SUPERPHOSPHATE PRODUCTION: THE INFLUENCE OF VARIOUS FACTORS ON THE SPEED OF REACTION AND THE COMPOSITION OF THE PRODUCT

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The effects of acid concentration, acid/rock ratio, and fineness of grinding on the interaction between Moroccan phosphate rock and sulphuric acid, in the preparation of superphosphate, have been studied.

An organic-solvent technique has been used to follow the course and the rate of the chemical reactions. This method has been adopted because the precise composition of the superphosphate cannot be found by the analysis of water extracts, since interaction occurs between unchanged phosphate rock and free acids during the extraction.

Results are given in the form of Tables and graphs.

Introduction

The factors that determine the speed of interaction between phosphate rock and sulphuric acid to make superphosphate, and the composition of the product, are as follows: (1) the nature of the phosphate rock; (2) the degree of fineness to which it is ground; (3) the concentration of the sulphuric acid; (4) the proportions in which acid and rock are mixed, which may be stated in lb. of H_2SO_4 per 100 lb. of phosphate rock; (5) the temperature of reaction.

The phosphate rock used throughout this investigation was that obtained from the Kourigha deposit in Morocco, usually referred to in this country simply as Moroccan phosphate. We have found that the chemical composition of this material varies only slightly. The fineness of grinding of the rock, the concentration of the acid and the acid/rock ratio have been varied over considerable ranges.

One difficulty in imitating, in the laboratory, conditions obtained on a large scale, is to get the correct reaction temperature. On a large scale, the surfaces of the mixer and den are far smaller per unit weight of reactant than they are when the reaction is carried out on a laboratory scale, and in consequence losses of heat are much smaller and the reaction temperature tends to be much higher. The method we have adopted is to carry out in each case a preliminary experiment in a Dewar flask. Suitable quantities of the reactants are mixed in this flask, and the temperature attained is noted. The temperature in the mixing vessel for the later experiment has generally been fixed at this adiabatic temperature.

The operating conditions in continuous superphosphate plants are determined by the physical conditions of the product that are necessary (*a*) at the entrance of the den and (*b*) at the den exit. The slurry entering the den must be fluid enough to run across it, but it must not be so fluid that it would for instance run between the slats of a Broadfield den. At the exit the superphosphate must have set to an extent sufficient for the cutter and conveyor to deal with it. In order to obtain a comparative measurement of the fluidity of the slurry during mixing, the laboratory reaction vessel was mounted on a turn-table. A stirrer was arranged to be operated by a motor mounted above the vessel, and independently of the turn-table. The force exerted by the motor, through the slurry, on the turn-table, was measured by observing the extension of a spring.

When the desired fluidity had been obtained, the reaction vessel was unclamped from the turn-table, covered with a clock-glass, and placed in a cupboard, maintained at 15° below the reaction temperature, for 45 minutes, thus imitating roughly the period of retention in a Broadfield den.

To imitate as nearly as possible the conditions below the surface of a large storage pile, the superphosphate, after removal from the reaction vessel, was placed in a bottle, which was provided with a rubber bung and a Bunsen valve, and this was kept for three weeks at a temperature 35° below that of the reaction vessel.

For many of the experiments, it was sufficient to analyse the products by the ordinary methods of chemical analysis used in the fertilizer industry. Such methods, however, are not suitable when, for research purposes, the extent of interaction is to be measured during its early stages. It is well known that the interaction that takes place in the superphosphate after it has left the den is a slow one, so that, even after say three weeks, there are considerable proportions of free acid and of unchanged phosphate rock still present. When analysis is preceded by extraction of the superphosphate with water, interaction between the acid and previously

unchanged rock is comparatively rapid after the superphosphate is dispersed in the water. Thus the result of the analysis, although useful for comparative or practical purposes, does not indicate the precise composition of the superphosphate.

The discrepancy between the results obtained by analysis after extraction with water and the true composition of the superphosphate is much greater during the early stages of the reaction, so that aqueous extraction is quite unsuitable for determining the extent of interaction. For this purpose, it is necessary to bring the reaction to a stop before analysis is carried out. A technique with this object has been developed by Mr. R. F. Knight, working in the Research Department of Fisons Ltd.

The organic solvent, dioxan, has the property of combining with sulphuric and phosphoric acids to form 'loose' compounds, the speed of interaction of which with phosphate rock is very low. When working with triple superphosphate it was found that dispersion of a sample in dioxan caused further interaction to be so slow that separation of the dioxan solution of the acid from the solid matter could be carried out without interaction proceeding simultaneously to any appreciable extent. In the presence of sulphuric acid, however, dioxan alone was unsuitable, since, with certain proportions of sulphuric acid, water and dioxan, the liquid separated into two layers. With a mixture of equal volumes of acetone and dioxan this separation into two layers did not occur, and the rate of interaction between acid and rock was again lowered to such an extent that their separation could be carried out.

After separation of the free acids in this way, the solution was analysed by normal methods for the proportions of sulphuric and phosphoric acids. The solid residue from the extraction was treated with a saturated solution of sodium oxalate at boiling point to extract the monocalcium phosphate as sodium dihydrogen phosphate, the proportion of which could then be determined. The phosphate remaining in the solid residue was determined.

Experimental

Series 1

In this series of experiments the fineness of grinding of the phosphate rock was fixed, namely such that 68% of the material passed through a 100-mesh B.S. sieve; a more detailed sieve analysis is given below. Four concentrations of sulphuric acid were used, namely 70, 60, 55 and 45% H_2SO_4; each of these concentrations was used in acid/rock ratios of 60, 55, 50 and 45 g. of H_2SO_4 per 100 g. of phosphate rock. In these experiments the organic-solvent technique was not used, and the results given in Table II show only the analysis of the product after 21 days' storage and its physical condition at that time. Mixing was carried out at a temperature that is shown in Table II, and was generally continued until a fluidity was reached that was equal to that obtained with this rock in three minutes when used with acid of concentration 70% H_2SO_4 in an acid/rock ratio of 60 g. of H_2SO_4/100 g. of phosphate rock. The times required are also shown in Table II; in three cases the fluidity had not fallen to the desired value in 15 minutes, but mixing was stopped at that time.

Series 2

In this series the main object was to study the speed of the interaction between phosphate rock and sulphuric acid, but the chemical composition of the products was also determined. The acid/rock ratio was fixed at 60 g. of H_2SO_4/100 g. of rock throughout the series, and there were two sub-series, one with acid of concentration 70% H_2SO_4 and the other with acid of concentration 76% H_2SO_4. In each sub-series the fineness of grinding of the rock varied to give from 21 to 93% passing a 100-mesh sieve.

In order to determine the speed and course of the reaction, 11 or 12 samples of the reacting mass were taken in each experiment at times ranging from five minutes up to three weeks after mixing. Each sample was immediately dispersed in a weighed amount of acetone–dioxan and analysed as previously indicated.

For purposes of comparison, samples were taken from the exits of mixer and den of a Broadfield superphosphate plant. Portions of these samples were at once dispersed in acetone–dioxan. The remainder was stored under the same conditions as for the laboratory products, and portions were taken for analysis at various intervals of time.

Raw materials

The acid was initially of concentration 76% H_2SO_4, produced in a chamber plant. Except where used at this concentration, it was diluted with water and used at the adiabatic temperature of dilution.

The Moroccan rock used had the chemical and sieve characteristics shown in Table I.

Table I

Characteristics of Moroccan rock

Sample	Cumulative percentage passing B.S. mesh No.			Mean particle size, μ	Total P_2O_5, %
	170	100	60		
Series 1	44	68	92	109	33·2
Series 2					
1	5	21	70	215	33·6
2	27	51	91	150	33·2
3	47	70	93	100	33·8
4	56	93	100	75	34·0

For ease of comparison with other sieve analyses, the key figures chosen are the cumulative percentage passing a 100-mesh B.S. sieve, together with the cumulative percentages passing 60 and 170 mesh, to indicate the ' slope ' of the curve. The mean particle size is also given. Values were determined with nine consecutive freshly checked sieves, and were plotted as shown in Fig. 1, smooth curves drawn, and the figures required were taken from these.

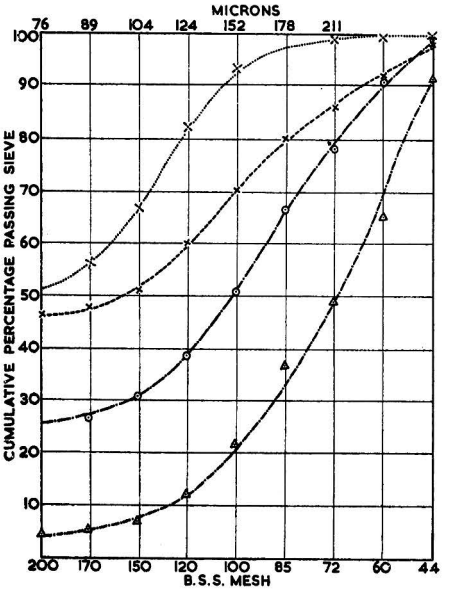

FIG. 1.—*Sieve analysis of samples of Morocco phosphate rock*

FIG. 2.—*Reaction vessel, turn-table and scale*

Experimental details

The reaction vessel (Fig. 2) consisted of a stainless-steel beaker of one litre capacity placed concentrically in another of 2½ litres capacity. The space under and around the inner beaker was packed with asbestos wool, in which was embedded a 400-w heating element ; the top of the annular space was sealed with a cement made with litharge and glycerol. The heater was only needed when the time of mixing exceeded five minutes, and was then controlled by a Sunvic energy regulator.

The stirrer was of Monel, with three blades, and was driven by a constant-speed electric induction motor, geared to give 70 rev./min.

For each experiment the required amount of sulphuric acid was placed in the mixer, the stirrer was started, and 400 g. of phosphate rock added. As the fluidity of the slurry decreased, more of the power of the stirrer was transferred to the turn-table to which the reaction vessel was fastened. This stretched the coil spring, and a pointer attached to the turn-table moved over a scale. The point was found on the scale that was reached in three minutes when acid of concentration 70% H_2SO_4, at 35°, was used with rock ground to give 70% through a 100-mesh B.S. sieve, in the ratio 60 g. of H_2SO_4/100 g. of rock. Other mixtures were stirred until the indicator came to this same point on the scale.

Results

Series 1

Fig. 3 illustrates the results, which are given in more detail in Table II.

Table II

Effect of concentration of sulphuric acid, and ratio of sulphuric acid to phosphate rock
Rock ground for 68% to pass 100 B.S. sieve; analysis of product after 3 weeks

Concn. of acid, % H_2SO_4	Mixing temp., °C	Time of mixing, min.	Total P_2O_5 in product, %	Moisture content Losses at 100°	Moisture content Losses at 50°	P_2O_5 as % of total P_2O_5 Water-soluble	P_2O_5 as % of total P_2O_5 Available †	P_2O_5 as % of total P_2O_5 Free acid	Yield, g. of P_2O_5/100 g. of H_2SO_4 Water-soluble	Yield, g. of P_2O_5/100 g. of H_2SO_4 Available	Physical condition of product after 3 weeks' storage
					60 g. of H_2SO_4/100 g. of phosphate rock						
70	105	3	19·4	12·7	9·8	88·4	95·8	21·9	48·8	52·9	Ordinary superphosphate
60	90	10	18·6	15·6	12·2	86·9	93·3	30·0	48·0	51·5	Comparable with ordinary superphosphate except for slightly more damp appearance
55	80	15*	17·4	20·9	15·1	84·2	93·1	32·7	46·5	51·4	Fairly good condition, but damp appearance
45	78	15*	15·9	28·2	17·0	77·2	93·2	39·4	42·7	51·5	Fairly good condition, but damp appearance
					55 g. of H_2SO_4/100 g. of phosphate rock						
70	104	2	19·9	11·4	9·3	78·0	88·1	12·1	47·0	53·1	Very good; free-flowing powder
60	90	7½	19·4	20·8	19·5	75·9	81·5	23·2	45·8	49·2	Similar to ordinary superphosphate
55	82	11½	18·2	19·4	15·9	75·6	82·4	30·6	45·6	49·7	Fairly good condition, but damp appearance
45	78	15*	16·8	26·0	15·8	72·7	86·1	30·2	43·8	51·9	Fairly good condition, but damp appearance
					50 g. of H_2SO_4/100 g. of phosphate rock						
70	104	1	20·8	10·7	8·9	74·8	82·8	9·0	49·6	54·9	Very good; free-flowing powder
60	90	1	20·4	14·4	10·5	71·8	78·1	19·2	47·6	51·8	Similar to ordinary superphosphate
55	83	2½	18·7	20·0	16·5	66·9	75·8	25·8	44·4	50·3	Fairly good condition, but rather damp appearance
45	78	12	17·5	25·7	15·2	68·0	77·9	24·2	45·1	51·6	Good condition, comparable with ordinary superphosphate
					45 g. of H_2SO_4/100 g. of phosphate rock						
70	102	0·5	24·1	4·9	3·1	62·2	73·9	4·7	45·8	54·4	Very good; free-flowing powder
60	90	0·5	21·6	14·6	12·3	59·8	70·4	17·3	44·0	51·8	Similar to ordinary superphosphate
55	83	0·5	19·0	19·2	15·9	63·4	68·8	22·6	46·7	50·7	Fairly good condition, but damp appearance
45	80	15·0	18·9	22·1	12·9	58·0	72·1	20·5	42·8	53·1	Good condition, comparable with ordinary superphosphate

* Standard fluidity not obtained after 15 minutes

† Official and Tentative Methods of Analysis of the A.O.A.C., 1950, 7th edn., p. 11 (Washington, D.C.: Association of Official Agricultural Chemists)

From Table II it is seen that, with any one of the ratios (g. of H_2SO_4/100 g. of rock) used in the experiments, 70% H_2SO_4 gives the best results of the concentrations tried. Fig. 3 shows that a high water-soluble/total P_2O_5 value is associated with a high proportion of free acid in the product; also that, for a given water-soluble/total P_2O_5 percentage, the free acidity of the product rises sharply as the concentration of sulphuric acid used is lowered from 70% H_2SO_4.

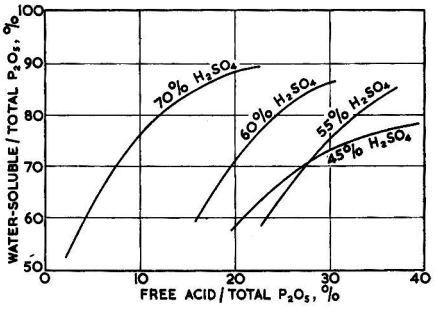

FIG. 3.—*Relation between free acidity of product and conversion*

In Table II, attention may be drawn to the values for the moisture contents of the products. Losses at 100° include some water of crystallization, whereas those at 50° are believed to represent fairly accurately the free-water contents. The difference between the two values increases slowly between concentrations of 70 and 55% H_2SO_4, then sharply as the concentration falls to 45% H_2SO_4. At this concentration it is probable that a high proportion of the calcium sulphate in the superphosphate is present as gypsum, which accounts for the good physical condition of material made with acid of this concentration and acid/rock ratios of 45 to 50. At the higher concentrations, calcium sulphate will be formed mainly as anhydrite and hemihydrate.

