	0·56	0·05	−0·05
21 ,, ,, ..	2·53*	1·73*	2·55*				1·57	0·92	0·73	0·22	0·06	0·32
Panel members' means	0·84	0·98	1·51	0·64	0·72	1·08	0·94	1·07	1·13	0·41	0·27	0·23
		1·11			0·81			1·05′			0·30	

Panel members ..	5			6			7			Batch means			Mean of batch means
	R	O	F	R	O	F	R	O	F	R	O	F	
1st batch† ..	0·32	0·69	0·71	1·22	0·86	2·40*	1·10	3·18	0·92	0·68	1·21	1·26	1·05
2nd ,, ..	0·38	0·28	0·57				0·26	0·49	0·40	0·30	0·45	0·49	0·41
3rd ,, ..	0·74	0·61	2·26*	1·64*	1·44	2·02				1·15	0·71	1·35	1·07
4th ,, ..	0·52	0·91	0·85	1·16	1·99*	2·51*				0·77	1·19	1·22	1·06
5th ,, ..	0·43	0·44	0·27				0·43	0·95	1·19	0·45	0·66	0·44	0·52
7 days batch ..	0·50	0·26	0·17	0·36	0·39	0·37				0·43	0·32	0·27	0·34
†7 ,, ,, ..	0·73	1·02	0·86	0·54	1·96	2·97*	0·31	1·96	2·59*	0·51	1·70	2·30	1·50
11 ,, ,, ..	0·27	0·36	0·39	1·16	0·58	1·00				0·60	0·26	0·46	0·44
15 ,, ,, ..	0·80	1·14	0·11	0·60	2·10	4·23*				0·82	1·17	2·01	1·33
21 ,, ,, ..	0·09	0·21	0·27	0·64	0·44	2·04*				1·01	0·67	1·20	0·96
Panel members' means	0·48	0·59	0·65	0·92	1·22	2·19	0·53	1·65	1·27	0·68	0·86	1·13	0·89
		0·57			1·44			1·15					

Considering all the data for the ten batches, the average difference, 0·8, in error variance between panel members 1 and 4 may be regarded as statistically significant; it is clearly due to the differences for very stale fish, the 3rd and the 15- and 21-day-old batches, only. (Compare also the ' spurious zero ' asterisks in Table II.) The variances of panel member 4 appear to be significantly smaller, by 0·3, than those of 5, but the difference of 0·4 between the more inaccurate members 3 and 6 is hardly significant.

Panel members 8 *and* 9.—The scores of panel members 8 and 9 have been considered separately, as they were aware of the age of the samples. Here the error of scoring is estimated from the discrepancy between their scores and the mean score of the rest of the panel; the two values are therefore independent and the statistical approach is easier (cf. Appendix II). Both panel members have error variances of about 0·5, and only the bias of number 8 for ' cooked ' odour (0·2) is statistically significant.

Conclusions on panel reliability.—To sum up, bias has been found, and although it is not negligible the establishment of fairly large differences in quality (e.g. 1 unit or more) should not be very much affected. Most panel members were considerably more erratic than the best, but special causes for some of the worst aberrations seem to exist. Unless biases are negligible panels will have to be large and cannot be used as absolute instruments of assessment with much confidence. Residual errors should clearly be small, and not differ much from occasion to occasion. So long as panels have to be large, it is perhaps important to note that no panel member here is so inaccurate that his inclusion in a panel consisting even of the more accurate members only would not improve the reliability of the panel's mean score.

The standard error of a single score in this set of experiments must on the average be taken as not much less than one unit. In addition there will be the sampling error of fish of the same age to take into account (see below). In practice this would mean that under the same panel conditions the difference of the means of two sets of n scores would have a standard error of about $\sqrt{(2/n)}$, and only a difference at least twice as large would be judged statistically significant. For example, to be able to establish a difference of half a unit as significant at

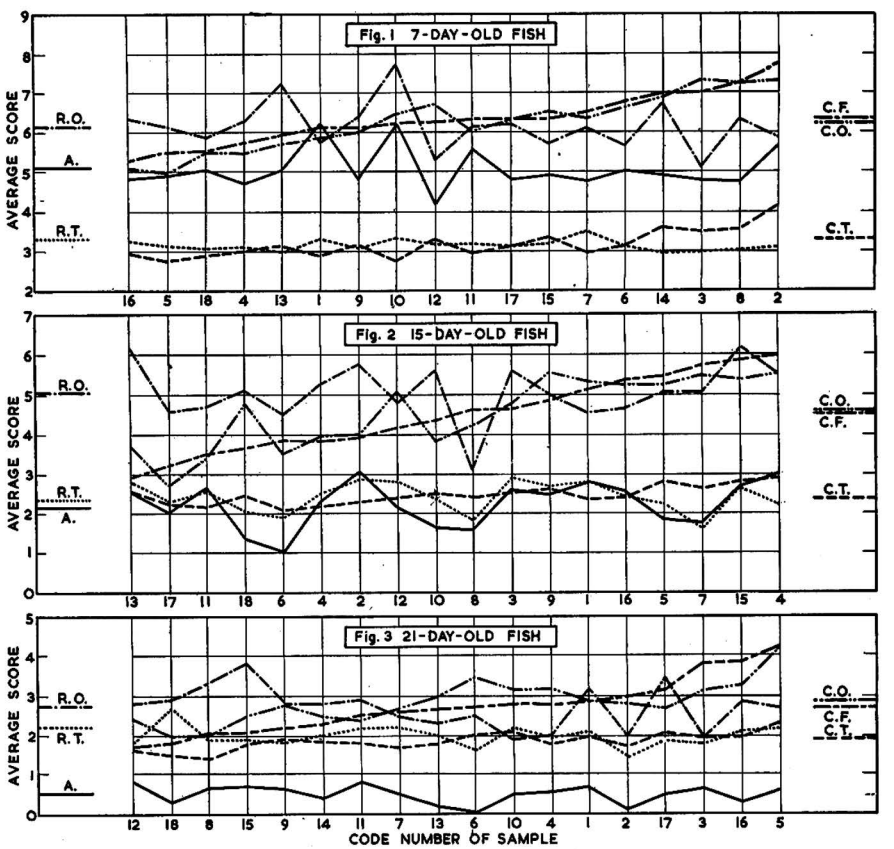
least 30 scores would be needed in each set, e.g. a panel of five scoring two batches of six samples each.

The relationship between the quality-factors

In investigating the relationships between the quality-factors of fish as evaluated in this set of experiments, only the panel's *average* scores for each quality-factor have been examined and no attempt to look into the behaviour of individual panel members from this point of view has been made. (The score sheet originally designed as a result of the period of intensive training by the panel has undergone several modifications in the light of subsequent experience. The one given in Appendix I is the revised version now in use.)

It is of little use here to compute the traditional correlation coefficients, for such coefficients could not be readily interpreted. The batches of fish usually scored under experimental conditions are not random samples from some population in which the scores are approximately normally distributed (the basis of the usual descriptive interpretation of correlation coefficients and tests of significance etc.); instead, fish of different ages have been deliberately selected.