At the time of acute acid shortage it was thought that some saving might be achieved by using low acid/rock ratios, making superphosphate containing a high proportion of dicalcium phosphate, and selling the product on the basis of the proportion of P_2O_5 soluble in ammonium citrate solution. The experimental results, however, showed only a rather small difference between citrate-soluble and water-soluble P_2O_5, which did not increase as the acid/rock ratio fell.

Taking into account other experimental values, some obtained on the plant, we consider that Table III represents roughly the results to be expected in a continuous plant using Moroccan rock ground so that about 70% passes a 100-mesh B.S. sieve, and acid of concentration 70% H_2SO_4.

Table III

Acid/rock ratio, lb. of H_2SO_4/100 lb. of rock	Water-soluble/total P_2O_5, %, in 3-week-old superphosphate	Quantities, tons, required for one ton of water-soluble P_2O_5	
		Rock	H_2SO_4, 100%
50·0	72·0	4·18	2·09
52·5	76·2	3·95	2·08
55·0	81·0	3·72	2·05
57·5	86·3	3·49	2·01
60·0	91·0	3·31	1·99
62·5	93·5	3·22	2·01
65·0	94·7	3·18	2·07

With an acid/rock ratio higher than 60, the free acidity of the product begins to be excessive, and this value is considered to be the optimum.

Series 2

The results are conveniently represented in Figs. 4–7 by plots of the logarithm of time (in minutes plus one) against percentages of the initial P_2O_5 present as free acid, monocalcium phosphate, water-soluble phosphate and insoluble P_2O_5. The percentage of the initial H_2SO_4 present is also plotted. In order to save space, only half of the graphs are reproduced here, but the others are similar.

The results obtained on the samples from the Broadfield plant are shown in Fig. 8.

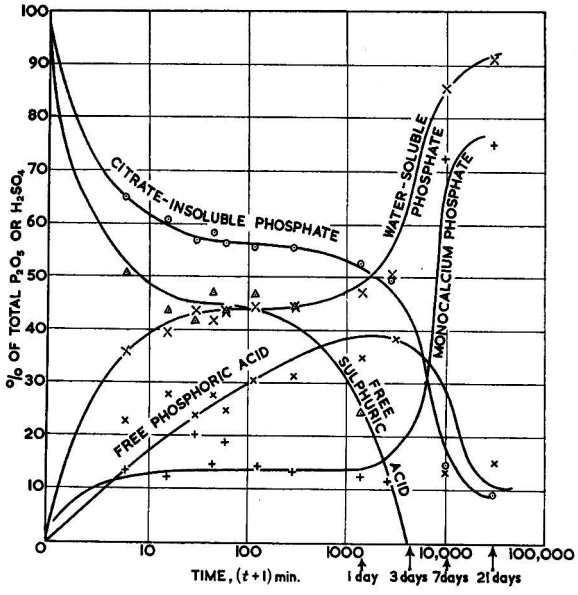

FIG. 4.—*Interaction of 70% sulphuric acid with Morocco phosphate (22% through 100 B.S. sieve)*

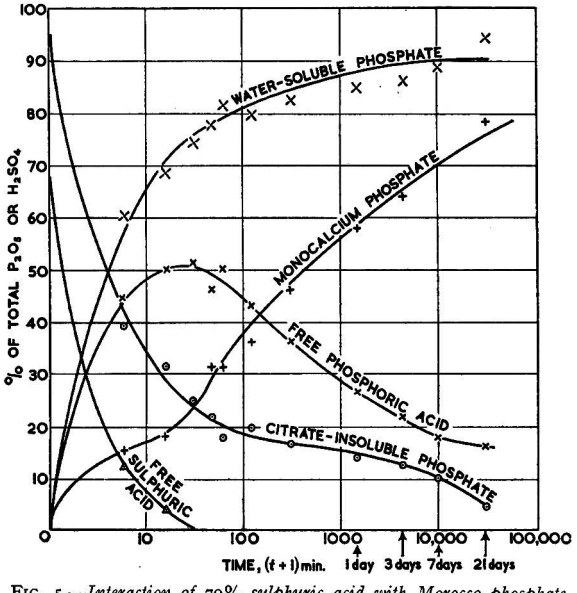

FIG. 5.—*Interaction of 70% sulphuric acid with Morocco phosphate (70% through 100 B.S. sieve)*

It is seen that the general course of the reaction is as follows. While free sulphuric acid is present a little monocalcium phosphate is formed but the principal reaction is the formation of free phosphoric acid. With the disappearance of sulphuric acid (which, with rock of the usual fineness of grinding and acid of concentration 70% H_2SO_4, takes place in 30 to 40 minutes) formation of free acid P_2O_5 ceases. This free acid now forms 40 to 50% of the total P_2O_5, and

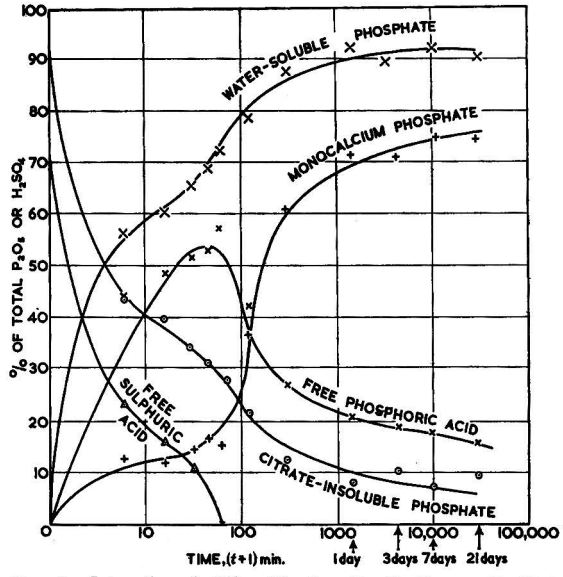

FIG. 6.—*Interaction of 76% sulphuric acid with Morocco phosphate (70% through 100 B.S. sieve)*

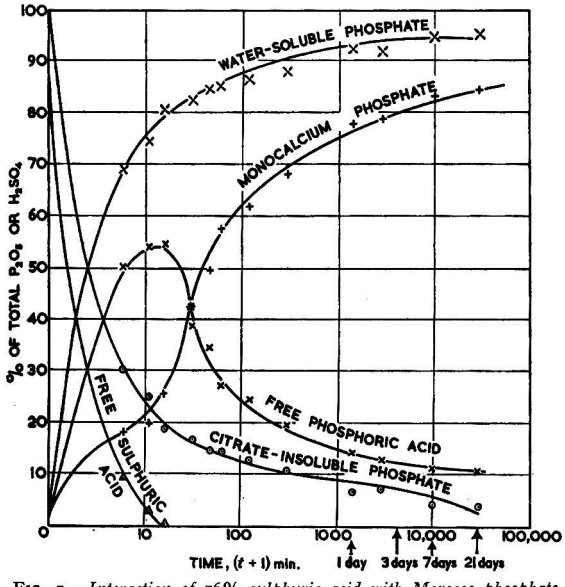

FIG. 7.—*Interaction of 76% sulphuric acid with Morocco phosphate (93% through 100 B.S. sieve)*

further interaction is between this and previously unchanged rock, mainly to form monocalcium phosphate. It is seen that this reaction gradually loses speed, and that, with rock of the usual fineness of grinding, an almost stationary state has been reached in three weeks, with substantial proportions of free acid and insoluble phosphate still present. The figures shown for water-soluble P_2O_5 represent, in each case, the sum of the acid P_2O_5 and the monocalcium phosphate.

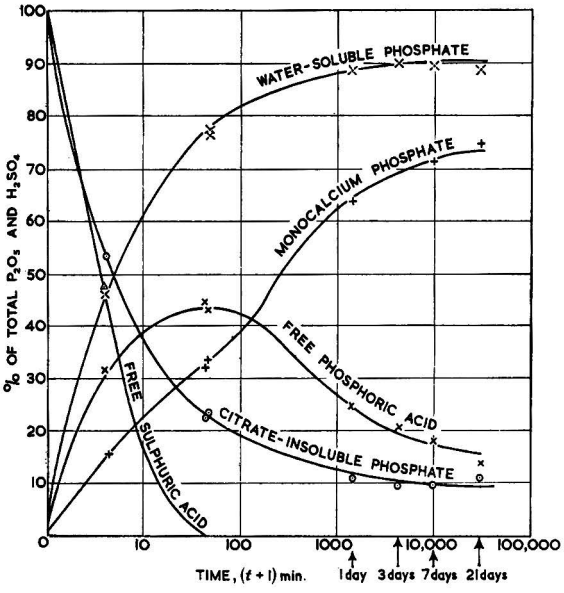

FIG. 8.—*Samples taken from continuous Broadfield plant*

The gradual slowing down of the interaction between phosphoric acid and phosphate rock is largely caused by the coating of the residues of the original rock particles with calcium sulphate.

Visual observation showed that the superphosphate was wet in appearance and sticky as long as appreciable proportions of sulphuric acid were present. In fact, it appeared that the ideal time of retention in the plant, i.e. the time at which the product should reach the cutter, practically coincided with the disappearance of sulphuric acid.

In Fig. 9, the time for disappearance of sulphuric acid is plotted against the fineness of grinding of the rock. (In drawing the curves, the point obtained with acid of concentration 76% H_2SO_4 and rock ground for 51% to pass 100 mesh has been ignored. The points obtained for rock ground for 21% to pass 100 mesh with the two concentrations of acid were practically identical.) It is suggested that this graph gives the minimum fineness of grinding of Moroccan rock for a plant of which the time of retention is known. For instance, with a Broadfield plant that retains the material three minutes in the mixer and 30 minutes in the den, and with acid of concentration 70% H_2SO_4, it should suffice to grind so that about 73% passes a 100-mesh B.S. sieve (always assuming that the sieving graph is similar in slope to those of the samples of rock used in these experiments).

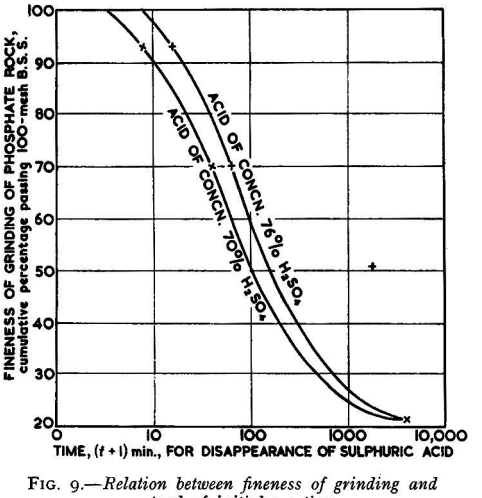

FIG. 9.—*Relation between fineness of grinding and speed of initial reaction*

Other conclusions from this series of experiments are (i) that interaction is more rapid with acid of concentration 70% H_2SO_4 than with acid of 76% H_2SO_4, and (ii) that 'curing' time can be reduced with finer grinding, which speeds up the formation of water-soluble P_2O_5.

Table IV gives both the experimental conditions and the chemical analyses of the products (by the usual methods) after three weeks' storage. The analysis is seen

to vary but little with the fineness of grinding of the rock and concentration of acid, with the important exception that percentages for both water-soluble and available P_2O_5 were exceptionally high with the finest rock.

Table IV

Effect of fineness of grinding of phosphate rock
Acid/rock ratio : 60 g. of H_2SO_4/100 g. of rock ; product analysed after three weeks

Fineness of rock, % passing 100-mesh sieve	Mixing temp., °C	Time of mixing, min.	Total P_2O_5 in product, %	Moisture content Losses at 100°	Losses at 50°	P_2O_5 as % of total P_2O_5 Water-soluble	Available	Free acid
			Acid of 70% H_2SO_4, at 35°					
21	75	5·0	18·9	13·0	10·6	92·0	97·6	16·7
51	99	3·5	19·6	9·6	8·0	91·7	98·4	15·5
70	105	3·0	20·1	8·9	6·8	91·6	97·3	14·5
93	105	0·5	20·3	8·2	7·0	93·0	99·7	15·9
			Acid of 76% H_2SO_4, at 15°					
21	90	5·0	19·9	8·9	7·3	91·4	95·0	16·3
51	85	3·5	19·7	9·2	7·0	91·2	95·2	15·9
70	85	3·0	20·1	8·8	6·8	91·4	96·8	16·1
93	110	0·5	20·9	4·6	3·7	95·3	99·9	10·3

Appendix

Method of separating various P_2O_5 fractions from a sample of superphosphate

Dioxan and acetone are dried by standing over anhydrous calcium chloride. A mixture of equal volumes of the two solvents is prepared. For each determination, 25 ml. of the mixture is placed in a weighing bottle, the ground-glass stopper of which has been greased to minimize evaporation. The bottle, with its contents, is weighed. A sample of the superphosphate of about 5 g. weight is taken, and immediately immersed in the solvent. The bottle is then well shaken to extract the free acid, and again weighed.

The mixture is filtered, using a Whatman No. 12 filter paper, and the residue is washed with 25 ml. of acetone–dioxan mixture, and then with 75 ml. of acetone. The filtrate is diluted with 100 ml. of distilled water, neutralized with approximately normal caustic soda to B.D.H. 4·5 indicator, and evaporated nearly to dryness to remove the organic solvent. Concentrated nitric acid (25 ml.) is then added, and the solution boiled for ten minutes, diluted with 80 ml. of distilled water, and again boiled. The solution is filtered through a paper-pulp pad into a 250-ml. graduated flask. Aliquots of this solution are analysed for phosphate and sulphate, giving measures of the free acids in the sample.

The residue is transferred, together with the filter paper and weighing bottle, to a 600-ml. beaker, and boiled for five minutes with 200 ml. of a saturated solution of sodium oxalate. The solution is then filtered through a Whatman No. 12 paper into a 1000-ml. graduated flask, the residue being washed with hot water. An aliquot of this solution is analysed for phosphate, the result indicating the monocalcium phosphate content of the sample.

The residue is transferred, together with filter paper, to a conical flask containing 100 ml. of neutral ammonium citrate solution (A.O.A.C.) at 65°. Digestion at this temperature is continued for one hour, the flask being shaken at intervals of five minutes. The solution is then filtered through a Whatman No. 5 paper on a Buchner funnel ; the residue is washed with water at 65° until the volume of filtrate measures 350 ml.

After ignition of the filter paper, the residue is dissolved in 25 ml. of concentrated nitric acid, boiled for ten minutes, diluted with 80 ml. of distilled water, and boiled again. The solution is filtered through a paper pad into a 250-ml. graduated flask. An aliquot of this solution is analysed for phosphate, giving the citrate-insoluble P_2O_5 content of the sample.

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THE MINERAL CONSTITUENTS OF SOME NYASALAND TEA LEAVES AND TEA SOILS

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Analyses of the mineral constituents of some Nyasaland tea leaves and tea soils were made during investigations into the cause of a mal-growth of tea bushes on one estate. The analytical work was supported by injection and spraying experiments in the field. Results from both investigations are given and indicate that it is unlikely that a serious ill-balance of any mineral exists. The prime purpose of this paper is to place on record a catalogue of analytical results, since there is no previous record of this kind for Nyasaland.

Introduction

For some years past an area of originally vigorous and healthy tea growing in Nyasaland has showed progressive mal-growth which reached alarming proportions during October and November, 1952. The symptoms, primarily loss of newly developing leaves and die-back of new shoots, could not be reconciled with any of the usual pathological or entomological causes. A comprehensive programme of investigation into possible causes was put under way, including analyses of the major and minor elements in the leaf and soil. In the end no single specific cause could be found to account for the trouble, and it appears that a physiological disturbance to normal tea-growth is resulting from the failure of the usual cultural methods of Nyasaland to overcome the effects of abnormally high rainfall periods alternating with hot droughts acting upon an unusual type of soil.

There is a lack of published data on the major or minor mineral constituents of tea leaf and no published data at all of those in Nyasaland leaf or soils. The chief purpose of this paper is to record a catalogue of analytical results. Since there is a lack of comparable data from Nyasaland or from other areas, discussion will be kept to a minimum.

In the middle 1930s great difficulty was found in fermenting leaf plucked from the badly affected area, and empirical observation showed that a 1% Bordeaux spray applied at about two-yearly intervals cured the condition. At the same time analytical figures of made teas from this area showed a low manganese and copper content. Work in Ceylon showed the enzyme responsible for fermentation of the leaf to be a copper-protein. A copper deficiency, for fermentation purposes at least, was therefore known to exist, though it was doubted that this could be a cause of mal-growth by itself owing to the Bordeaux spray which is regularly applied.

The worst-affected area and that chosen for intensive investigation lies at the foothills of Mlanje mountain at an altitude of about 3000 ft. above sea level and receives an average of 125 inches of rain per year. The soil type is a black highly organic loam of pH 4·8 and a carbon to nitrogen ratio of 25 : 1, overlying a normal red subsoil about three feet below. Progressing away from the mountain base into the average tea area of Mlanje, some eight miles south, the altitude drops to about 2500 ft. and rainfall to an annual average of about 75 inches; the soil grows less and less dark, finally becoming the usual red lateritic type with a pH of about 5·4 and a carbon to nitrogen ratio of about 14 : 1.

Experimental

It was realized that since comprehensive analytical results of mineral constituents from previous investigations were lacking, the interpretation of results obtained only from the affected area would be of little value in itself. Resort was therefore made to analysing samples from five locations of affected and healthy tea in the hope that any serious deficiency or excess of mineral would be reflected in the analytical results between locations. The five locations chosen and described below lie approximately on a straight line running north to the mountain base, and therefore provided samples of both healthy and affected tea growing on soil type and in climates merging from the normal conditions of the average Mlanje tea belt into the abnormal of the badly affected areas.

Location I: Field 8A, Tea Research Station, Swazi. Tea healthy and in good heart, mature, newly pruned and growing on the usual red lateritic soil type receiving 72 inches of rain per annum. Conditions typical of the Mlanje tea belt and eight miles south from the base of the mountain.

Location II: An area on the affected estate with newly pruned mature tea showing symptoms in sporadic small patches. Soil dark red and showing some signs of erosion. Rainfall estimated at 80 inches per annum; the location is about five miles south of the mountain.

Location III: Soil type equivalent to that of location II, mature tea newly pruned and in good health. Rainfall estimated to be slightly higher than in location II and area is about three-quarters of a mile north and nearer the mountain than location II.

Location IV: Very badly affected area, tea mature, newly pruned and growing on a deep, black highly organic soil receiving 125 inches of rain per annum. Conditions not typical of the Mlanje tea belt and about one mile away from the mountain base. This location was the one chosen for intensive investigation.

Location V: Soil type, climate and position equivalent to those of location IV, tea mature though five years younger, newly pruned and in fair to good health. This area is about a quarter of a mile nearer to the mountain than location IV.

Locations II and III form one comparable group, IV and V another, and location I was chosen as a yard-stick by virtue of its being average for the Mlanje tea area. All comparisons are between mature and newly pruned tea.

Samples of leaf were taken from bushes chosen at random and at each location each sample was replicated five times. Apical buds (200) with the first two immature leaves attached formed the first sampling variate, and for the second sample 200 of the fourth leaf below the apical bud were taken; the latter sample was chosen because it is a comparatively newly formed leaf, fully expanded and presumably photosynthetically active.[1] Both types of sample were partially dried overnight and consigned to England next day by air, where they were received in fair condition, though some of the immature apical buds and leaves were found to be mildewed.

Five soil samples at each location were drawn at random points from the top nine inches by using a constant-volume sampling cylinder. The five were air-dried, bulked, quartered and the sample sent to England for analysis.

Owing to the urgency of the problem the apical buds and two leaves were first analysed by chemical methods at the Hammersmith Laboratories of J. Lyons & Co. Ltd. As the weight of the material was insufficient to allow analysis of separate replicates the five from each location were bulked and the mineral constituents, as recorded in Table I, were determined on the bulked sample. Dr. R. L. Mitchell of the Macaulay Institute for Soil Research kindly undertook the spectrographic analysis of the samples of fourth leaf. The smaller amounts of material required for this type of work enabled analyses of replicates to be made and the mean value of each mineral constituent with its standard error is recorded in Table II.

Table I

The mineral constituents of the apical bud with two immature leaves attached

Constituent	Location I	II	III	IV	V
	Red soil, healthy tea	Dark-red soil, affected tea	Dark-red soil, healthy tea	Black soil, affected tea	Black soil, healthy tea
Ash, %	6·9	8·9	6·8	6·8	6·4
N, %	6·0	6·5	6·3	7·3	6·6
P_2O_5, %	1·13	1·24	1·02	1·10	1·03
K_2O, %	2·6	3·3	2·6	2·4	2·3
CaO, %	0·8	1·1	0·8	0·8	0·7
MgO, %	0·51	0·46	0·53	0·46	0·43
Fe, p.p.m.	83	79	110	90	96
Al, p.p.m.	583	1000	760	800	600
Mn, p.p.m.	950	1110	870	295	470
Ni, p.p.m.	5	3	4	4	3
Zn, p.p.m.	34	36	28	34	35
Cu, p.p.m.	12·0	16·5	12·5	14·0	12·0
Mo, p.p.m.	<0·1	<0·1	<0·1	<0·1	<0·1
B, p.p.m.	50	42	31	40	37

Soil samples were analysed spectrographically at the Macaulay Institute, and the results are presented in Table III.

In support of the analytical work a series of spraying and injection experiments were carried out at location IV.[2–6] A randomized block layout was chosen to compare the effects of the sprays listed below. Before treatments were applied each bush was calibrated for healthiness after the manner evolved by Storey & Leach during their work on a sulphur deficiency in tea.[5] Sprays used and their percentage by weight in aqueous solution are given in Table IV.

Table II

The mean mineral constituents of the fourth leaf below the apical bud, with standard errors (S.E.)

Constituents	Location I: Red soil, healthy tea		II: Dark-red soil, affected tea		III: Dark-red soil, healthy tea		IV: Black soil, affected tea		V: Black soil, healthy tea	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Ash, %	7·6	—(*a*)	10·1	—	7·7	—	6·2	—	6·5	—
N, %	4·4	±0·10	5·4	±0·10	5·1	±0·10	6·9	±0·23	5·4	±0·20
P_2O_5, %	0·96	±0·04	1·03	±0·06	0·79	±0·05	0·72	±0·03	0·70	±0·01
K_2O, %	2·0	±0·07	2·8	±0·07	1·9	±0·11	1·5	±0·14	2·0	±0·11
CaO, %	1·3	±0·03	1·7	±0·04	1·3	±0·06	1·4	±0·07	1·1	±0·05
Fe, p.p.m.	128	±10·3	—	—	—	—	135	—	—	—
Mn, p.p.m.	1246	±96	1670	±107	1184	±42	564	±24	930	±51
Ni, p.p.m.	5	±0·25	3	±0·78	3	±0·54	3	±0·42	3	±0·32
Zn, p.p.m.	45	±2·68	32	±2·67	60	±5·40	35	±2·36	30	±3·50
Cu, p.p.m.	10	±0·39	18	±0·63	13	±0·32	13	±0·57	16	±2·30(*b*)
Mo, p.p.m.	<0·2	—	0·2	—	0·2	—	0·1	—	0·1	—
Co, p.p.m.	0·22	±0·03	0·36	±0·04	0·35	±0·08	0·10	—	0·08	±0·13
Pb, p.p.m.	3·4	±0·50	2·4	—	15·2	±9·12(*c*)	3·1	±0·42	<2·0	—
Sn, p.p.m.	2·8	±0·22	<2·0	—	<4·0	—	<2·0	—	<2·0	—
V, p.p.m.	0·4	±0·07	0·3	±0·09	0·3	±0·04	0·3	—	0·2	±0·04
Ti, p.p.m.	13	±2·10	12	±0·84	17	±1·86	16	±1·23	10	±0·96
Cr, p.p.m.	2·8	±0·47	1·5	±0·21	1·1	±0·28	0·6	±0·84	0·6	±0·09
Ag, p.p.m.	<0·2	—	0·2	—	<0·3	—	0·2	—	0·2	—
Sr, p.p.m.	39	±2·6	156	±9·0	109	±7·6	88	±10·1	105	±6·0
Ba, p.p.m.	58	±6·9	135	±8·5	89	±7·8	106	±7·6	85	±9·6

(*a*) A dash is inserted where there is no analytical result or where it was not possible to work out the standard error

(*b*) The comparatively high standard error (2·30) for copper at location V might be due to high residual copper on the leaf surface after spraying with Bordeaux mixture six weeks before sampling

(*c*) The mean value for lead and its standard error at location III are comparatively high. A mean of nearer 4 might perhaps be more acceptable as the individual replicate values for this location are 4, 4, 4, 13 and 51

Table III

The mineral constituents of tea soils

	Location I: Red soil, healthy tea	II: Dark-red soil, affected tea	III: Dark-red soil, healthy tea	IV: Black soil, affected tea	V: Black soil, healthy tea
pH	5·4	5·0	4·9	4·8	4·9
Content of 2·5% acetic acid extract					
K_2O, mg./100 g.	29	48	17	19	17
Na_2O, mg./100 g.	5	6	6	9	7
Cu, p.p.m.	0·25	0·04	Nil	Nil	Nil
Ni, p.p.m.	0·24	0·35	0·19	0·27	0·74
Mo, p.p.m.	<0·1	<0·1	<0·1	<0·1	<0·1
Zn, p.p.m.	42	40	42	34	30
Co, p.p.m.	0·67	0·83	0·04	0·08	0·06
Pb, p.p.m.	<2·0	<2·0	<2·0	<2·0	<2·0
Sn, p.p.m.	1·3	1·7	1·3	2·0	1·6
V, p.p.m.	0·3	0·3	0·2	0·2	0·2
Ti, p.p.m.	0·4	0·4	0·3	0·5	0·3
Cr, p.p.m.	0·2	0·5	0·3	0·3	0·4
Ag, p.p.m.	<0·1	<0·1	<0·1	<0·1	<0·1

Note: For copper for locations III, IV and V the analytical report states: 'The other three soils gave what were, in effect, negative values in that they removed some of the very slight trace of copper present in the extracting solution!'

Half-bush injections and single-branch injections were made with aqueous solutions of 0·5% urea, 0·1% manganese sulphate and 0·1% aluminium sulphate in factorial combination. Before the injection of mineral salts, their expected distribution was plotted by the use of a 0·5% aqueous acid fuchsin solution.[3]

Table IV

	Wt., %		Wt., %
Unsprayed control	—	A mixture of manganese and aluminium sulphate, each at	0·25
Urea	1·0	Nickel sulphate	0·25
Sodium dihydrogen phosphate	1·0	Zinc sulphate	0·5
Potassium chloride	1·0	Ammonium molybdate	0·25
Calcium chloride	1·0	Cobalt sulphate	0·10
Ferrous sulphate	0·5	Magnesium sulphate	0·5
Manganese sulphate	0·5	Aluminium sulphate	0·5
A mixture of manganese and ferrous sulphate, each at	0·25	Boric acid	0·25

No visual benefits were obtained from either the sprays or the injections.

With the sprays no significant differences ($P = 0{\cdot}05$) in yields of plucked leaf were produced, after adjusting the yields by regression on original healthiness calibration; but manganese sulphate alone, the mixture of manganese and aluminium sulphates and the mixture of manganese and iron sulphates occupied first, third and fifth places respectively out of the 16 treatments used. Ferrous sulphate alone was eighth and aluminium sulphate alone was tenth in order.

Discussion

In less urgent circumstances it would have been advisable to work out beforehand methods of sampling leaf that would be expected to provide mean values within desired limits of accuracy. As it was, this could not be done; nevertheless the range of standard errors between locations for each constituent of Table II are, for most minerals, sufficiently close to make comparisons possible. No serious major differences are shown between mineral constituents of the leaf excepting perhaps the contents of manganese. Manganese and aluminium are known to be present in tea in large quantities but the essential lower limit of either is not known.[7, 8] If manganese had been in limiting supply the sprays and injections would have been expected to produce more decisive results, and the conclusion is drawn that manganese is not seriously limiting growth, either by itself or in association with aluminium.

The absence of copper in the top nine inches of soil at locations III, IV and V is not reflected in an absence of copper in either the apical buds and immature leaf or in the mature leaf. This may be because all these areas receive Bordeaux sprays and because copper might be obtained from the deeper layers of the soil which were not sampled. It is of interest to note that the three locations with no top-soil copper are the three nearest to the mountain, two being within a mile of the mountain and the third some four miles away. This distribution fits fairly closely with the areas that are found empirically to require Bordeaux sprays.

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None of the analytical results in this paper were produced in Nyasaland and the author is entirely in the debt of the laboratories of J. Lyons & Co. Ltd. for the figures quoted in Table I; of Dr. R. L. Mitchell of the Macaulay Institute of Soil Research for Tables II and III, and of both for allowing their results to be published. The author is also in their debt for the urgency with which they treated his request for analyses when he was faced with an alarming situation in Nyasaland with few research facilities and with no previous experience of this particular type of problem.

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ANALYTICAL STUDIES ON THE CARBOHYDRATES OF GRASSES AND CLOVERS. V.*—Development of a Method for the Estimation of Cell-wall Polysaccharides

By V. D. HARWOOD

The cell-wall polysaccharides, i.e. the carbohydrate fraction of herbage that is insoluble in alcohol and cold water, are determined by a two-stage acid-hydrolysis procedure. In the first stage, treatment with N-sulphuric acid effects solution and hydrolysis of the greater part of the hemicellulose fraction, with a maximum variability in any one sugar of 7% of the total hydrolysed. The remaining 'cellulose' residue is saccharified with 72% sulphuric acid during the second stage, with agreement within 5%.

Introduction

In a review of the carbohydrate components of herbage Percival[1] divides this fraction into the non-structural components comprising monosaccharides, oligosaccharides and fructosan, and the structural carbohydrates cellulose, pentosans, galactan, pectin and glucosan. Two of the earlier papers in this series[2, 3] have dealt with the estimation of the monosaccharides and oligosaccharides soluble in 80% ethanol and the fructosan soluble in cold water. The present paper deals with the remaining structural or cell-wall polysaccharides.