Raw and cooked odour, and flavour.—The scores for cooked flavour and cooked odour, as determined by the present technique, are in close agreement. These factors were scored simultaneously, but actual duplications of two scores for one sample are not particularly frequent. The panel's scores for single fish have been plotted graphically, and typical graphs for three batches of 18 fish, 7-day, 15-day and 21-day-old respectively, are shown in Figs. 1–3.



FIGS. 1–3.—*The six quality-factors for representative batches of 18 samples of fish, arranged in order of increasing cooked-flavour scores. Mean values marked in the margins* (A. = *appearance*, R.O. = *raw odour*, R.T. = *raw texture*, C.O. = *cooked odour*, C.T. = *cooked texture*, C.F. = *cooked flavour*)

Even comparatively slight changes in cooked flavour are accompanied by corresponding variations in the cooked-odour scores, and the differences between the two scores for any fish are in fact no larger than might be expected from their experimental errors. On each graph the fish have been deliberately arranged in order of increasing cooked-flavour scores to show clearly that the raw-odour score for a fish differs on the whole significantly from the cooked quality scores: for a number of fish of fairly similar quality the raw-odour scores lie within the same range as the cooked scores but do not increase with the cooked quality. The mean scores for the three characteristics for groups of fish of the same age are generally not significantly different. Raw odour and cooked odour-and-flavour are therefore about equally well correlated with the age of the fish, but they are not so closely correlated with each other.

A similar effect can be observed from the estimation of bias (cf. Tables I and III); individual panel members have very similar biases for cooked odour and cooked flavour, but the raw-odour values appear to be quite independent. Regarding the whole panel's accuracy in scoring for these quality-factors, average consistency as indicated by the existence of biases is about the same for each quality-factor (Table I or III). However, the panel seems to be more erratic for cooked flavour (average variance 1·1) than for cooked odour (0·9), and most accurate for raw odour (0·7) (cf. Table II). These differences are due partly to the ' spurious zero ' scores discussed earlier and have not been tested for statistical significance.

External appearance.—Each of the five external factors—eyes, gills, slime, flesh including belly flaps, and general appearance—was scored 0, ½, 1, 1½ or 2 [according to the initial form of the score sheet (cf. above)]. As the five scores for one fish were found to differ almost always by less than half a unit, and as there is no obvious regularity as to which property is scored higher or lower, the five scores have with some justification been added in this instance to give an ' external appearance ' score with a possible range from zero to ten. This composite score has been plotted against the raw- and cooked-odour and -flavour scores (e.g. Figs. 1–3); it seems to be correlated with the raw-odour score and, as is apparent from the graphs shown, there is no trend in the appearance scores corresponding to the increase for fish at the same age in cooked odour-and-flavour quality. For any batch of not very stale fish (e.g. scores for cooked factors greater than five), the average composite score is about one unit less than the odour and flavour averages; for stale fish there is a sudden relative deterioration in external appearance as evaluated here, the composite score being about two units less.

Texture.—Raw and cooked texture are fairly constant for a batch of fish of one age, as is shown on the three representative graphs. The fish, which are, as already mentioned, plotted in order of increasing cooked-flavour scores, show a slight similar trend in their cooked-texture scores. The raw-texture scores appear to be correlated with the other raw properties. Especially for fairly fresh fish, the texture scores decrease rather slowly as the fish age and eating-quality otherwise deteriorates. Texture in this set of experiments would therefore not seem to be a striking criterion for distinguishing between fish that are not definitely stale.

Chemical data.—A brief examination of the data of the chemical analysis for trimethylamine content of the fish suggests that this ' non-sensory ' test does not provide a very precise indication in these experiments either of age-in-ice or of quality, as evaluated by scoring for cooked odour and flavour or raw odour. However, there are definite regressions of trimethylamine content on both age and the panel scores, but the scatter of values is considerable. Thus thirty-nine 7-day-old fish give a mean measurement of 10·8, with a standard deviation of 7·4, and twenty 21-day-old fish give a mean of 40·1 and a standard deviation of 11·8. Further investigation of the relationship between this chemical test and sensory scoring may lead to more precise and useful results.

The validity of the scoring technique

Certain criteria should be developed at this stage in addition to those for the internal consistency of the panel which have been discussed above. But in these experiments the panel have scored a sample only once for each quality-factor, so that one cannot examine directly whether they would score a given sample similarly on different occasions, or how far it is justifiable to represent each quality-factor on a one-dimensional scale, or whether scoring errors are independent, and so on.

However, one can conclude from the fairly good internal consistency of the panel and other such indirect indications that the whole procedure appears to be valid in most respects. Thus, the individual panel members agree sufficiently so as to appear to be evaluating a ' real ' effect. Again, fish of the qualities which the panel is expected to score (up to 21-days-on-ice) were tested; in scoring these fish practically the whole possible range of scores for any one property, e.g. 0 to 10, or 0 to 5, etc. has been used, and with increasing age-on-ice the scores generally

decrease (cf. mean values of Figs. 1–3). There is also agreement between the panel's scores for some of the different quality-factors, either for groups of fish (e.g. the raw-odour and cooked-odour means for sets of fish of the same age) or even for single fish (e.g. the cooked-odour and -flavour scores).

The averaging of numerical scores.—A different question that has to be answered is: What operations may be performed on the scores? In particular, is it 'valid' to average a set of scores, or, what interpretation can such an average score be given?

It is found (see Appendix II) that on the whole the variance of residual scoring errors does not vary with the scoring level and that the errors appear to follow normal (Gaussian) distribution laws. If, then, biases are negligible, or if the constitution of the panel has remained constant, the average of the individual panel members' scores for a sample would differentiate the sample completely from any other sample specified by its average score.

In the same way it is of considerable interest that the average variance of fish of the same age-in-ice, which is about 0·3, appears not to vary systematically between raw odour, cooked odour and cooked flavour, or with number of days in ice, nor to depend on whether it is estimated from a set of 18 fish tested on one day or from a set of three fish served in a random order with another such set. (There is the exception of the second batch of 7-day-old fish mentioned earlier, for which there existed, however, some *a priori* suspicion that the fish might not all have had the same treatment. These quite unusual values have therefore been excluded from the average figure of 0·3.)

Scores and stowage period in ice.—A different point may also be mentioned under the heading of validity, since it bears on the interpretation of the scoring system. It is the relationship between the scores and the period of stowage in ice of the fish.

For raw and cooked odour and cooked flavour, which are considered in some respects the most important quality-factors, two roughly linear relationships between scores and the number of days in ice under the best conditions appear to hold good. Thus, above a score of 8 unit fall is produced in about four days in ice, and between scores of 8 and 2 it is produced by from two to three days in ice.

Conclusions of the analysis

It has been suggested that each panel member's scoring-reliability within the panel can be described by his 'biases', i.e. his consistent discrepancies, and by his more random 'residual errors'. Various factors may introduce biases and residual errors, such as differing sensory discrimination, training, fatigue, etc. on the one hand and variability in the material tested on the other. It appears that all members tended to score similarly within limits of about half a unit; large biases would, of course, have made the data difficult to interpret, and indeed, the purpose of training the panel is to help them to agree amongst themselves. Formulae for the estimation of the variances of the residual errors have been developed, and it has been shown that certain panel members were more accurate than others, the average variances varying from a figure of 0·3 to one of 1·4 for the most erratic panel member.