Schemes for the analysis of the cell-wall polysaccharides fall into two classes: those involving extraction of the pectins and hemicelluloses by various solvents ranging from hot water to strong alkalis, and those involving the complete hydrolysis of the whole polysaccharide fraction. Extraction procedures are of more benefit where a structural investigation is envisaged and generally follow a delignification step. Since there is some evidence that carbohydrates are lost during delignification,[4–6] such a scheme appears unlikely to lead to a quantitative method of analysis. Hydrolysis procedures have the main drawback that a specific sugar may be destroyed by the acid conditions used. Such is the case with fructose-containing oligosaccharides.[3] This destruction may be avoided by a pre-hydrolysis with a weaker acid, the effect of which is to remove and hydrolyse many of the carbohydrate components present in smaller quantity[7, 8] and to leave a residue consisting mainly of cellulose.

The present investigation has led to a method involving a pre-hydrolysis with N-sulphuric acid followed by the complete hydrolysis of the carbohydrates in this solution. The residue is saccharified with 72% sulphuric acid and an acid-insoluble residue comparable with herbage lignin is determined. Since the method adopted involves hydrolysis of mixtures of polysaccharides, the results can be expressed only in terms of quantities of the various anhydro-sugars present in the herbage.

The simultaneous hydrolysis and extraction of rye-grass hemicelluloses

The complete hydrolysis of cellulose-containing mixtures of polysaccharides can readily be effected with 72% sulphuric acid, an observation that is the basis of the Klason method of lignin determination. In this method a secondary hydrolysis at a concentration of 3 to 5% sulphuric acid dissolves the material that reprecipitates upon dilution of the 72% sulphuric acid. The main disadvantages of the application of this procedure to a mixed-pentosan cellulose polysaccharide are that some destruction of pentoses occurs during either the primary or secondary hydrolyses[9] and the large quantities of glucose produced interfere with the chromatographic estimations, particularly of galactose. These difficulties are avoided by a preliminary treatment with a dilute acid, the action of which is to dissolve and simultaneously hydrolyse a considerable portion of the pentosan polysaccharides. The residue remaining is mostly cellulose.

Whereas Björkqvist & Jörgensen used 0·5N- to N-sulphuric acid at 95° for 15 hours in this preliminary treatment,[7] the author has found that N-sulphuric acid for one hour at the temperature of a boiling-water bath dissolves all the galactose and arabinose polysaccharides, leaving a grass residue containing only cellulose and xylan. The N-sulphuric acid solution was found to contain xylose and glucose as well as considerable quantities of oligosaccharides. Further hydrolysis with N-acid eliminated the oligosaccharides after four hours at reflux temperature. This was established by determining the ratios of the various sugars chromatographically and by following the increase in reducing value. For periods of longer than four hours, destruction of the monosaccharides becomes significant (Table I). It is also apparent from

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Table I

Change in content and composition of the sugars in a N-sulphuric acid extract of rye-grass during further heating under reflux

Time of heating, h.	Total sugar in solution, mg.	Amount removed in aliquot, mg.	Net gain or loss, mg.	Composition of solution remaining + aliquots removed, mg.			
				Arabinose	Xylose	Glucose	Galactose
0	812	113	—	237	393	138	44
2	1115	181	1115 − (812 − 113) = + 416	268	427	465	68
4	954	205	954 − (1115 − 181) = + 20	267	435	479	67
6	712	226	712 − (954 − 205) = − 37	294	408	451	58

the results in Table I that the main component of the oligosaccharides was glucose, which suggests that the preliminary treatment with N-sulphuric acid degraded cellulose to glucose and glucose oligosaccharides. There is no evidence that the cell-wall polysaccharides contain a soluble glucosan, though the author has isolated a mixture of polysaccharides containing glucose, galactose, arabinose and xylose by a hot-water extraction of an ethanol- and cold-water-extracted sample of S24 perennial rye-grass.

The possibility exists that some destruction of the sugars occurred at the same time that the oligosaccharides were hydrolysed. Various investigators have studied the action of sulphuric acid on monosaccharides. Sundman, Saarnio & Gustafsson[9] treated xylose, arabinose and glucose with 72% sulphuric acid at 25° for 4 hours, then diluted the acid to 3·5% and boiled the mixture for 6 hours. Removal of acidic reducing decomposition products on ion-exchange resins enabled them to calculate a relative destruction of 9–11% for xylose and 10% for arabinose related to glucose. Ekelund[10] performed a similar experiment but without the treatment with resins. From the reducing values he calculated a loss of 4–7% of xylose, 0–3% of arabinose, 0–4% of glucose and 0–3% of galactose. Saeman, Bubl & Harris[11] heated glucose in sealed tubes at 100° with 4% sulphuric acid for up to three hours and found a maximum loss of 2·5% based on determinations of reducing power. In the present investigation the monosaccharides were not treated with 72% sulphuric acid but instead were dissolved in 5% sulphuric acid and heated in a boiling-water bath. After varying intervals the reducing values were determined and related to those of the original solutions. From the results in Table II it is concluded that no more than 5% of the monosaccharides is destroyed during the five hours' total time of contact of herbage sugars with N-sulphuric acid. No correction factor is applied since this experiment does not duplicate that of the Finnish workers.[9]

Table II

Decomposition of sugars on heating with N-sulphuric acid for varying times at 100°

Time, h.	Recovery of reducing power of sugars, %			
	Arabinose	Xylose	Galactose	Glucose
1	95·1	99·5	100·5	100·3
2	94·4	101·0	98·5	—
3	94·4	—	100·9	98·8
4	—	95·4	97·9	—
5	95·3	—	—	101·2
6	—	96·6	93·0	—

The saccharification of cellulose- and xylan-containing residues

After the N-sulphuric acid treatment a residue from the grass is obtained that contains ash, protein, lignin and polysaccharides hydrolysable to glucose and xylose only. Numerous investigators have studied the saccharification of such residues; the general technique is a primary hydrolysis at room temperature with 72% sulphuric acid followed by a secondary hydrolysis at the boiling point after dilution to 3–5%. By using cotton and a high α-cellulose pulp, Saeman *et al.*[11] obtained a reducing value of 94·5–97·2% of the theoretical; the conditions were 45 minutes at 30° for the primary and 4·5 hours at 100° and 4% acid concentration for the secondary hydrolysis. Ritter, Mitchell & Seborg[12] treated spruce-wood cellulose with 72% sulphuric acid for varying times at various temperatures, then boiled the product at 4% acid

concentration for 4 hours. From the reducing value the recovery of cellulose (uncorrected for mannan or xylan) was 98·0 ± 0·4% for 2 hours at 35°, and 99·7 ± 0·9% for 6 hours at 16°.

In the present investigation an oat-straw xylan, an acid-pretreated sample of S24 rye-grass and a similarly treated sample of ensiled rye-grass were each treated with 72% sulphuric acid at 18 ± 2° for varying lengths of time, diluted and boiled (see Table III). The results show that xylan yields a 95% recovery of reducing power when the primary hydrolysis is of four hours' duration. The lower yield at 6 hours can be explained on the basis of xylose decomposition in 72% sulphuric acid; the low yield after 2 hours in the strong acid cannot be explained on the basis of retention of oligosaccharides during the secondary hydrolysis[12] since qualitative chromatograms showed oligosaccharides only in the 1-hour secondary hydrolysate. With the silage cellulose the secondary hydrolysis at 3% acid concentration is insufficient to decompose the cellulose dextrins[12] or oligosaccharides. It is concluded therefore that a 4-hour primary and a 3-hour secondary hydrolysis are the optimum conditions for saccharifying residues containing xylan and cellulose. Both xylose and glucose are decomposed to the same extent, namely 5–7%, so, whatever is the quantity of xylan associated with the grass cellulose, no correction factor need be applied for the loss of a specific sugar, e.g. xylose.

Table III

Conditions for saccharifying the carbohydrates associated with lignin

Material	Time in contact with 72% sulphuric acid, h.	Concn. of diluted acid	Time of boiling, h.	Recovery of carbohydrate, %
Oat-straw xylan	4	1N	1	96
			3	95
			6	94
	2	1N	1	83
			3	81
			6	79
	6	1N	1	82
			3	81
			6	85
Silage cellulose*	4	1N	1	78
			3	99
			6	89
	4	3%	1	53
			3	76
			6	76
Grass cellulose†	4	1N	1	78
			3	93
			6	92

* Prepared from rye-grass silage by pretreatment with N-sulphuric acid

† Prepared from rye-grass by extraction with 4N-potassium hydroxide solution and by pretreatment with N-sulphuric acid

So far it has been assumed that a 95% recovery of the calculated reducing power of these polysaccharide preparations is evidence that these materials yield sugars and no acidic reducing substances as postulated by Sundman, Saarnio & Gustafsson.[9] The validity of this assumption has been shown throughout many months of the application of this method to grass and silage samples in this Laboratory. In all these analyses the recovery of the glucose and xylose from the chromatograms accounted for 90–105% of the calculated carbohydrate content of the samples analysed.

Determination of lignin in grass samples

The determination of lignin by the Klason (72% sulphuric acid) method on an alcohol- and water-extracted grass sample might lead to high lignin values because of furfuraldehyde condensation products resulting from the action of the strong acid on pentosans.[13] The acid-pretreatment method proposed in this paper effectively removes about 80% of the furfuraldehyde-producing carbohydrates. As a result the value for the acid-insoluble residue obtained during the secondary hydrolysis of the lignocellulose and xylan residues approaches the Klason lignin figure as obtained by the method of Moon & Abou Raya[14] (see Table IV). However, it may

Table IV

Comparison of acid-insoluble residues from hydrolysis of acid- and pepsin-treated grass residues

Sample and pretreatment	Protein in pretreated residue, % of dry grass	Acid-insoluble residue after saccharification, % of dry grass
A		
N-H_2SO_4	5·3	8·5
N-H_2SO_4 and pepsin	2·1	7·3
B		
3% Nitric acid	6·1	4·3
3% HNO_3 and pepsin	3·2	3·6
C		
Pepsin and cold hydrochloric acid	1·3	6·2

be noted that if the acid pretreatment is made with 3% nitric acid instead of 5% sulphuric acid, a value for an acid-insoluble residue is subsequently obtained which is less than the Klason lignin value. It is concluded therefore that the smaller acid-insoluble residue in the nitric acid experiment suggests either some lignin solubility or else a truer lignin value because of less furfuraldehyde formation during pentosan hydrolysis with nitric acid.[15] Further investigation of this anomaly is suggested.

The other main contaminant of lignin residues is due to protein[16] and, as a result, Moon & Abou Raya[14] use a pepsin digestion at 30° in 0·1N-hydrochloric acid followed by a washing with cold hydrochloric acid; the washing with acid is not used specifically to remove the peptides produced. The effectiveness of this digestion can be seen from the results in Table IV; the reduction in the acid-insoluble residue is 14 to 16%. However, even with this treatment the acid-insoluble residue by the N-sulphuric acid and pepsin pretreatments does not equal the lignin value obtained by the method of Moon & Abou Raya. For this reason the residue from this method is not considered to be a true lignin value. In the calculation of the results (see below) the reduction of the acid-insoluble residue by 15% has the effect of only changing the correction factor.

Experimental

Grass samples were supplied by the Experimental Farm, Boghall, Midlothian. The general methods of sugar analysis are based on chromatographic separations on paper, followed by elution with hot or cold water and estimation by the 1945 Somogyi reagent.[17] Sugar factors in terms of 0·005N-thiosulphate were determined from time to time and remained constant within 5%. Acid hydrolysates were neutralized with barium carbonate and concentrated *in vacuo* below 40°, so that no epimerization was noted (cf. Laidlaw & Reid[2]).

Method for analysis of total carbohydrate in grass

The grass sample (20·0 g.) is extracted for 7 h. in a Soxhlet apparatus with 80% ethanol in the boiling flask and the free sugars are determined by the method outlined in Part I.[2] Fructosan is dissolved by shaking the alcohol-extracted grass with two successive lots of cold water and is analysed for fructose by a colorimetric method.[3] The grass residue is dried at 100° overnight after washing with acetone, and generally amounts to 1·5 to 2·0 g., according to the moisture content of the original 20-g. sample.

This material is heated in a boiling-water bath with N-sulphuric acid (20 ml./g.) for one hour, recovered on a Buchner funnel and washed well with water. A final washing with acetone is discarded and the residue dried overnight at 100°. The acidic extract and washings are adjusted to 1N by the addition of 6N-sulphuric acid. An aliquot equivalent to 0·2–0·3 g. of the ethanol- and water-extracted residue is boiled under reflux for 4 hours, and 15 to 20 mg. of ribose is accurately weighed and added. After neutralization and concentration, duplicate pairs of paper chromatograms are run in a mixture of ethyl acetate, acetic acid and water (3 : 1 : 3) for 40 hours at 23° to determine xylose and the ribose standard, and in a mixture of benzene, butanol, pyridine and water (1 : 5 : 3 : 3) for 40 hours at 23° to determine galactose, glucose and arabinose. Each paper is spotted with the same quantity of sugar solution (about one-fifteenth). The results are calculated as percentages of anhydro-sugars based on the dry weight of the original grass sample.

Protein and ash determinations are made respectively on 50-mg. and 100-mg. samples of

the acid-treated and dried residue, and the remainder of this material is hydrolysed with 72% sulphuric acid (1·5 ml./0·1 g.) at 18 ± 2° for 4 hours, followed by dilution to 1N (23·5 ml. of water for each ml. of 72% acid) and by boiling under reflux for 3 hours. This solution is filtered through an asbestos-lined Gooch crucible and the ash-free acid-insoluble residue determined. An aliquot of the filtrate, equivalent to 0·1 g., is removed, a weighed quantity (about 20 mg.) of ribose is added and the solution neutralized, concentrated and spotted on a pair of chromatogram papers. These are irrigated with a mixture of ethyl acetate, acetic acid and water (3 : 1 : 3) for 40 hours at 23° and the quantities of xylose and glucose are estimated. The method of calculating the results is as follows.

From the weight of sample taken for hydrolysis the ash, protein and acid-insoluble residue are subtracted. The xylose and glucose are calculated as xylan and cellulose and related to the calculated carbohydrate hydrolysed. This relationship is generally 90 to 105% and shows whether or not the results are reliable. The xylan and cellulose values are then calculated as percentages of the original dry weight of grass subjected to the alcohol extraction and subsequent treatments, with due allowance for the 90 to 105% recovery.

Reproducibility of results

In an experiment to check the reproducibility of the method, 2 g. of an alcohol- and water-extracted sample of rye-grass leaf was divided into two equal portions and subjected to the above treatment with N-sulphuric acid. The two hydrolysates were analysed; the following results, calculated as percentages of the dry grass, were obtained.

Galactan	0·69	0·53
Araban	1·81	2·18
Xylan	4·20	3·71
Glucosan	0·94	1·21

The totals are identical but variations in the individual components are attributed to difficulties in obtaining exact separations between adjacent sugars on the chromatograms.