Such biases and residual errors would permit one to say that samples of fish differing by one unit are clearly distinguishable; smaller differences can also be detected, although they may lack precise meaning because of the existence of the scoring biases. It can, for example, be shown that certain roughly linear relationships between the scores and the number of days in ice under the best conditions appear to hold good. Again, close relationships appear to exist between the scores for the various quality-factors, particularly cooked odour and cooked flavour. There is also a definite relationship between the chemical tests and the panel scores, but the scatter of values is very considerable.

From the data available it was not possible to test explicitly whether the panel members, individually or as a whole, are consistent from one day to the next, or, therefore, whether it is valid to compare scores obtained on different occasions, but no reason has become apparent from the data for believing otherwise. In particular, it was concluded from the empirical evidence that for each quality-factor of odour, flavour and so on, the individual fish, as well as batches of fish of the same age-in-ice, can on the whole be compared simply by the averages of the panel members' scores.

Further experience with the scoring technique

The scoring technique described above has been in continuous use, both in the laboratories of the Scientific Adviser's Division, Ministry of Food, and at Torry Research Station (Department of Scientific & Industrial Research), since its development in 1948. At Torry it has been used,

together with chemical tests, as a tool in various laboratory experiments, such as those concerned with the effects of temperatures in the region of 0° on the rate of spoilage of cod[10] and in assessment of the improvement in quality possibly accruing from modifications in the practice of handling and stowage on commercial fishing vessels, e.g. ' shelving ' compared with ' bulking '.[10] In these latter experiments it is tacitly assumed that the intrinsic quality of cod, which may vary somewhat owing to a variety of factors such as season, environment etc., has little effect on the quality factors as given in the score sheet. The laboratories of the Scientific Adviser's Division, Ministry of Food, have also used these techniques on wet fish and, with slight modifications, in experiments on frozen fish.

It is not suggested that this technique, which appears to have obvious advantages as a laboratory tool, should, or even could, be used generally in market inspection, where there is usually no dubiety about the majority of fish landed, particularly if it is obviously fresh or putrid. It is in doubtful cases that difficulties arise, and then the use of a scheme such as outlined above, perhaps supplemented by appropriate physicochemical measurements, might possibly be of practical value.

To conclude, the technique consists essentially in describing the various sensorily perceptible quality-factors, such as odours, flavours etc. of the material. The descriptions are put into arbitrary numerical forms for the convenience of statistical analysis and the derivation of empirical mathematical laws, and in this way do not seem to differ from classical instrumental measurements. The peculiar importance of the method is that the perception of these quality-factors affects the ordinary consumer in his judgment of the food. Its actual usefulness depends on the calibration or interpretation of the scales against factors such as the effect of storage in ice under the best conditions and consumer acceptance and preference.

Critical analysis of the results so far obtained has shown that various aspects of the panel procedure require closer scrutiny and a series of experiments is now under way with this object in view. Although the present system may well require considerable alteration in the future, it does appear to have advantages over any so far published and has already yielded results which show that it is a valuable instrument in the assessment of fish quality.

Appendix I: Score sheet for the organoleptic characteristics of white fish

Raw fish

	Score marks
General appearance (5 *marks*)	
Eyes perfectly fresh, convex black pupil, translucent cornea; bright red gills (colour depending on species); no bacterial slime, outer slime water-white or transparent; bright opalescent sheen, no bleaching	5
Eyes slightly sunken, grey pupil, slight opalescence of cornea; some discoloration of gills and some mucus; outer slime opaque and somewhat milky; loss of bright opalescence and some bleaching	3
Eyes sunken; milky-white pupil, opaque cornea; thick knotted outer slime with some bacterial discoloration	2
Eyes: completely sunken pupil; shrunken head covered with thick yellow bacterial slime; gills showing bleaching or dark brown discoloration and covered with thick bacterial mucus; outer slime thick yellow-brown; bloom completely gone; marked bleaching and shrinkage	0
Flesh, including belly flaps (5 *marks*)	
Bluish translucent flesh, no reddening along the back-bone and no discoloration of the belly flaps; kidney bright red	5
Waxy appearance, no reddening along backbone, loss in original brilliance of kidney blood, some discoloration of belly flaps	3
Some opacity, some reddening along backbone, brownish kidney blood and some discoloration of the flaps	2
Opaque flesh, marked red or brown discoloration along back-bone, very brown to earthy-brown kidney blood, and marked discoloration of the flaps	0
Odours (10 *marks*)*	
Fresh ' seaweedy ' odours	10
Loss of fresh ' seaweediness ', shellfish odours	9
No odours, neutral odours	8
Slight musty, acetamide-like, milky or caprylic acid-like odours	7
' Bready ', ' malty ', ' yeasty ' odours	6
Lactic acid, ' sour milk ', or oily odours	5
Some lower fatty acid (e.g. acetic or butyric acids), or ' grassy ', slightly sweet, fruity odours	4
Stale, sour, ' cabbage water ', ' turnipy ', or phosphine-like odours	3
Ammoniacal (trimethylamine and other lower amines) with strong *o*-toluidine-like odours	2
Hydrogen sulphide, other sulphide and strong ammoniacal odours	1
Nauseating, putrid, faecal odours; indole, ammonia, etc.	0

	Score marks
Texture (5 *marks*)	
Firm, elastic to the finger touch	5
Softening of the flesh, some grittiness on skin	3
Softer flesh, definite grittiness and scales easily rubbed off the skin	2
Very soft and flabby, retains the finger indentations, grittiness quite marked and flesh easily torn from the back-bone	1

Cooked fish (*approx.* 6–8 *oz. middle cut of fish steamed en casserole in resistance-glass dishes* (7 *in. in diameter*) *over boiling water for* 35 *minutes*

Odour (10 *marks*)*	
Strong fresh 'seaweedy' odours	10
Some loss of fresh 'seaweediness'	9
Lack of odour, or neutral odours	8
Slight strengthening of the odour but no sour or stale odour; 'wood shavings', 'woodsap', vanillin or terpene-like odours; slight salt-fish or cold storage odours	7
'Condensed milk', caramel or toffee-like odours	6
'Milk jug', 'boiled-potato' or 'boiled clothes', or metallic odours	5
Lactic acid, 'sour milk' or *o*-toluidine-like odours	4
Some lower fatty acid (e.g. acetic or butyric acids) 'grassy', 'soapy', 'turnipy' or 'tallowy' odours	3
Ammoniacal (trimethylamine and lower amines) odours	2
Strong ammoniacal (trimethylamine etc.) and some sulphide odours	1
Strong putrid and faecal odours (ammonia, indole etc.)	0
Texture (5 *marks*)	
Firm thick white curd; bluish-white in appearance, no discoloration	5
Firm, but woolly; loss of bluish whiteness, some yellowing	3
Softer, cheesy; marked discoloration	2
Sloppy, soapy; very marked browning along the back-bone	1
Flavour (10 *marks*)*	
Fresh, sweet flavours characteristic of the species	10
Some loss of sweetness	9
Slight sweetness and loss of the flavour characteristic of the species	8
Neutral flavour, definite loss of flavour but no off-flavours	7
Absolutely no flavour, as if chewing cotton wool	6
Trace of off-flavours, some sourness but no bitterness	5
Some off-flavours, and some bitterness	4
Strong bitter flavours, some rubber-like and slight sulphide-like flavours	3
Strong bitter flavours, but not nauseating	1
Strong putrid flavours (e.g. sulphides) tasted with difficulty	0

* The descriptive terms, although capable of irrelevant associations, are those used spontaneously and agreed upon by the original panel at the Torry Research Station. Other panels may tend to use other, but probably similar, terms. A more precise terminology, replacing ordinary descriptive terms by suitable chemical analogues, is being continuously pursued.

Appendix II: Statistical considerations

The statistical methods used in the analysis of the experimental data, and in some cases the data themselves, are considered in more detail in this Appendix.

The idea underlying the analysis is to consider the score x_{ij} of the jth panel member for the ith sample in the form

$$x_{ij} = a_i + b_j + e_{ij}, \quad (i = 1, \ldots, p;\ j = 1, \ldots, q),$$

where a_i is the characteristic value of the ith sample, b_j is the value of the jth panel member's bias, and e_{ij} is the residual error, distributed independently with zero mean and variance s_j^2. This is a generalization of the traditional analysis-of-variance kind of model.

Biases.—The jth panel member's bias b_j has been estimated as the difference between his own and the panel's mean score for a set of fish of the same age in ice (i.e. either eighteen or three fish). The estimated standard error of such a value, formed from p fish, is $\sqrt{(sj^2/p + s^2/pq)}$, where sj^2 and s^2 are the error variances of the jth panel member and of the q panel members' mean score respectively.

In mathematical notation, the estimate of bias is $x_{.j} - x_{..} = b_j + e_{.j} - b_{.} - e_{..}$, where the second suffix is summed over the q panel members present. (The notation $x_{.j}$ represents the mean of the values indicated by the period in the suffix, e.g. $x_{.j} = \sum_{i=1}^{p} x_{ij}/p.$) Clearly, the estimate is not independent of the other persons who are present on the panel (represented by the terms $b_{.}$ and $e_{..}$), and is therefore affected by the varying constitution of the panel for

the different batches of fish. But Table I, giving the simple averages for all the ten batches, should indicate the constant biases of the panel quite well.

Table III

Bias estimated by least squares from 39 sets of three fish

Panel member	1	2	3	4	5	6	7
No. of samples tested	117	69	111	114	117	72	66
Raw odour	0·36*	0·11	− 0·14	0·15	0·08	− 0·79*	0·21
Cooked odour	− 0·06	0·15	0·47*	0·21	0·09	− 0·27	− 0·58*
Cooked flavour	0·02	0·27	0·42*	0·11	− 0·16	− 0·25	− 0·41*

* Significant at 5% probability level

The biases have also been estimated more elaborately by ' least squares ', allowing for the effect of the varying panel-composition (Table III). The data used are batches 1–5 and the additional nine sets of three fish mentioned earlier, taking each panel member's mean for each set of three fish, after fitting the panel's mean scores. The resulting seven linear equations in the unknowns b_j are not linearly independent, and one more relation is necessary to solve them, which has somewhat arbitrarily been chosen as $\sum_{j=1}^{7} b_j = 0$ (see also below, under ' The distribution of errors '). The solution of these equations involves the inversion of the eight-by-eight matrix of their coefficients, a lengthy procedure. However, the same matrix applies to each of the sets of equations for cooked flavour, cooked odour and raw odour ; at the same time the inverted matrix gives the sampling variances and covariances of the bias estimates (i.e. $\mathrm{cov}(b_r b_s) = c_{rs}s^2$, where c_{rs} is the element in the rth row and sth column of the inverted matrix, and s^2 is the variance of the residuals after fitting panel means and biases). The only entry very different from Table I, nearly significant in both Tables (number 7 for raw odour), is due to a batch of eighteen 7-day-old fish for which this panel member has a decided negative bias, and which is of course not included in the data on which Table III is based. On the whole the two Tables agree well, supporting the view that the error of averaging bias estimates from panels of somewhat varying constitution is in this case not very great.

The entries in Tables I and III represent average biases. Occasionally one person's bias estimates vary significantly from one set of fish to another. It has not been possible to identify any causes for this variable bias. The divergencies remaining after fitting the average bias estimates and the panel's means to the panel member's scores are considered as more or less random residual errors.

Error variances.—The variances $s_j^2 (j = 1, \ldots, q)$ of the residual errors have to be estimated from data which do not contain repeated scores. Clearly, the error is $e_{ij} = x_{ij} - a_i - b_j$, but as the values of a_i and b_j are unknown, estimates for them such as $x_{i.}$ and $(x_{.j} - x_{..})$ have to be substituted, giving the following expression :

$$x_{ij} - x_{i.} - x_{.j} + x_{..} = e_{ij} - e_{i.} - e_{.j} + e_{..}$$

The expected value (denoted by E) of the square of this expression, summed over p samples,

$$E\sum_{i=1}^{p}(x_{ij} - x_{i.} - x_{.j} + x_{..})^2 = E\sum_{i=1}^{p}(e_{ij} - e_{i.} - e_{.j} + e_{..})^2$$

is

$$\frac{(p-1)(q-1)^2}{q^2}s_j^2 + \frac{p-1}{q^2}\sum_{t\neq j}^{q}s_t^2 \qquad \text{(for all } j = 1, \ldots, q)$$

i.e. the expression is a statistically biased estimate of s_j^2.

These sums of squares, when summed over the panel of q members, are identical with the residual sum of squares in an analysis of variance of the data ; if all the panel members have the same error variance, s^2, such an analysis of variance gives, of course, an unbiased estimate of it :

$$E\sum_{j=1}^{q}\sum_{i=1}^{p}(x_{ij} - x_{i.} - x_{.j} + x_{..})^2 = (p-1)(q-1)\sum_{t=1}^{q}s_t^2/q = (p-1)(q-1)s^2$$

From the last two equations it follows that in the general case an unbiased estimator of s_j^2 is

$$\frac{q}{(p-1)(q-2)}\left\{\sum_{i=1}^{p}(x_{ij}-x_{i.}-x_{.j}+x_{..})^2-\frac{1}{q(q-1)}\sum_{i=1}^{p}\sum_{t=1}^{q}(x_{it}-x_{i.}-x_{.t}+x_{..})^2\right\}$$

As this estimator is unbiased, one can average the values from different samples to give the best estimate, if the panel are scoring with the same accuracy on each occasion. In the present set of experiments, however, this was fairly clearly not the case, owing in particular to the ' spurious zero ' scores given occasionally (cf. main text). Moreover, the accuracy of estimates based on different sample numbers, i.e. $p = 3$ and $p = 18$, will differ. However, the straightforward ' panel members means ' in Table II are useful and reliable condensations of the data. The two very small negative estimates (Table II, panel numbers 3 and 4, cooked flavour) have arisen because the particular panel member is relatively accurate, and the negative term in the estimator of s_j^2 is quite large, compared with the first term.

It should perhaps be mentioned that since the unbiased variance estimator is the difference of sums of squares, it is not distributed in a χ^2 distribution. One cannot therefore apply a logarithmic transformation to normalize the distribution before performing an analysis of variance on the variance estimates themselves,[19] as this would give a very much more skew distribution than the original one. (The effect is very marked on the small values, let alone the negative estimates.) However, assuming the normality of the distribution of the residual errors (see below), fairly simple expressions for the sampling variances and covariances for the variance estimates can be calculated.