Duplicate analyses of aliquots of the 72% sulphuric acid hydrolysates were found to agree within 5%. It has also been noted that the addition of ribose to the latter solutions can be dispensed with since the ratio of xylose to glucose is directly related to the xylan and cellulose in these residues.

Comparison with the results obtainable by isolation of 'hemicellulose' and 'cellulose' fractions.

Two samples of S23 perennial rye-grass were extracted with 80% ethanol and cold water to remove free sugars and fructosan. One was analysed by the method described above and the results are recorded in Table V (*a*). The second sample was extracted with 4N-potassium hydroxide solution for 48 hours at room temperature with occasional stirring. From the alkaline liquors a 'hemicellulose' fraction was recovered after neutralization with acetic acid and precipitation with ethanol. This product was hydrolysed with N-sulphuric acid in a boiling-water bath for 6 hours. An 82% recovery of pentosan and hexosan, based on the calculated content of carbohydrate, was obtained. The individual anhydro-sugars were corrected by the factor 100/82 and recorded in Table V (*b*). The alkali-extracted grass residue was hydrolysed by 72% sulphuric acid in the usual way and the results are also noted in Table V (*b*). The results are in good agreement and establish the fact that the N-acid treatment removes practically the same material as does 4N-potassium hydroxide solution, leaving in each case a constant ratio of cellulose to xylan.

Preliminary experiments leading to the development of the method adopted

Rehydrolysis of a N-sulphuric acid grass extract.—A 1·0-g. sample of perennial rye-grass, free of sugars and fructosan, was heated with 30 ml. of N-sulphuric acid for one hour in a boiling-water bath. The acid was removed by filtration and the grass residue washed well with water. The acid extract and washings were then concentrated *in vacuo* at 30–40° to 30 ml. and then boiled under reflux. At intervals 2-ml. aliquots were removed, neutralized with barium carbonate, filtered and washed to give a volume of 35 ml. Total reducing values were estimated and quantitative chromatograms were run on these solutions. The ratio of the component sugars was determined from the chromatograms, and the amount of sugar in the hydrolysate, and in the 2-ml. aliquots removed, was calculated from the reducing values. In Table I the total sugar at each interval, the quantity of the sugars removed in the aliquots and the change in composition of solution plus aliquots are recorded.

The decomposition of monosaccharides during heating with sulphuric acid.—Solutions of the various monosaccharides were made in N-sulphuric acid and 5-ml. aliquots containing about

Table V

Cell-wall polysaccharides of S23 perennial rye-grass

(*a*) By two-stage sulphuric acid method

	Components, % of dry grass samples			
	Araban	Xylan	Galactan	Cellulose
Stage 1	2·3	8·7	3·1*	—
Stage 2	—	2·7	—	24·4
Total	2·3	11·4	3·1	24·4

(*b*) By separation of component polysaccharides

'Hemicellulose'	2·1	10·8	1·8	0·3
'Cellulose'	—	2·3	—	24·3
Total	2·1	13·1	1·8	24·6

* Containing some glucosan

0·8 mg. were placed in a boiling-water bath under reflux condensers for varying periods. The aliquots were neutralized with 4N-potassium hydroxide solution and reducing values were determined. In Table II the reducing power is recorded as a percentage of that of the original solutions.

The saccharification of lignin–cellulose–xylan mixtures.—Samples of an alkali- and acid-extracted rye-grass, an acid-extracted rye-grass silage and an oat-straw xylan were treated with 1·5 ml. of 72% sulphuric acid per 0·1 g. of material at 18 ± 2° for varying times, diluted and boiled under reflux. The reducing values were determined. The results are recorded in Table III in terms of recovery of anhydro-sugars based on the calculated content of carbohydrate in the starting material (i.e. by subtraction of ash, protein and acid-insoluble residue).

The determination of acid-insoluble residues and Klason lignin in a sample of S24 rye-grass.—A sample of S24 perennial rye-grass collected on 4 June, 1953, was extracted with ethanol and water for the removal of free sugars and fructosan. The oven-dried residue was divided into three parts, A, B and C. Portion A was treated with N-sulphuric acid at 100° for one hour, lot B with 3% nitric acid under reflux for one hour and lot C with 1% pepsin in 0·1N-hydrochloric acid at 30° for 16 hours, followed by washing with cold concentrated hydrochloric acid and water.[14] Portions A and B were then subdivided and half of each was treated with the pepsin as above but washed with water only. All five residues were then hydrolysed with 72% sulphuric acid for 4 hours at 18°, and, after dilution to 1N, for 3 hours at reflux temperature. Each hydrolysate was filtered through an asbestos-lined Gooch crucible and an ash-free acid-insoluble residue was determined. Protein and ash were estimated on a sample of each of the five residues. The results are recorded in Table IV as percentages of the original extracted and dried grass residues.

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ANALYTICAL STUDIES ON THE CARBOHYDRATES OF GRASSES AND CLOVERS. VI.*—Changes in the Cell-wall Polysaccharides during the Ensilage of Perennial Rye-grass with a High Protein and Low Soluble-Carbohydrate Content

By V. D. HARWOOD

When high-protein grass containing only small amounts of water-soluble carbohydrates is ensiled a product with high pH is obtained. In this type of silage a decrease in pentosan carbohydrates is observed.

In Part III of this series[1] it was suggested that the main requirement for making silage with low pH, i.e. 3·5 to 3·8, is a plentiful supply of reserve polysaccharide. It was found that monosaccharides still remained after eight months in this silage prepared from a mixed herbage containing 24·8% (dry weight) of water-soluble carbohydrate, not including oligosaccharides, and 13·3% of crude protein[2] (N × 6·25). Some decomposition of cell-wall polysaccharides was inferred from the appearance of free arabinose, xylose and galactose, presumably because of hydrolysis at this low pH.

During further investigation of the ensilage process a sample of S24 perennial rye-grass was harvested at a time when the relationship of protein to carbohydrate was at the opposite extreme, that is 27·2% of crude protein (N × 6·25) and 6·2% of total water-soluble carbohydrate, each on the basis of the dry matter in the grass. Silages prepared from this grass with and without the addition of mixed lactobacilli underwent a decrease in pH but, after 8 days, returned to pH 5; continued breakdown of protein then occurred during the next two months and the pH increased to 6·5. All the water-soluble carbohydrates disappeared but the only losses suffered by the cell-wall polysaccharides were noted in the pentosans. These losses were 35% of the araban and 26 to 58% of the xylan.

Experimental

On 17 June, 1953, about 40 lb. of S24 perennial rye-grass was cut from a plot at the Experimental Farm, Boghall, Midlothian. Within three hours the grass was brought to King's Buildings, Edinburgh, mixed on sterile paper and each of 10 quart milk-bottles was filled with 500 g. of grass, some compression being required. The remainder of the grass was inoculated with a mixed culture of lactobacilli under the supervision of members of the staff of the East of Scotland College of Agriculture and another 10 bottles were filled as above. The bottles were capped with rubber stoppers containing Bunsen valves and placed in an incubator at 30°. Bottles of the inoculated and control silages were removed at intervals, bacterial counts were made by the staff of the College of Agriculture and the carbohydrates analysed by the procedures described in Parts I, IV and V of this series.[3–5] Results are shown in Tables I and II. Amino-acids, volatile base and proteins were analysed by Dr. A. R. Kemble and are the subject of a future publication.

Discussion

A normal silage is one in which the pH has rapidly fallen to a value between 3·5 and 4·5. The amount of protein breakdown is therefore small and the growth of butyric acid-producing bacteria is at a minimum. In practice this can be achieved without the addition of lactobacilli, though such additions do hasten the attainment of the necessary low pH conditions. The lactobacilli and other acid-producing bacteria require a ready source of carbohydrate in their nutrients and for grasses this source appears to be glucose and fructose derived from the free monosaccharides, oligosaccharides and fructosan.[1]

A second important factor in the preparation of a normal silage is the ratio of crude protein to water-soluble carbohydrates. In the experiment described by Wylam[1] this ratio was approximately 1 : 2. These experiments of summer, 1953, show the effect of the opposite conditions where the ratio was approximately 4·5 : 1.

The results in Table I with respect to pH, and the rapid breakdown of protein to such an extent that the silages became putrefied after five days, show that in these experiments a 'bad' silage was the result. This is attributable to the low water-soluble-carbohydrate content of the grass. The effect of this low carbohydrate content is manifest in Table I, which shows the almost complete disappearance of carbohydrate, the rapid fall in bacterial count and the rise

* Part V: preceding paper

Table I

Changes in water-soluble carbohydrates during ensilage

Material	pH	Lactobacilli, millions per g. of dry matter	Glucose	Fructose	Sucrose	Fructosan	Total
Fresh grass	6·4		0·2	0·8	3·5	0·6	6·2*
Control silages							
after 1 day	6·3	3,631	2·1	2·4	nil	0·4	4·9
after 3 days	4·9	1,549	1·2	0·8	nil	0·3	2·3
after 8 days	4·9	490	nil	nil	nil	0·2	0·5*
after 2 months	6·5	0·003	nil	nil	nil	nil	0·2*
Inoculated silages							
after 1 day	4·4	12,590	0·3	0·3	nil	0·4	1·0
after 3 days	4·4	5,754	nil	nil	nil	0·1	0·3*
after 8 days	5·0	1,349	nil	nil	nil	0·1	0·2*
after 2 months	6·6	11·2	nil	nil	nil	nil	nil

* Containing values attributed to oligosaccharides

Table II

Analyses of cell-wall polysaccharides in grass and silages

Material	Galactan	Araban	Xylan	Cellulose	Total
Fresh grass	0·6	1·7	6·2	20·2	28·7
Control silage (8-day)	0·6	2·1	6·1	19·1	27·9
,, ,, (2-months)	0·5	1·1	4·6	22·7	28·9
	0·5	0·8	3·6	23·8	28·7
Inoculated silage (8-day)	0·2	1·8	6·2	20·7	28·9
,, ,, (2-months)	0·4	1·1	2·6	20·5	24·6

in pH to 5, after eight days in both control and inoculated silages. During the remainder of the two-month period the pH continued to rise and the bacteria to decrease. A perusal of Table II enables the following hypotheses to be proposed:

(1) Since the pH is approaching neutrality it is apparent that little or no acid hydrolysis of the cell-wall polysaccharides takes place, which is corroborated by the failure to observe free arabinose or xylose in the free-sugar extracts.

(2) Significant losses of araban and xylan have occurred during the two months, whereas no change in cellulose content, except the anomalous value in the two-month control silage, is noted. It is concluded therefore that the lactobacilli attack the pentosans after the depletion of the free sugars, a conclusion that is borne out by the greater loss of xylan in the inoculated silage, where a higher count of lactobacilli was noted after eight days.

The change in galactan is of doubtful significance and is within the limit of experimental error. The apparent increase in cellulose is difficult to understand particularly since duplicate determinations are recorded.

It is concluded that, in the absence of sufficient water-soluble carbohydrates, lactobacilli survive by attacking araban and xylan. This action occurs in silage near the neutral point whereas in acidic silages hydrolysis of araban and xylan is the destructive mechanism. Further study of the action of lactobacilli on samples of hemicellulose and cellulose is proposed and also the hydrolysis of similar materials by acid solutions of pH 3·5–4·5 at 30°.

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THE SEED OILS OF *CLITORIA TERNATEA* AND OF *ENTADA PHASEOLOIDES*

By D. N. GRINDLEY, E. H. W. J. BURDEN and A. A. AKOUR

The seed oils of the species *Clitoria ternatea* and *Entada phaseoloides* have been examined in detail and their fatty acid composition determined. Like other species belonging to the Leguminosae group of families previously examined, they are characterized by the presence of higher saturated fatty acids.

The plant *Clitoria ternatea* (family Papilionaceae) is being introduced into the Gezira area of the Sudan as a fodder crop, and the possibility of using the seeds as a human foodstuff is also under consideration. This species is known commonly as the butterfly bean, or Kordofan pea.

Although the root, like jalap, is said[1] to be a powerful cathartic, and an infusion of leaves is used for eruptions and also as an antidote to scorpion stings, nothing of a poisonous nature could be detected in the seeds. They were examined for the presence of alkaloids, with negative results, though Folkers & Unna[2] have reported the presence of an alkaloid having a curare-like action in the related species *C. arborescens*. The powdered seeds were fed to monkeys in 5-g. doses which the animals ate voluntarily, and no toxic effects were observed. Particular attention was directed to the possibility of the presence of hydroxyanthraquinones or other purgative principles in view of an earlier report[3] claiming purgative properties for these seeds, but no effect of this nature could be observed on the animals nor could any such compound be detected by chemical means. The seeds do, however, like other parts of the plant, contain a blue dye with indicator-like properties which has been referred to before[4] and used in chemical analysis.

Though this plant is indigenous to the Sudan, the sample analysed was from locally grown stocks originally obtained from the Bureau of Tropical Agriculture, South Johnstone, Queensland, where *Clitoria* is a constituent of natural grazing. The Australian strain is more vigorous than the local one, and will be used for large-scale cultivation.

The plant is a strong-growing bushy creeper with dark-blue flowers, having a deep-rooting habit that enables it to withstand the rigours of a very dry hot climate. It is a perennial and the cattle of the Gezira have found it very palatable and graze it heavily.

The plant bears a large number of nitrogen-fixing nodules; it thus increases the fertility of the soil and is important as a rotational crop as well as for fodder. In addition it is a very effective controller of weeds, for once *Clitoria* is fully established watering may be stopped. The species does not die, but continues to absorb water from the soil and dry out the soil very thoroughly, making conditions unfavourable for the growth of perennial weeds. *Clitoria* revives very quickly with the early rains and smothers both annual and perennial weeds before they can establish themselves. This crop is particularly suitable for cultivation in the Gezira as it is resistant to many of the common insect pests and diseases.

The species *Entada phaseoloides* is a member of the family Mimosaceae, and occurs in Equatoria Province in the Sudan, but not very abundantly. It is of more frequent occurrence in Uganda. It is a lofty woody climber, with very stout stems, and has a large woody pod of up to 3 ft. long and $3\frac{1}{2}$–4 in. broad. The seeds, which are the largest of any of the Leguminosae group of families known, are about $2\frac{1}{2}$ in. in diameter and $\frac{5}{8}$ in. broad; the husk is dark brown, enclosing a white kernel.

The seeds of the two species were analysed; the results are shown in Table I.

The high protein content of *C. ternatea* is worthy of note, and is the main point to commend its use as a feeding-stuff.

The ground seeds, whole for *C. ternatea* and kernels only for *E. phaseoloides*, were exhaustively extracted with light petroleum and the oils analysed. The oil content of both species is rather low. After removal of the unsaponifiable matter, the fatty acids were recovered and their iodine and thiocyanogen values were determined, thus enabling the composition of the fatty acids to be calculated, using the empirical value of 96 for the thiocyanogen value of pure linoleic acid (cf. Hilditch & Murti).[5] In view of the difficulty experienced by some workers with this method, all thiocyanogen values were determined in duplicate; excellent duplication was obtained in all cases. In the dry climate of the Sudan we have never experienced any difficulty with this method, which we regard as highly reliable and useful. The fatty acids were next brominated in ethereal solution, and the absence of any precipitation

indicated the absence of linolenic acid. On removal of the ether and twice recrystallizing the residues from light petroleum, tetrabromides were obtained corresponding to that derived from *cis-cis-*$\Delta^{9:12}$-octadecadienoic acid, the linoleic acid commonly present in seed fats, whose presence in these two species is thus proved.