Apart from the effect of the ' spurious zero ' scores, the variation of the variance estimates in Table II from one batch to another appears to be irregular and has not been further investigated. On the other hand, some panel members appear to be generally more accurate than others, and it is of interest to see what variance differences can be taken to represent significant differences in scoring accuracy. The variance of the estimated difference between the jth and kth panel members' variances s_j^2 and s_k^2 for a group of p samples has therefore been obtained :

$$\frac{2}{(p-1)}(s_j^4+s_k^4)+\frac{4}{(p-1)(q-2)^2}(s_j^2+s_k^2)\sum_{t\neq j,\,k}^{q}s_t^2.$$

In the application of this formula, summarized in the main text, the estimates of the variances s_t^2 ($t = 1, \ldots, q$) were substituted for the population values. Variance differences between any two panel members for a single batch of 18 fish were not found significant, but on combining the data of different batches a more sensitive test was obtained. This is a rigorous test of significance (as far as the ' standard error ' is at all applicable here), and is not to be confused with the fact that averaging the variances for different groups does not necessarily give the ' best ' estimates.

The formulae here developed break down for a panel of two members only ($q = 2$) ; the whole problem, including a solution for $q = 2$, has been more fully discussed elsewhere.[20, 21]

Panel members 8 and 9.—The analysis of the scores of panel members 8 and 9 is more straightforward, as their agreement with the rest of the panel was tested. The estimate $\bar{x}_i$ of a_i which is used is independent of the actual score x_i, and so the bias for a set of p samples is estimated as $\sum_{i=1}^{p}(x_i-\bar{x}_i)/p$. The residual error variance s_j^2 can be estimated from the variance of these quantities,

$$\mathrm{var}(x_i-\bar{x}_i)=s_j^2+s^2/q$$

where s^2 is the known error variance of the panel of q members whose estimates $\bar{x}_i$ of the a_i we are using. This simpler form of analysis might be suitable in general to compare a new person with a reliable panel.

The distribution of residual errors.—It is of interest to investigate the distribution of residual errors e_{ij}. For the 39 sets of three fish of one age, the following estimate of the residual errors has been used : $x_{ij} - x_{i.} - b_j$, where b_j is the least-squares estimate of the jth panel member's bias (Table III). In estimating these values, it was assumed that $\sum_{j=1}^{7} b_j = 0$. Since the composition of the panel varied for the 39 sets of fish, the panel mean $x_{i.}$ is not generally a summation over the seven panel members and should have been adjusted in each case for the biases of the

panel actually present, to be quite accurate. An apparent consequence of the omission of this slight adjustment is mentioned below.

The distribution of the errors of 0–7, 8–14 and 15–21-day-old fish for each of the raw-odour, cooked-odour and cooked-flavour quality-factors have been considered. The mean values of the distributions are not significantly different from zero, showing that the bias estimates as a whole do not depend on the age of the sample. The variances for the three quality-factors indicate, as did the unbiased variance estimates (Table II), that raw odour has been scored most accurately, and cooked flavour most erratically. For each quality-factor the error variances of the three age-groups are practically equal. The histogram and best normal fit for cooked flavour is given in Fig. 4, which is typical also of the other two quality-factors. It is apparent that the fit is on the whole good. The error estimates are not independent, as they are constrained to add to zero for any one sample; one cannot therefore test the goodness of fit by the usual χ^2 test. Such a precise test is, however, not really essential at this stage. The excess of large negative errors, which also occurs for cooked odour, is clearly due to 'spurious zero' scores and to the slight statistical bias, mentioned above, of the error estimates caused by the varying constitution of the panel; for example, in the case of cooked flavour the bias 0·42 (cf. Table III) has been subtracted from 111 scores, and — 0·41 from only 66 scores. The excess of very small errors is, for these data, due to the superposition of differently sized samples from seven distributions (one for each panel member) with different variances.

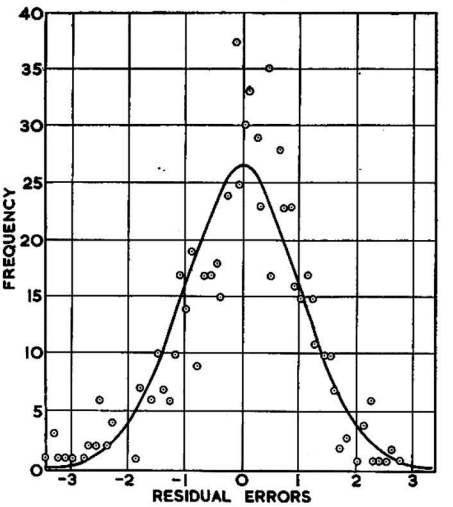


FIG. 4.—*Frequency distribution and best normal fit for residual-error estimates from the cooked-flavour data*

Finally, the variation of the scores of fish of one age can be estimated by subtracting from the mean square of the panel's average scores the effect of the panel's residual errors, i.e. the estimate of var a_i is

$$\frac{1}{(p-1)}\sum_{i=1}^{p}(x_{i.}-x_{..})^2-\frac{1}{q^2}\sum_{t=1}^{q}s_t^2$$

where the s_t^2 have been calculated previously. The distribution of such scores appears to be approximately normal.

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THE MINERALIZATION OF NITROGEN IN A SOIL ACIDIFIED WITH SULPHUR, ALUMINIUM SULPHATE OR FERROUS SULPHATE

By A. H. CORNFIELD

The mineralization of nitrogen in a soil which had been acidified to different degrees by applying sulphur, aluminium sulphate or ferrous sulphate at three equivalent rates was studied. With low and medium applications of the acidifying materials pH was reduced to a similar extent at each rate by all materials, but with heavy applications sulphur had the least potent, and ferrous sulphate the most potent, effect in reducing pH. During incubation of the treated soils in the absence of added chalk, nitrate and total mineral nitrogen (ammonia plus nitrate) accumulation decreased, whereas ammonia accumulation increased with the amount of acidifying agent used. When the acidified soils were incubated with excess of added chalk, nitrate accumulation returned to normal, except where heavy application of sulphur and ferrous sulphate had been made; total mineral-nitrogen accumulation returned to normal, except where heavy ferrous sulphate application had been made; and ammonia accumulation occurred only where heavy ferrous sulphate application had been made.

The reduction of soil pH by the application of acidifying materials such as sulphur has been practised in order (*a*) to increase the availability of trace elements such as manganese,[1] (*b*) to reduce disease incidence in plants (e.g. Hooker[2] found that commercial control of potato scab was obtained by applying sulphur to the soil) and (*c*) to produce suitable soil conditions for the growth of acid-loving species such as rhododendrons and azaleas.

Apart from a mention by Fraps & Sturges[3] that the application of sulphur to a soil resulted in decreased nitrification, little attention has been paid to changes in ammonification and nitrification that may result in soils which have been acidified in this way. Since there is a

general shortage of sulphur, ferrous and aluminium sulphates are likely to come into more general use for acidifying soils. The purpose of this paper is to report results obtained on the mineralization of nitrogen during incubation of a soil which has been acidified under laboratory conditions with sulphur, aluminium sulphate or ferrous sulphate applied at three rates on the basis of sulphur equivalence.

Methods

A field-moist soil (14·7% water) was passed through a ¼-in. mesh sieve. The soil was of medium-high fertility and had the following characteristics: mechanical composition—coarse sand 9·3%, fine sand 56·7%, silt 18·7% and clay 12·6%; total nitrogen 0·19%; organic carbon 2·1%; pH 6·65. 500-g. samples of the sieved soil were placed in amber-glass flower pots. Sulphur, aluminium sulphate or ferrous sulphate were added at the rate of 0·03%, 0·1% and 0·3% of sulphur-equivalent, the acidifying material being finely ground and mixed with the top 100 g. of soil. The amounts added were approximately equivalent to 600, 2000 and 6000 lb. of sulphur-equivalent per acre. A control soil, i.e. one to which no acidifying material has been added, was prepared at the same time. After sufficient distilled water had been added to saturate the soils, further water was added at the rate of 2 in. every two weeks over a period of 22 weeks. All pots were allowed to drain freely and the volume of drainage water was noted. Two weeks after the final addition of water the soil in each pot was tipped out and mixed thoroughly. pH was determined, the soil-water ratio being 1:2. The accumulation of ammonia, nitrite and nitrate before and after incubation of the soils at 28° for 21 days both in the absence and presence of 1% of added chalk was determined in the manner described in a previous publication by the author.[4]

Results

In Table I are shown the effects of treatments on pH and on nitrate, ammonia and total mineral-nitrogen accumulation during incubation, both in the absence and presence of added chalk. The ammonia present before incubation is also shown. Negligible amounts of nitrate were present before incubation, and nitrite could not be detected in any of the soils either before or after incubation.

Table I

Treatment	Rate (S-equiv., %)	pH	Nitrate-N accumulated (p.p.m.) during incubation		Ammonia-N (p.p.m.) in non-incubated soil	Ammonia-N (p.p.m.) accumulated during incubation		Total mineral-N accumulated (p.p.m.) during incubation	
			no chalk added	1% chalk added		no chalk added	1% chalk added	no chalk added	1% chalk added
Sulphur ..	0·03	5·94	36·2	52·0	9·7	− 4·0	− 5·7	32·2	46·3
,, ..	0·1	5·20	16·0	56·0	10·1	17·2	− 6·5	33·2	49·5
,, ..	0·3	4·86	0·0	40·4	13·3	18·0	− 3·3	18·0	37·1
$Al_2(SO_4)_3$ ..	0·03	6·02	36·0	51·1	9·4	− 6·4	− 5·8	29·6	45·3
,, ..	0·1	5·17	23·4	56·2	12·6	1·4	− 10·0	24·8	46·2
,, ..	0·3	4·23	0·0	56·2	18·1	9·7	− 15·1	9·7	41·1
$FeSO_4$ ..	0·03	6·17	33·6	56·6	12·3	− 5·2	− 7·9	28·4	48·7
,, ..	0·1	5·22	17·6	60·0	19·0	1·7	− 15·0	19·3	45·0
,, ..	0·3	3·93	0·0	19·0	34·4	6·0	11·6	6·0	30·6
Untreated ..	nil	6·54	40·0	54·0	14·0	− 7·2	− 9·9	32·8	44·1

The results show that, for each acidifying material, soil pH was reduced approximately in proportion to the amount of material added. It is also seen that, although there was little difference in pH produced between the three materials when added at the low (0·03%) or medium (0·1% S-equiv.) rates, there were fair differences in pH when the heavy (0·3% S-equiv.) rates of addition were made; sulphur had the least potent, and ferrous sulphate the most potent, effect in decreasing pH.

The amount of drainage water obtained from the pots ranged from an equivalent of 15·1 to one of 16·5 in. of water. There appeared to be no consistent differences due to type or quantity of acidifying material added. The rate of entry of water into the soil was somewhat slower where the heavy dressing of iron or aluminium sulphates was given, in comparison with the rate of entry of water with the other treatments.

For each acidifying material the amount of nitrate accumulated during incubation in the absence of added chalk was inversely related to the amount of acidifying material used. No nitrate at all accumulated with the heaviest application of either of the three materials. Nitrate accumulation during incubation in the presence of added chalk was as high or somewhat higher than that in the control soil, except where the heavy dressings of sulphur or ferrous sulphate had been made.

The ammonia present in the soils before incubation increased with increasing application of acidifying material, a particularly large amount being present where the heavy dressing of ferrous sulphate had been made. Ammonia accumulation during incubation in the absence of added chalk was greater with the medium and heavy dressings of sulphur than with corresponding dressings of the two sulphates. The negative amounts of ammonia obtained with the lowest dressings indicate that there was a net loss of ammonia as a result of incubation. When the soils were incubated with added chalk there was a net loss of ammonia in all soils, except where the heavy dressing of ferrous sulphate had been made.

The total mineral nitrogen (i.e. the sum of the ammonia- and nitrate-N) that accumulated during incubation in the absence of added chalk was very similar with all materials where the low dressing had been used, but decreased with the medium and heavy dressings; ferrous sulphate had the greatest, and sulphur the least, effect in this respect. In the presence of added chalk total mineral-nitrogen accumulation during incubation was somewhat higher with the low and medium dressings of all the acidifying materials than it was with the control soil. Accumulation was particularly low, in comparison with the control, in the soil receiving the heavy dressing of ferrous sulphate.

Discussion

It was thought that the poorer acidifying action noted with heavy applications of sulphur may have been due to incomplete oxidation to sulphuric acid. The acidifying action of the two sulphates is independent of microbial activity. It was found, however, that when samples of the sulphur-acidified soils, as well as of the other soils, were subjected to leaching for a further 24 weeks under the same conditions as those obtaining during the first 24 weeks little further reduction of pH occurred, indicating that oxidation had been complete during the first 24 weeks. The presence of aluminium and iron in the two sulphates and the high base-displacing power of these two cations, especially at the low pH induced by heavy additions, probably accounts for the greater acidifying action of these compounds as compared with sulphur. The different effects of the three materials on microbial activity of the soil during the 24 weeks of leaching should not be overlooked as a possible factor in bringing about different final pH values.

The fact that there were no great differences, due to the different acidifying materials, in nitrate accumulation during incubation indicates that the three materials had a similar effect on the activity of the nitrifying organisms when the soils were acidified to a moderate degree by the low and medium applications. When soil pH was considerably reduced by the heavy applications of acidifying materials, nitrate accumulation was completely inhibited where no chalk was added, and returned to normal, as compared with the control, when chalk was added only with the soil that had received aluminium sulphate. This indicates that the nitrifying power of a soil may be more than temporarily impaired when sufficient sulphur and iron sulphate are added to cause a drastic fall in pH.

Values for ammonia accumulation during incubation are of interest mainly for indicating the effects of treatments on the relative rates of the ammonifying and nitrifying actions. When low pH-reductions were obtained owing to applications of the low dressings of the acidifying materials, the rate of nitrification exceeded that of ammonification. With the heavier dressings the reverse was true. When incubation was carried out in the presence of added chalk nitrification occurred more rapidly than did ammonification with all soils except that which had been acidified with the heavy dressing of ferrous sulphate. This accumulation of ammonia, together with the low nitrate-accumulation, confirms that even the addition of sufficient chalk to neutralize the acidity in this soil has not overcome the deleterious effect of the initial low pH on the activity of the nitrifying organisms.