The saturated fatty acids were determined by Bertram's oxidation method,[6] and the results obtained agree closely with the values derived thiocyanometrically. After the oxidation mixture had been extracted with light petroleum to remove the saturated acids, the aqueous layer, after concentration, was further extracted with diethyl ether; large yields of a dibasic acid whose melting point and equivalent weight corresponded closely with those of azelaic acid were obtained in each case. It is thus evident that in each of the species there is no unsaturated acid present having a double bond nearer to the carboxyl group than in the 9 : 10-position, as in oleic acid.

Table I

Composition of seeds

	Entada phaseoloides (Mimosaceae)	*Clitoria ternatea* (Papilionaceae)
Wt. of seeds	41·6 g. each	4·44 g./100 seeds
Kernel, %	53·61	
Analysis	(of kernel)	(of whole seed)
Moisture, %	6·22	5·68
Oil, %	7·00	11·76
Protein, %	19·54	41·19
Ash, %	3·08	3·48
Crude fibre, %	0·98	0·66
Carbohydrate (diff.), %	63·18	37·23
	100·00	100·00

The high saponification values of the oils and the high molecular weight and melting point of the saturated acids separated in Bertram's process suggested the presence of higher saturated acids containing 20–24 carbon atoms per molecule, whose presence in the seed fats of the families Papilionaceae, Mimosaceae and Caesalpiniaceae appears to be characteristic.[7, 13] We have found that Bellier's turbidimetric method (Evers' modification)[8] can give a very useful approximate estimate of the amount of such higher acids present in a seed oil, if one assumes that the figure given for 100% groundnut oil represents 6·5% of higher acids. The percentage of higher acids appears to be remarkably constant in groundnut oil.[9–11] Should turbidity be obtained at a temperature higher than that given by pure groundnut oil, i.e. 40° (as was so with *E. phaseoloides*), the oil of unknown composition may be diluted with an equal volume of pure olive oil and the turbidity process repeated. We have found that this process works well and is very simple and rapid, and results agree closely with those from the gravimetric method.

In order to be able to examine in more detail these higher saturated acids, recourse was had to Renard's method,[12] with slight modifications, that is the saturated acids obtained in the Bertram process were used instead of those obtained by extracting the mixed lead salts with ether and using the insoluble fraction, as recommended by Renard. The total saturated acids (from 5 g. of total fatty acids) were therefore dissolved by warming in 25 c.c. of 90% (v/v) ethanol (sp. gr. 0·8340 at 15·5°), and cooled for one hour at 15°. The deposited crystals were rapidly filtered off on a sintered-glass funnel, sucked dry and washed with three successive quantities of 5 c.c. each of 90% alcohol at 15°. The final residue was dissolved in ether, evaporated and weighed, and was examined for molecular weight, melting point etc. A correction has to be added for the solubility of ' arachidic acid ' in the 40 c.c. of 90% alcohol used at 15°, but it is small compared with the large corrections necessary to be applied in Evers' modified quantitative method,[8] which is based on the total fatty acids and not the separated saturated acids. This method is recommended, especially when the elaborate and more orthodox ester-fractionation methods are not available, or when there is insufficient material for their employment, and it is of interest to note the close agreement obtained by this method and the turbidimetric method of Bellier.

The constants and fatty acid compositions of the two oils are given in Table II.

It is thus seen that the oils of these two species exhibit the characteristic of their respective families in that they both contain appreciable quantities of higher saturated fatty acids.[7, 13]

Table II

Properties of the oils

	E. phaseoloides	*C. ternatea*
Colour	Golden yellow	Greenish yellow
Saponification value, mg.KOH/g.	185·1	178·7
Unsaponifiable matter, %	1·34	2·99
Total fatty acids, %	94·60	91·01
Refractive index at 40°	1·4622	1·4589
Iodine value of oil	87·83	72·91
Thiocyanogen value of oil	63·63	57·51
Iodine value of fatty acids	91·42	77·13
Thiocyanogen value of fatty acids	65·77	63·13
Melting point, ° c, of total fatty acids (slip-point)	36	35
Molecular weight of total fatty acids	288·1	287·3
Hexabromides	nil	nil
Tetrabromides, m.p., ° c	113	113
Total saturated acids (Bertram), %	28·00	32·12
Melting point, ° c, of saturated acids (slip-point)	52½	52½
Molecular weight of saturated acids	284·7	284·5
Bellier's test (direct), ° c	40·9	38·0
,, (+ 50% of olive oil), ° c	36·0	31·5
As higher saturated acids, %	8·5	5·2
Higher acids (Renard), %	8·2	5·5
Melting point, ° c, of higher acids (closed tube)	72½	71
Molecular weight of higher acids	344·7	358·4
Dibasic acids recovered, %	42·97	40·42
Melting point, ° c, of dibasic acids	106	106
Equiv. wt. of dibasic acids	95·3	94·9

Fatty acid composition (thiocyanometrically)

	E. phaseoloides	*C. ternatea*
Linoleic acid, %	30·6	16·7
Oleic acid, %	40·5	52·3
Saturated acids, $C_{16} + C_{18}$, %	20·4	25·8
Higher saturated acids, C_{20}–C_{24}, %	8·5	5·2

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A CHEMICAL STUDY OF THE FRUITS OF THREE SOUTH AFRICAN *XIMENIA* SPECIES, WITH SPECIAL REFERENCE TO THE KERNEL OILS

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The average composition of the fruits was found to be: fruit pulp 81%, hull 4% and kernel 15%. The high acidity of the fruit juice, 8·6%, was accounted for entirely by citric acid. The kernels contained 64–65% of oil, 18–20% of protein and 4% of fibre. In addition to the acids already found by other workers, a tetracosenoic and an octacosenoic acid were isolated from the kernel oils and a hydroxyoctadecanoic acid was found in the hydrogenated acid mixture from one species. The component acids of the oils from the three species, which were similar in composition, included oleic acid (32·5–40·5%), ximenynic acid (22–24·3%), tetracosenoic acid (3·0–7·0%), hexacosenoic (ximenic) acid (3·5–8·7%), octacosenoic acid (4·7–12·2%) and triacontenoic (lumequeic) acid (3·0–7·0%).

The genus *Ximenia* belongs to the Olacaceae or hog-plum family. The most common species, *X. americana*, is found in the tropical regions of America, Africa and Asia. It is of economic importance since the wood yields a fair timber, the flesh of the fruit is used for making jam and jellies, and the kernels are prized for their high oil content.[1] Localized species are found in Australia, South Africa, India and parts of America. The following species, known locally as 'sour plum' (English), 'suur pruim' (Afrikaans), 'magwenya' (Zulu), occur in South Africa: *X. caffra* Sond., *X. caffra* var. *natalensis* Sond. and *X. americana* var. *microphylla* Welw. (= *X. rogersii*, Burtt-Davy).

After earlier examinations of the kernel oils of several species,[2] detailed chemical investigations of the oil of *X. americana* were made by Puntambekar & Krishna,[3] who showed the presence of a new hexacosenoic acid (ximenic acid) as well as palmitic, stearic, oleic and linoleic acids. Later, Boekenoogen[4] confirmed these results and isolated in addition a triacontenoic acid (lumequeic acid). The new unsaturated acids were shown by him to be *n*-hexacos-17-enoic acid and *n*-triacont-21-enoic acid respectively. More recently, Ligthelm & Schwartz[5] have shown the presence of octadec-11-en-9-ynoic (ximenynic) acid in the kernel oil of the South African *Ximenia* species. In the present work the oils from the three South African species have been investigated in more detail.

Composition of the fruits and kernels

Ripe fruits of *X. caffra* and *X. caffra* var. *natalensis* were collected from the Middelburg–Groblersdal districts of the Transvaal. The composition of the fruits and kernels is shown

Table I

Composition of the fruits and kernels of South African Ximenia *species*

	X. caffra	*X. caffra* var. *natalensis*	*X. americana* var. *microphylla*
Average weight of fruit, g.	12·4	11·8	—
Average weight of seed, g.	2·4	2·1	1·1
Kernel in seed, %	79	72	64
Fruit pulp			
Moisture, %	77·6	77·8	—
Oil, % of fresh weight	0·3	0·4	—
Citric acid, % of expressed juice	8·7	—	—
Kernels			
Moisture, %	26·2	22·5	—
Oil, % of dry weight	65·7	64·1	68·4
Protein, % of dry weight	19·9	18·8	17·8
Fibre, % of dry weight	3·7	4·2	3·9
Ash, % of dry weight	1·6	2·0	1·8

in Table I. The total acidity of the fruit juice, determined potentiometrically, was equivalent to a citric acid content of 8·5%, and the percentage of citric acid found by the method of Gray[6] was 8·7%. The high acidity of the fruit is thus entirely accounted for by citric acid.

Table II

Characteristics of Ximenia *kernel oils*

	X. caffra	*X. caffra* var. *natalensis*	*X. americana* var. *microphylla*
Specific gravity (25°/25°)	0·9190	0·9152	0·9154
Refractive index at 25°	1·4734	1·4710	1·4712
Acid value	2·5	1·0	1·5
Iodine value (Wijs, 1 h.)	83·0	79·7	77·4
,, ,, (from hydrogen absorption)	129·0	—	—
Saponification value	170·4	172·8	165·2
Acetyl value	13·1	—	—
Reichert-Meissl value	0·2	0·4	0·2
Polenske value	0·3	nil	nil
Unsaponifiable matter, %	1·4	1·3	1·4
$E^{1\%}_{1\,cm.}$ (229 mμ)	142·3	121·5	123·7
$E^{1\%}_{1\,cm.}$ (268 mμ)	11·8	0·7	0·9

The kernel oils

The oils were extracted from the kernels of the fresh fruits referred to above, and also from sun-dried fruits from the following localities: *X. caffra* from Kanye, Bechuanaland; *X. caffra* var. *natalensis* from Ixopo, Natal; and *X. americana* var. *microphylla* from Pongola.

The oils were extracted from the minced kernels either with acetone or with light petroleum. The mobile oil extracted with acetone (viscosity E in Gardner–Holt tubes[7] at 25°) became viscous on standing (viscosity Y after one week) and a white gelatinous solid separated out. It was insoluble in light petroleum and could be easily removed by diluting the oil with 3–4 volumes of this solvent and filtering the cold solution. The precipitate constituted about 1% of the oil. It yielded, after removal of saponifiable impurities and crystallization from ethanol, an alcohol, m.p. 307–308° (decomp.) (Found: C, 75·5; H, 10·6%); melting point of acetate, 283–284°. This material is apparently the 'phytosterol fraction' of Puntambekar & Krishna.[3] However, we were not able to obtain an insoluble digitonide. After removal of this material, the oil regained its original viscosity (viscosity E). Oils purified in this way were used in the present study.

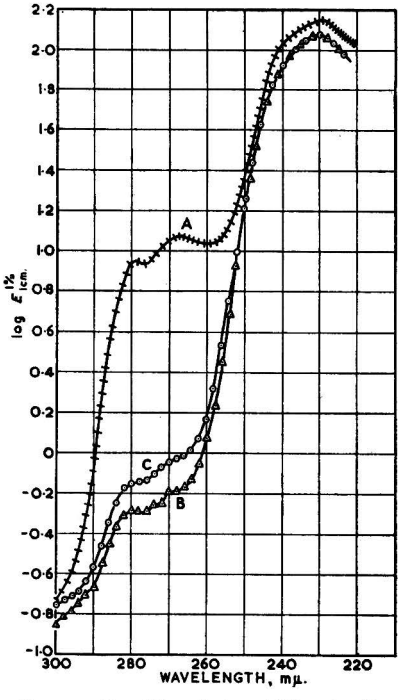

FIG. 1.—*Absorption spectra of* Ximenia *oils*
A *X. caffra* Sond.
B *X. caffra* var. *natalensis* Sond.
C *X. americana* var. *microphylla* Welw.

Extraction of the kernel oils with light petroleum yielded a viscous oil directly. In this instance, the high viscosity, also noted by other workers,[3, 4] was found to be due to the presence of 2·5% of rubber, which could be removed from the oil by diluting it with acetone, in which it is insoluble.

The characteristics of the oils, determined according to 'The Official and Tentative Methods of the American Oil Chemists' Society',[8] are given in Table II. Both the yields (Table I) and the characteristics of the oils from the three South African *Ximenia* species lie within the range of values reported for *X. americana* by other workers.[2–4] The absorption spectra of the oils in *cyclo*hexane solution (Fig. 1) show the presence of 20–25% of ximenynic acid ($E^{1\%}_{1\,cm.}$ 583 at λ_{max} 229 mμ) together with a small amount (up to 0·6%) of a conjugated trienoic acid.

Component acid analyses

By distillation through Longenecker column

The mixed fatty acids from the kernel oil of each species were crystallized from acetone at − 25° to give a soluble fraction, which consisted mainly of ximenynic and oleic acids, and an insoluble fraction, which contained the bulk of the saturated and high-molecular-weight unsaturated acids. In preliminary experiments, the mixed acids were crystallized first from ether at − 40°, followed by crystallization of the soluble acids at − 60°. The fractionation achieved by this procedure was, however, only slightly better than that obtained by a single crystallization from acetone at − 25°; so the latter procedure was used in all subsequent work. The following details illustrate the methods as applied to the *X. caffra* fatty acids. The mixed acids [407 g.; neutralization equivalent (N.E.), 313·0; $E^{1\%}_{1\,cm.}$ (229 mμ), 148·3] obtained by hydrolysis of 450 g. of oil were crystallized from acetone (10 ml. per g.) at − 25°, yielding two fractions with the following characteristics:

Fraction	Wt., g.	% of total acids	Iodine value	N.E.	$E^{1\%}_{1\,cm.}$ (229 mμ)
A Insoluble in acetone at − 25°	140·8	34·7	47·5	373	42·1
B Soluble in acetone at − 25°	265·7	65·3	97·0	288	196·9

Each group of acids was converted into the methyl esters and fractionally distilled through a Longenecker column.[9] The distilled ester fractions were analysed for saponification equivalent, iodine value and ultra-violet absorption at 229 mμ and 268 mμ.

The percentage of methyl ximenynate and that of conjugated trienoic esters (calculated as methyl β-elaeostearate) in each fraction were calculated from the extinction at 229 mμ and 268 mμ respectively (the $E^{1\%}_{1cm.}$ of methyl ximenynate and methyl β-elaeostearate were taken as 549·2 and 2000 respectively[10–12]). The remaining components of the fractions were calculated from the saponification equivalents and iodine values in the usual way.[13] In calculating the contributions of the methyl ximenynate and methyl elaeostearate to the iodine value of the fraction, the iodine values of these were taken as 124·8 and 160·0 respectively.[10] Assumptions on the nature of the higher-molecular-weight acids present were based on the results of the work described below. The calculated composition of the oils is shown in Table III.