If it is assumed that none of the nitrogen mineralized during incubation is lost by microbial fixation or by denitrification, then the values obtained for total mineral nitrogen accumulation are a direct measure of the ammonifying activity of the soils. The results obtained show that ammonifying activity was directly related to soil pH for each acidifying material, and also when the soils are considered as a whole. When chalk was added, ammonifying activity was of the same order for all soils, with the exception of that receiving the heavy dressing of ferrous sulphate. It must be remembered that this soil had an exceptionally low pH, and it appears that under

these conditions both nitrifying and ammonifying capacity are impaired to such an extent that they are not brought back to normal by neutralizing the soil.

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THE VITAMIN-B_{12} (COBALAMIN) CONTENT OF THE MILK OF LOWLAND AND HILL SHEEP

By D. H. SHRIMPTON and J. DUCKWORTH

The vitamin-B_{12} (cobalamin) content of the milk of ewes, on pasture with supplementary feeding, declines gradually during lactation. Individual values fell within the range of 1 to 6 μg./l., with mean values at different stages of lactation generally between 2 and 3 μg./l.

In lowland and hill-farm flocks the mean values ranged from 1·3 to 5·0 μg./l., with considerable variation from one animal to another.

The provision of supplementary cobalt to lowland and hill flocks had no effect on the vitamin-B_{12} content of the milk.

Introduction

Collins, Harper, Schreiber & Elvehjem[1] have shown that the vitamin-B_{12} (cobalamin) content of the milk of cows and goats decreases as lactation progresses. With cows, the vitamin-B_{12} content of the milk fell from an initial value of 16 to 7·5 μg./l. during the first 10 weeks of lactation. The decrease was more striking with goats, when the values were 1·2 μg./l. on the first day *post partum* and 0·11 μg./l. 10 weeks later. These workers also record a mean value of 1·4 μg./l. for the vitamin-B_{12} content of ewes' milk, but with no study of the changes during lactation. In this work, the cows received mineral mixtures containing cobalt, but the sheep and goats did not.

Harper, Richard & Collins[2] found that giving mineral mixtures containing cobalt to ewes fed in dry lot increased the vitamin-B_{12} content of the milk about sixfold. Their values of 1·38 μg./l. for the vitamin-B_{12} content of the milk of ewes not receiving cobalt was almost identical with the value obtained by Collins *et al.* A particularly high value for the vitamin-B_{12} content of ewes' milk was reported by Sreenivasamurthy, Nambudripad & Iya,[3] who gave 14·0 μg./l. as an average (range 8–20 μg./l.).

In all these the animals were stall-fed or pen-fed. Only Hartman, Dryden & Cary[4] and Hartman & Dryden[5] have compared stall-feeding in winter and grazing in summer, with and without supplementary feeding of trace-element (Co, Fe, Mn, Cu, I, Zn) mixtures. In their studies with cattle they found that dosage with trace elements had no effect on the vitamin-B_{12} content of the milk either in summer or winter feeding, and that there was no difference between stall-fed and grazing animals in the vitamin content of their milks.

The present investigation was undertaken to determine the changes taking place during lactation in the vitamin-B_{12} content of the milk of ewes on pasture with supplementary feeding of concentrates, and to determine the content of the vitamin in the milk of sheep under natural conditions of grazing, including hill grazing.

Experimental

Lactation study.—Five Halfbred ewes of the Institute flock were selected. They had lambed within three days of each other. They were grazing on good-quality pasture throughout the study, with a daily allowance of 1 lb. per head of a mixture of 3 parts of crushed oats and 1 part of linseed-cake meal. Water, and a commercial common-salt lick containing trace elements (Co, Cu, Fe, I, Mn), were available at all times.

Milk samples were taken from the ewes at regular intervals, as shown in Fig. 1. The milk sample of each ewe was assayed individually, all assays being started within two hours of milking, to avoid risk of biosynthesis of vitamin B_{12} in the milk on standing.

Field studies.—Milk samples were collected from flocks on 10 farms in Scotland and the North of England.

Farms 1 and 2 were in lowland areas of Inverness-shire; both had a pining history and had not been dressed with cobalt, and neither had carried sheep for several years. In both areas, the flocks on which the tests were made had been bought in the autumn of 1951 from good stock rearing areas. Four weeks before lambing, 12 ewes were drawn at random from each flock, 6 from each group of 12 being dosed by mouth with 21 mg. of cobalt as cobalt sulphate. All the test ewes were milked at one-month and two-month intervals after lambing.

The remaining farms were all in upland areas of northern England, Farms 3, 4, 5, 6 and 7 having little or no heather in their grazing. On Farm 3, a common salt–calcium phosphate mineral supplement with trace elements (Co, Cu, Fe, I) and containing 10% magnesium was fed with oats to the ewes during a trough-feeding period from January until lambing in mid-April. Of the 24 ewes selected at random for sampling, 12 had received in addition a trace-element drench (Co, Cu, I, Mn) on 28 February and 13 and 27 March, 1952. Twenty-four ewes were similarly selected from Farm 4, 12 of which received 21 mg. of cobalt as cobalt sulphate three weeks before lambing, but no other supplement. Twelve ewes were selected from each of the remaining three farms in this group; no supplements of any kind were fed.

Farm 8 extends over two fells, one 'white' land and one 'black' land, and in this case 12 sheep were tested from each fell. Ewes on the 'white' land received a mineral mixture containing magnesium and cobalt, those on the 'black' land mineral mixture containing cobalt.

Farm 9 was on 'black' fells and on Farm 10 there was some heather in the grazing. No mineral supplement was fed on either farm. Twelve ewes were selected at random from each farm.

The test ewes from Farms 3, 4 and 8 were milked one month after lambing, and those from Farms 5, 6, 7, 9 and 10 two months after lambing. To prevent biosynthesis of vitamin-B_{12} in the milks during transport to the laboratory, they were steamed after collection, with addition of potassium cyanide up to a concentration of 0·1%. At the laboratory they were stored at − 20° while awaiting analysis. In some cases the milks of the hill flocks were assayed individually, and in others bulk samples were prepared and analysed in duplicate, as shown in Table I.

Assay method for vitamin B_{12}.—Two 1-ml. aliquots of the individual or bulk samples were each steamed for 30 minutes with 20 ml. of 1% acetate buffer, pH 4·5, in the presence of potassium cyanide at a concentration of 0·1%. After centrifuging and dilution to an approximate vitamin-B_{12} content of 0·1 mμg./ml., they were assayed according to the method of Skeggs, Nepple, Valentik, Huff & Wright,[6] using *L. leichmannii* ATCC 4797.

Results

The changes in the vitamin-B_{12} content of the milks during lactation are given in Fig. 1. Values for each sheep are plotted individually. The heavy curve in the figure is the mean lactation trend.

The average content of vitamin-B_{12} was about 3 μg./l. during early lactation and fell irregularly to about 2 μg./l. by the end of lactation. Two sheep, number 1 and number 2, tended to have a higher vitamin-B_{12} content throughout lactation, but one, number 5, tended to remain low. Irregular changes occurred in all sheep during the lactation period.