Table III

Acid	*Component acids of* Ximenia *oils, wt.* %				
	X. caffra	*X. caffra* var. *natalensis*	*X. americana* var. *microphylla*	*X. americana* Indian [3]	*X. americana* W. African [4]
Palmitic	0·4	0·3	0·3	—	—
Stearic	2·6	2·4	1·4	1·2	4·0
Eicosanoic	0·6	1·0	0·4	—	—
Docosanoic	0·6	0·9	trace	—	—
Tetracosanoic	2·2	2·6	1·3	—	—
Hexacosanoic	3·8	2·6	3·0	15·2	2·0
Octacosanoic	1·0	0·8	1·1	—	—
Hexadecenoic	1·5	1·0	0·5	—	—
Oleic	32·5	40·5	35·8	60·8	54
Linoleic	—	—	—	6·7	10·0
Ximenynic	24·3	22·0	21·9	—	—
as β-Elaeostearic	0·4	0·2	0·2	—	—
as Eicosenoic	2·5	2·3	1·8	—	—
Docos-13-enoic	2·3	1·3	0·2	—	—
Tetracos-15-enoic	5·7	7·3	3·0	—	—
Hexacos-17-enoic	3·5	7·0	8·7	14·6	25·0
Octacos-19-enoic	9·6	4·7	12·2	—	—
Triacont-21-enoic	5·4	3·0	7·0	—	5·0
as Dotriacontenoic	1·0	—	0·9	—	—

By distillation in spinning-band column

The acids from *X. caffra* oil were also examined by distilling the methyl esters before and after hydrogenation in a modified Piros & Glover micro-spinning-band fractionating

column.[14] The efficiency of this column was very much better than that of the Longenecker column and it was possible to obtain a sharp separation of the esters of different chain length (see Fig. 2).

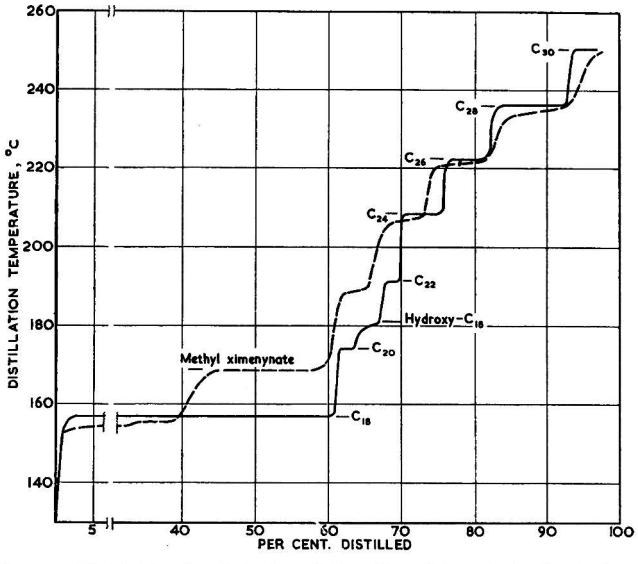

FIG. 2.—*Distillation of methyl esters of* X. caffra *oil in spinning-band column*
– – – Esters before hydrogenation
——— Hydrogenated esters

(*a*) *Distillation of hydrogenated esters.*—The methyl esters (70 g.) of the mixed *X. caffra* acids were dissolved in methyl alcohol (1 litre) and hydrogenated at 60–70° and 100 lb./sq. in. in the presence of a palladium–calcium carbonate catalyst until the iodine value of the product was 0·5. The hydrogenated esters (66 g.) were distilled in the spinning-band column with a boil-up rate of 30–40 ml. per hour, a pressure of 1·0 (± 0·05) mm. and a rotor speed of 1200 r.p.m. Fractions of 1 ml. were collected. Methyl esters of the saturated acids from octadecanoic to triacontanoic acid were isolated by crystallization from *iso*hexane of appropriate fractions of the distillation plateaux. The properties of these compounds and of the corresponding acids are given in Table IV.

Table IV

Characterization of esters isolated by distillation of hydrogenated X. caffra *esters*

B.p. at 1 mm. of fraction, °C	Chain length of acid	Methyl esters M.p., °C Found	Methyl esters M.p., °C Literature[16]	Analysis Found C, %	Analysis Found H, %	Analysis Calc. C, %	Analysis Calc. H, %	M.p. of free acids, °C Found	M.p. of free acids, °C Literature[16]
156·5	C_{18}	39·0	39·1	76·8	12·9	76·5	12·8	69·5	69·6
191	C_{22}	53·0–53·4	53·3	78·0	12·7	77·9	13·1	79·7–80·0	79·9
208	C_{24}	58·5	58·4	78·7	13·3	78·5	13·2	83·8–84·1	84·2
222	C_{26}	63·5–63·7	63·5	79·1	13·3	79·0	13·2	87·6–87·8	87·7
236	C_{28}	67·5–67·8	67·5	79·7	13·4	79·4	13·3	90·7–91·0	90·9
250	C_{30}	70·8	71·7	80·0	13·5	79·7	13·4	93·0–93·3	93·6

The distillation curve also indicated the presence of a compound with a boiling point between that of methyl eicosanoate and methyl docosanoate. Crystallization of fractions of b.p. 176–179° from *iso*hexane yielded the methyl ester of a hydroxyoctadecanoic acid, m.p. 55·6–56·0° (Found: C, 72·7; H, 12·2; $C_{19}H_{38}O_3$ requires C, 72·6; H, 12·2%). The free acid crystallized from a mixture of acetone and alcohol (1 : 1) at — 30° as a white amorphous

solid, m.p. 77·5–78·0°, $[\alpha]_D^{20}$ − 1·6 (*c*, 5·56 in chloroform) (Found: C, 72·2; H, 12·1; N.E. 301. $C_{18}H_{36}O_3$ requires C, 72·0; H, 12·0%; N.E. 301). Further work on this acid is in progress.

The sharpness of the separation of the esters achieved in this distillation allows the calculation of the relative proportion of the component esters with considerable accuracy. The results so obtained are shown in Table V, together with the values for the composition of the unhydrogenated acids as determined by low-temperature crystallization and distillation of the esters through the Longenecker column. This provides a check on the results of the latter method, which is desirable in this instance because of the unusual complexity of the *Ximenia* oils. No attempt was made to calculate the percentage of hydroxy-acid in the unhydrogenated oil. However, this introduced only a small error in the final results. Otherwise, the agreement between the values obtained by the two methods is very good.

Table V

Component fatty acids of X. caffra *oil*, % (*mol.*)

Acid	Hydrogenated acid (spinning-band distillation)	Unhydrogenated acid (Longenecker column)	
Palmitic	0·3	0·5	2·4
Hexadecenoic	—	1·9	
Stearic	66·3	2·8	66·8
Oleic	—	36·1	
Ximenynic	—	27·5	
as β-Elaeostearic	—	0·4	
Hydroxystearic	4·1	—	
Eicosanoic	3·0	0·6	3·1
as Eicosenoic	—	2·5	
Docosanoic	2·2	0·5	2·6
Docosenoic	—	2·1	
Tetracosanoic	5·3	1·9	6·8
Tetracosenoic	—	4·9	
Hexacosanoic	5·3	3·0	5·8
Hexacosenoic	—	2·8	
Octacosanoic	8·0	0·8	8·0
Octacosenoic	—	7·2	
Triacontanoic + still residue	5·5	—	
Triacontenoic	—	3·8	4·5
as Dotriacosenoic	—	0·7	

(*b*) *Distillation of unhydrogenated esters.*—Unhydrogenated esters (66 g.) were distilled in the spinning-band column (see Fig. 2). The fractions boiling at 168° (1·0 mm.) had an $E_{1\,cm.}^{1\%}$ at 229 mμ of 542, and were thus substantially pure methyl ximenynate ($E_{1\,cm.}^{1\%}$ at 229 mμ = 549·2). The boiling points and iodine values of the fractions of the other plateaux indicated the presence of the esters of the normal even-numbered saturated acids from stearic to octacosanoic, and of the monoethenoid acids from oleic to triacontenoic. Most of the esters present in the mixture were isolated and characterized as below. A spectrophotometric examination of selected fractions, before and after alkali isomerization, showed that polyunsaturated acids, other than ximenynic, were not present in more than very small amounts.

Identification of acids present in X. caffra *oil*

The isolation of ximenynic acid from *Ximenia* oils has already been described.[5, 10]

Palmitic acid.—The first fractions from the distillation of the unhydrogenated esters in the spinning-band column were combined and kept at 0° for 8 hours. The crystals that separated were collected, washed with cold *iso*hexane and recrystallized from *iso*hexane at − 15°. This ester was saponified. The liberated acids crystallized from *iso*hexane in the form of plates, m.p. 62·0°, undepressed on admixture with pure palmitic acid.

Stearic acid.—Methyl stearate was isolated in a similar manner from fractions boiling at 154–156°/1 mm. The ester melted at 38·6–39·0° (Found: C, 76·9; H, 12·9. Calc. for $C_{19}H_{38}O_2$: C, 76·5; H, 12·8%). The free acid obtained by saponification had m.p. 69·6–70·0°, undepressed on admixture with pure stearic acid.

Oleic acid.—Fractions with a saponification equivalent of 295–296 and iodine value of 84–85 (from the distillation in the Longenecker column) were saponified, the acids dissolved

in aqueous potassium hydroxide and oxidized with potassium permanganate according to the method of Kaufmann & Fiedler.[15] The hydroxy-acids, after removal of unoxidized acids by extraction with light petroleum, were recrystallized from acetone as white plates, m.p. 130–131°, undepressed on admixture with dihydroxystearic acid from pure oleic acid.

Eicosanoic (arachidic) acid.—Methyl eicosanoate, m.p. 45·8–46·2° (Ralston,[16] 46·6°) was isolated by crystallization of the fractions distilling at 169–172°/1 mm. (Found: C, 77·6; H, 13·0. Calc. for $C_{21}H_{42}O_2$: C, 77·2; H, 13·0%).

It was not possible to isolate the eicosenoic acid from these fractions because of the high content of ximenynic acid (approximately 50%).

Docosanoic (behenic) and docos-13-enoic (erucic) acids.—Crystallization of the fractions, b.p. 190–192°/1 mm., from *iso*hexane yielded plates, m.p. 53·3–53·6°, undepressed on admixture with pure methyl docosanoate.

The soluble methyl esters isolated from the mother liquors were saponified, the acids dissolved in aqueous potassium hydroxide and oxidized with potassium permanganate.[15] The hydroxy-acids were recrystallized from 80% aqueous ethanol in the form of white plates, m.p. 130·0–130·3°, undepressed on admixture with dihydroxydocosanoic acid prepared by oxidation of erucic acid (Found: C, 71·2; H, 11·9. Calc. for $C_{22}H_{44}O_4$: C, 70·9; H, 11·8%).

Tetracosanoic (lignoceric) acid and tetracos-15-enoic acid.—Methyl tetracosanoate was obtained by recrystallization of the first fractions boiling at 205–206·5°/1 mm. from *iso*hexane, m.p. 58·4–58·7°, undepressed on admixture with an authentic specimen (Found: C, 78·3; H, 13·1. Calc. for $C_{25}H_{50}O_2$: C, 78·5; H, 13·2%).

The soluble esters from the mother liquors were saponified and the acids crystallized from *iso*hexane at 5°. The insoluble acids, mainly tetracosanoic acid, were discarded. The soluble acids were recrystallized from *iso*hexane at − 15° as plates, m.p. 43·1–43·4° (Found: C, 78·8; H, 12·8; N.E., 365·0; iodine value, 68·2. Calc. for $C_{24}H_{46}O_2$: C, 78·7; H, 12·6%; N.E., 367; iodine value, 69·2). Oxidation of the potassium salt with dilute aqueous potassium permanganate[15] gave a dihydroxytetracosanoic acid, m.p. 129·7–130·1° (Found: C, 71·9; H, 12·3. Calc. for $C_{24}H_{48}O_4$: C, 72·0; H, 12·1%). Oxidation of the methyl ester of the original acid with potassium permanganate in glacial acetic acid[17] below 40° gave nonanoic acid (the *p*-phenylphenacyl ester had m.p. 69·5–69·7°, undepressed on admixture with the ester of pure nonanoic acid), and 1 : 13-tridecanedicarboxylic acid, m.p. 112·5° (reported[18] 114·6–114·8°) (Found: C, 66·1; H, 10·4; N.E., 139. Calc. for $C_{15}H_{28}O_4$: C, 66·2; H, 10·3%; N.E., 136). From these results it is concluded that this acid is tetracos-15-enoic acid (selacholeic acid, nervonic acid[19]), and an infra-red examination confirmed the *cis*-configuration assigned to this acid on chemical grounds.[20]

Hexacosanoic (cerotic) and hexacos-17-enoic (ximenic) acids.—Crystallization of the fractions, b.p. 220–221°/1 mm., from *iso*hexane at 4° and 0° yielded methyl hexacosanoate as plates, m.p. 63·0–63·3°, undepressed on admixture with an authentic sample (Found: C, 78·8; H, 13·2. Calc. for $C_{26}H_{52}O_2$: C, 79·0; H, 13·2%).

The esters from the mother liquors from the crystallizations at 0° were converted into the acids and crystallized from *iso*hexane at 0° and − 10°. The acids that crystallized between 0° and − 10° were recrystallized from *iso*hexane at − 10°, yielding hexacos-17-enoic acid, m.p. 50·5–50·9° (Found: C, 79·0; H, 12·9; N.E., 392; iodine value, 63·4. Calc. for $C_{26}H_{50}O_2$: C, 79·1; H, 12·8%; N.E., 395; iodine value, 64·3). Oxidation of the free acid with Milas' reagent in *tert.*-butyl alcohol[21] gave a dihydroxyhexacosanoic acid, m.p. 119–120° (Found: C, 73·2; H, 12·1. Calc. for $C_{26}H_{52}O_4$: C, 72·9; H, 12·2%). Oxidation of methyl hexacosenoate with potassium permanganate in glacial acetic acid[17] gave nonanoic acid (identified as described above) and 1 : 15-pentadecanedicarboxylic acid, m.p. 116·5–117° (Chuit[18] records 118°) (Found: C, 68·1; H, 10·9; N.E., 151. Calc. for $C_{17}H_{32}O_4$: C, 68·0; H, 10·7%; N.E., 150). These results confirm Boekenoogen's findings[4] that ximenic acid is hexacos-17-enoic acid, and from an infra-red examination it is assigned the *cis*-configuration.

Octacosanoic (montanic) acid and octacos-19-enoic acid.—Crystallization of the first fractions, b.p. 234–236°/1 mm., from *iso*hexane at 4° yielded methyl octacosanoate, m.p. 67·0–67·2° (Found: C, 79·2; H, 13·2. Calc. for $C_{29}H_{58}O_2$: C, 79·4; H, 13·3%). The free acid melted at 90·0–90·4° (Ralston[16] records 90·9°).