The results of the field trial are shown in Table I. On Farms 1, 2 and 4, there was no significant difference in the vitamin-B_{12} content of milk from ewes that had been dosed with cobalt and from those not dosed. On Farm 1 the average vitamin-B_{12} content of the milk increased between the first and second month of lactation, but on Farm 2 it fell. On Farm 3, the drenched ewes had milk with a slightly higher vitamin-B_{12} content.

On Farms 5, 6, 9 and 10, the vitamin-B_{12} contents of the milks are of the same order, ranging from 4·5 to 5·0 μg./l. Ewes from Farm 7 gave a lower value of 3·5 μg./l.

Table I

The vitamin-B_{12} content (μg./l.) of the milk of ewes on lowland or hill grazing, with or without cobalt dosage

Farm No.	Type of grazing	Breed of sheep	Stage of lactation, months	Treatment: Ewes dosed with cobalt — Average value	Ewes dosed with cobalt — Range	Ewes not dosed — Average value	Ewes not dosed — Range
1	Lowland	Suffolk × Halfbred	1	1·3[a]	0·9–3·6	1·3	0·9–3·4
1	,,	,, ,,	2	3·1[a]	—	3·3	
2	,,	Halfbred × Cheviot	1	2·3[a]	1·1–3·3	2·5	2·1–3·9
2	,,	,, ,,	2	1·7[a]	—	1·7	—
3	Upland, no heather	Cheviot	1	3·9[b]	—	—	—
3	,, ,, ,,	,,	1	4·5[c]	—	—	—
4	,, ,, ,,	Blackface × Grey face	1	1·3[d]	—	1·7	—
5	,, ,, ,,	Herdwick	2	—	—	4·5	—
6	,, ,, ,,	,,	2	—	—	5·0	—
7	,, ,, ,,	Swaledale	2	—	—	3·5	—
8	,, ,, ,,	,,	1	2·0[b]	0·67–7·1	—	—
8	Upland, heather dominant	,,	1	5·0[e]	3·3–8·3	—	—
9	Upland, heather dominant	,,	2	—	—	4·5	—
10	Upland with some heather	,,	2	—	—	5·0	—

[a] Dosed by mouth with 21 mg. of cobalt (as cobalt sulphate) 4 weeks before lambing
[b] Common salt–calcium phosphate mineral supplement containing 10% of magnesium and trace elements (Co, Cu, Fe, I)
[c] The same mineral supplement as in *b* but with three trace-element (Co, Cu, I, Mn) drenches at 14-day intervals between 28 February and 27 March
[d] Dosed by mouth with 21 mg. of cobalt (as cobalt sulphate) 3 weeks before lambing
[e] Fed on the same mineral mixture as in *b*, but without the added magnesium

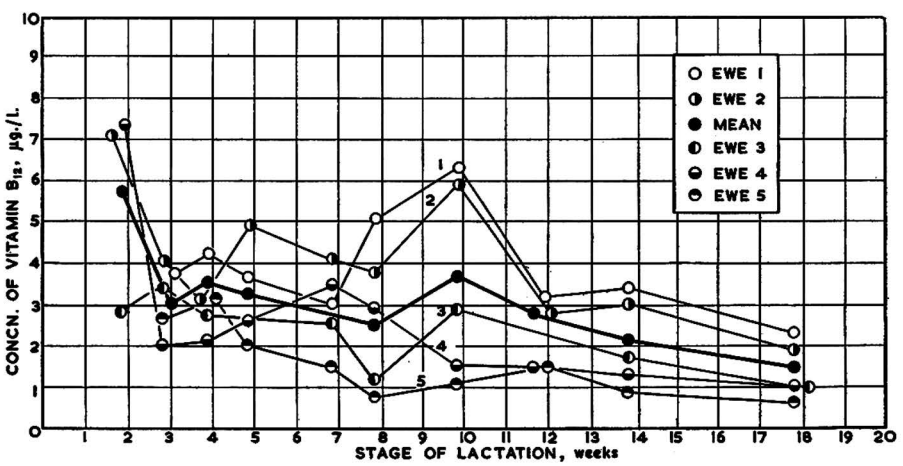

FIG. 1.—*Changes in the vitamin-B_{12} content of the milk of five ewes during lactation*

On Farm 8, the milk from ewes on 'black' land had a mean vitamin-B_{12} concentration of 5 μg./l. compared with only 2 μg./l. from those on the 'white' land. This difference was highly significant ($P = 0.01$).

Discussion

The finding that the vitamin-B_{12} content of the milk of ewes on pasture with supplementary feeding gradually declines during lactation agrees with the observations made on cattle and

goats confined in stalls. Over most of the lactation period the decline was fairly uniform, although erratic changes were observed in some ewes. Although it is possible that the transitory high values observed in some animals were the result of taking the cobalt-containing mineral lick, in view of the experience of Harper *et al.*, the results of the field studies do not suggest that this form of supplementation has any consistent effect on the vitamin-B_{12} content of the milk of grazing ewes.

Other workers have found a precipitous fall in the vitamin-B_{12} content of the milk of cattle and goats during the first few days *post partum*. There is some evidence that the same reduction takes place in ewes, although we have few data for this period.

The general finding of an initial sharp reduction, followed by a continued gradual falling off, in the vitamin-B_{12} content of the milk as lactation progresses suggests that a body reserve of vitamin B_{12} is being drawn upon, rather than that the milk derives all its vitamin B_{12} from alimentary synthesis. Comparable ewes of the Institute flock yielded about 1500 ml. of milk daily in early lactation, declining uniformly to about 400 ml. daily at the nineteenth week of lactation.[7] Combining these results with data from the assays it appears that the daily output of vitamin B_{12} at the end of lactation is about one-fifth of the output during the third week of lactation.

The values for vitamin-B_{12} concentration in the milk found in the field studies at the fourth and eighth weeks of lactation were distributed widely about the mean of 3 μg./l. observed in the Institute flock at that stage of lactation. Although some flock means were very low (Farms 1 and 4) almost all the mean values were higher than the average values reported by American workers. Great variation was found within each flock, in agreement with the observations made in the lactation study of the Institute flock.

Differences in assay technique may account for the differences between our results where the milk was heated with cyanide before dilution, and the American results, where no cyanide treatment was used. Denton, Kellogg & Bird[8] found that more of the vitamin B_{12} in eggs, body tissues and faeces was made available to test organisms when, as in our work, the materials assayed were hydrolysed with cyanide. Unfortunately, milk was not tested.

On the four farms where part of each flock had been dosed with cobalt, as a drench, as a mineral supplement, or as a combination of both, there was no evidence of an effect of the treatment on the vitamin-B_{12} content of the milk.

Farm 8 is of particular interest. Although the two flocks were of the same parent stock and under the same management there was a striking difference between the values for the ewes from the two fells. The flock on the ' white ' fell had a mean vitamin-B_{12} content almost as low as any observed in other flocks, whereas the flock on the ' black ' fell had a mean vitamin-B_{12} content as high as any observed in other flocks. However, it is unlikely that this difference arises solely from heather being available on one fell, since the mean values for three flocks on Farms 5, 6 and 7, with no heather, were closely similar.

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