The soluble esters from the mother liquors were crystallized at 0° and − 10°. The esters crystallizing between 0° and − 10° were recrystallized from *iso*hexane at − 10° (Iodine value, 57·2; methyl octacosenoate requires 58·1). A portion of the ester was converted into the

acid and crystallized from *iso*hexane at − 5°; m.p. 57·8–58·2° (Found: C, 79·2; H, 12·8; N.E., 422. Calc. for $C_{28}H_{54}O_2$: C, 79·6; H, 12·9%; N.E., 423). Oxidation of the methyl ester with performic acid,[22] followed by saponification, gave dihydroxyoctacosanoic acid, m.p. 108–108·4° (Found: C, 73·5; H, 12·5; N.E., 458. Calc. for $C_{28}H_{56}O_4$: C, 73·6; H, 12·4%; N.E., 457). Oxidation of methyl octacosenoate with potassium permanganate in glacial acetic acid[17] gave nonanoic acid (identified as described above) and 1 : 17-heptadecanedicarboxylic acid, m.p. 118–118·5° (Chuit[18] reports 119°) (Found: C, 69·5; H, 11·1; N.E., 162. Calc. for $C_{19}H_{36}O_4$: C, 69·5; H, 11·0%; N.E., 164). *X. caffra* oil thus contains a hitherto unreported octacos-19-enoic acid. An infra-red examination of the methyl ester showed it to be the *cis*-isomer.

Triacont-21-enoic (lumequeic) acid.—As the fractions boiling at 248°/1 mm. had iodine values above 50, and no methyl triacontanoate could be isolated from them, it appears that triacontanoic acid is absent or present in only very small amount.

The fractions were crystallized from *iso*hexane at 0° and 5° (Iodine value 53·8. Calc. for methyl triacontenoate 54·7). The free acid was crystallized from *iso*hexane at − 5° as white plates, m.p. 60·8–61·2° (Found: C, 79·7; H, 12·9; N.E., 445. Calc. for $C_{30}H_{58}O_2$: C, 79·9; H, 13·0%; N.E., 451). Oxidation of methyl lumequate with potassium permanganate in glacial acetic acid gave nonanoic acid and 1 : 19-nonadecanedicarboxylic acid, m.p. 116–118° (Chuit[18] reports 118–120°) (Found: C, 70·6; H, 11·6; N.E., 180. Calc. for $C_{21}H_{40}O_4$: C, 70·8; H, 11·2%; N.E., 178). The structure assigned to lumequeic acid by Boekenoogen,[4] namely that of a triacont-21-enoic acid, is thus confirmed. An infra-red examination showed it to be the *cis*-isomer.

Discussion of results

The oils of the three South African *Ximenia* species are very similar in composition (Table III). The content of high-molecular-weight unsaturated acids was highest in the oil from *X. americana* var. *microphylla*, where the sum of the percentages of tetracosenoic, hexacos-17-enoic (ximenic), octacos-19-enoic and triacont-21-enoic (lumequeic) acids was 30·9%, and lowest in that from *X. caffra* var. *natalensis*, where the sum was 22·0%. These differences are reflected in the differences in the saponification values of the oils (see Table II).

The most interesting result of the present investigations has been the discovery that South African *Ximenia* oils contain 3–7% of tetracos-15-enoic acid and 5–12% of octacos-19-enoic acid. Neither of these has been found before in seed oils, although the former acid has long been known as a component of elasmobranch-liver oils and brain cerebrosides.[19] It is interesting that the six monoethenoid acids isolated from the oils all have the formula $CH_3{\cdot}[CH_2]_7{\cdot}CH{:}CH{\cdot}[CH_2]_n{\cdot}CO_2H$, where n varies from 7 for oleic acid to 19 for lumequeic acid. A small amount of a C_{18} hydroxy-acid was also found in *X. caffra* oil. The similarity of the constants of our oils and those of *X. americana* kernel oils obtained from India and West Africa suggests that these oils also contained the new acids reported here, as well as ximenynic acid. The fact that the sum of the hexacos-17-enoic and triacont-21-enoic acid contents of *X. americana* oil reported by Boekenoogen (30% of the total acids, Table III) is roughly the same as the content of the higher-molecular-weight unsaturated acids (tetracosenoic and above) in our oils supports this view.

Both Boekenoogen and Puntambekar & Krishna reported a fair proportion of linoleic acid in *X. americana* oil; but this was not found to be present in more than traces in the South African oils. They also reported a higher content of oleic acid than we found. The total content of C_{18} unsaturated acids, however, shows reasonably good agreement with our values (Table III). Since ximenynic acid gives an iodine value of approximately 50% of the theoretical value (130·3 instead of 273·6 with the Wijs reagent[10]), it seems highly probable that the unsaturation attributed by the early workers to linoleic, and part of that attributed to oleic acid, was, in fact, due to ximenyic acid.

Although acetylenic acids have been reported in the kernel oil of other members of the Olacaceae family,[23] the *Ximenia* oils appear to be unique in containing high-molecular-weight monoethenoid acids.

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SOIL FUMIGATION. II.*—The Stability of Ethylene Dibromide in Soil

By PETER WADE

Liquid ethylene dibromide introduced into soils has been shown to be stable at moisture contents in the field range for periods of up to one week. Some evidence was found for the decomposition of the fumigant by an oven-dried clay. Attempts to induce soils to decompose ethylene dibromide by repeated fumigation of soil samples were unsuccessful.

Introduction

During the course of work described in the first paper in this series[1] little evidence was found for the irreversible sorption of ethylene dibromide vapour by soils, under a variety of experimental conditions. In practical fumigations ethylene dibromide is introduced into the soil

* Part I: *J. Sci. Fd Agric.*, 1954, **5**, 184

in the liquid phase. The work described in the present paper was carried out in order to determine if any decomposition of ethylene dibromide occurred when the liquid fumigant came into contact with soil, and to determine if it was possible to induce soils to decompose ethylene dibromide by repeated fumigations (possibly by building up a microbiological population capable of metabolizing the fumigant). The latter effect has been previously noted with carbon disulphide,[2] DD (dichloropropane–dichloropropene) mixture,[3] and with the plant-growth regulator 2,4-D.[4]

Methods

To investigate the stability of liquid ethylene dibromide in contact with soils the following procedure was employed: A small bulb of approximately 25 mm. diameter was blown on the end of a 5-in. × $\frac{5}{8}$-in. soft-glass test-tube. A sample (usually 5 g.) of soil passing a 2-mm. sieve was placed in the bulb, and the tube constricted about 2 cm. above the soil. A small piece of filter paper (Whatman No. 50) was placed in the tube above the constriction and a second constriction was made above the paper at a distance of about 3 cm. above the first constriction. A quantity of 86·4 mg. of liquid ethylene dibromide was measured on to the filter paper by means of a calibrated micrometer syringe,[1] the greater part of the liquid immediately running through the lower constriction on to the soil. The ampoule was sealed off at the upper constriction and the soil and fumigant were incubated at 25° for the required period of time. (The use of the filter-paper strip was found to be necessary in order to obtain reproducible conditions at the tip of the syringe needle.) At the end of the incubation period the ampoule was broken inside an all-glass unit and the unchanged fumigant removed by a two-stage aeration procedure consisting of one hour's ' dry ' aeration followed by one hour's ' wet ' aeration. This procedure was described in Part I.

The effect of repeated fumigation of a soil on its subsequent ability to decompose ethylene dibromide was studied in the following way: Portions of soil (600–800 g.) (passing a 2-mm.-mesh sieve) were placed in earthenware plant pots (5$\frac{1}{2}$ in. diameter and 5 in. deep). Water, in excess of that required to saturate the soil, was added to each pot and the soils were allowed to drain for 24 hours. Each pot was then injected with 1 ml. of liquid ethylene dibromide at a depth of 2$\frac{1}{2}$ in. below the surface and the injection hole closed by pressing the surrounding soil into it. The pots were set aside for one week. After this period had elapsed one of the pots was emptied and the soil passed through a 2-mm.-mesh sieve and spread out to air until no odour of ethylene dibromide was apparent. (This operation usually took 1–2 hours.) The remaining pots were again fumigated with 1 ml. of ethylene dibromide, and sufficient water (about 100 ml.) was added to the pots to keep the soil moist without removing any fumigant by leaching. Four 100-g. portions of the aired soil from the first pot were weighed out. Two of these portions were each suspended in one litre of distilled water and aerated under the conditions described in the previous Part in order to determine the amount of residual fumigant in the soil. The other two portions were placed separately in glass fumigation-units, dosed with 86·4 mg. of pure ethylene dibromide, and kept in a constant-temperature room at 20° for 24 hours. At the end of this period the unchanged fumigant was recovered by the two-stage process referred to above. The amount of fumigant recovered, corrected for the residual fumigant in the soil, was expressed as a percentage of the amount added (86·4 mg.). The whole procedure was repeated on the remaining pots of soil. Blank determinations were carried out on the original soil.

Results

The experiments on the stability of liquid ethylene dibromide in contact with soils were carried out on three soils, a sand (Ashurst Lodge), a clay (Bones Close) and a soil with a high content of organic matter (Black Fen). (An analysis of these soils has been given in the previous Part.) Tests were also carried out on a sample of peat and on samples of calcium hydroxide and calcium carbonate. In addition, tests were made on a sample of the clay soil after treatment with the soil conditioner ' Krilium '.

Tests were carried out on the soils and peat after oven-drying and at two moisture contents, one near the field capacity and the other corresponding approximately with the air-dried materials. The calcium hydroxide and calcium carbonate were tested at 40% moisture content only. Results are shown in Table I.

At the highest moisture contents recoveries of 99–100% of the fumigant added were obtained with all the samples, even after periods of contact of up to one week. At the lower moisture contents somewhat lower recoveries were obtained with the clay (Bones Close) and the peat. Of the oven-dried samples, the sand (Ashurst Lodge) still gave recoveries of 98–100% but the

other three soils showed much lower recoveries. The Black Fen soil and the peat both gave recoveries of the order of 96%, the actual amounts showing no systematic variations with time. The recoveries of fumigant from the clay, both before and after treatment with ' Krilium ',

Table I

Soil	Moisture content, % on dry weight	Period of contact, h.	Recovery of fumigant, %
Ashurst Lodge	Oven-dried	26	100·1
		74	98·7
		146	98·4
,,	2·1	1¾	98·1
		24¼	99·4
		72¼	98·8
		168	99·1
,,	12·9	23½	100·8
		167¾	99·3
Bones Close	Oven-dried	25	95·6
		73	94·1
		145	92·3
,,	2·9	22¼	97·6
		166½	97·4
,,	11·8	2¾	99·3
		23¼	100·6
		71	101·0
		167	100·0
Bones Close, treated with ' Krilium '	Oven-dried	27	94·1
		75	94·6
		147	93·4
,,	4·0	2¾	98·7
		25½	100·2
		73¼	99·1
		169½	98·7
,,	25·9	24¾	100·2
		169	99·6
Black Fen	Oven-dried	24	96·4
		72	96·0
		144	96·2
,,	33·4	1¾	100·2
		22	99·4
		70	98·9
		166	99·6
,,	51·0	21¼	100·3
		165½	99·5
Peat	Oven-dried	24	96·9
		72¼	95·0
		146½	96·4
,,	18·8	23¼	97·6
		71	95·3
		167¼	96·9
,,	175·7	24½	98·7
		72	98·1
		168½	99·1
Calcium hydroxide	Approx. 40	23½	100·1
		143½	98·3
Calcium carbonate	Approx. 40	24½	100·0
		144½	99·8

were considerably lower than with any of the other samples and showed a tendency to decrease with increasing time of contact.

The effect of repeated fumigations on the subsequent power of a soil to decompose ethylene dibromide was investigated on three soils, a sample of Ashurst Lodge soil, and two glasshouse

soils, one of which was ' tomato sick ', having had six successive crops of tomatoes grown in it. Fresh samples taken directly from the field and glasshouse were used.

Recoveries of 97–100% of the added fumigant were obtained, even after repeated fumigation. The results obtained with the Ashurst Lodge soil are given in Table II. Similar results were obtained with the other two soils.

Table II

Effect of repeated fumigations on Ashurst Lodge soil

No. of fumigations	' Blank ' aerations, mg.	Recovery of fumigant, % of amount added
0	0·2	98·0
	0·1	98·0
1	2·7	98·7
	2·7	99·4
2	2·8	98·6
	2·8	99·1
3	4·3	99·1
	3·8	99·7
4	6·6	99·9
	6·6	100·0

Discussion

The results given in Table I show that no decomposition of ethylene dibromide occurs in any of the soils at moisture contents in the region of the field capacity. The absence of decomposition of the fumigant in contact with the calcium hydroxide and calcium carbonate indicate that no decomposition may be expected in chalk soils or in soils fumigated immediately after liming.

The lower recoveries obtained with the clay and peat at the lower moisture contents, and with the oven-dried soils other than the sand, suggest that some slight decomposition of the fumigant may have occurred in these soils. Only with the dry clay is there indication of a progressive decrease in the amount of fumigant recovered with increasing time of contact with the soil. This is perhaps significant in view of the dehydrochlorination of DDT adsorbed on a dry lateritic clay, observed by Hadaway & Barlow.[5] Treatment of the clay with ' Krilium ' had no effect on the recoveries of fumigant obtained.

Soils taken freshly from the field and glasshouse were used in the second group of experiments, since Pauwels[6] has observed a decrease in the power of soils to decompose carbon disulphide on storage. The absence of breakdown of ethylene dibromide in soils, even after repeated fumigation, suggests that this fumigant may be used repeatedly in the same soil, unless, for example, the pest against which it is being used becomes resistant to it.

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THE SURVIVAL OF *BACILLUS SUBTILIS* SPORES IN THE BAKING OF BREAD

By F. J. FARMILOE,* S. J. CORNFORD,* J. B. M. COPPOCK* and M. INGRAM†

The survival of *Bacillus subtilis* spores at temperatures near 100° was determined in buffered suspensions. Known numbers (about 10^6/g.) of these spores were mixed into dough which was baked normally into bread. Temperatures taken near the centre of the loaf, near the crust, and midway between the two showed that the temperature within the loaf was 100–101° and this was only just attained at the centre. These temperatures were comparable with similar measurements in various baked products. Viable counts were made on the dough just before entering the oven and on baked samples taken at the thermocouple positions. Corresponding loaves were incubated at 37°.

In buffer solutions vegetative cells were killed in about 2 min. at 75°. The thermal death/time curves for spores were not exponential, a small proportion of the spores surviving for relatively long periods. In dough, about half the spores germinated between mixing the dough and putting it into the oven. Comparison of the survival of spores in the bread with that expected from the temperature treatment and from the experiments in buffer solutions suggested that the heat resistance in bread is roughly the same as, certainly no less than, that in phosphate buffer of pH 6·5. Only a small proportion of the surviving spores generated ropy patches within a week, owing perhaps to peculiarities in water distribution within the loaf.

(1) Introduction

That certain bacteria can survive baking in bread is plain, for the well-known defect, 'rope', is caused by their development from spores usually present in the original flour[1] or in the yeast.[2]

Most workers agree that some spores can resist baking. Thus, Aubertin *et al.*[3] confirmed that sporing bacteria (*Bacillus mesentericus*, *Clostridium perfringens*) survive, and although Edmonson, Thom & Giltner[4] failed to recover *Clostridium botulinum* that had been included in doughs and baked at 220° for 35 min., more recent workers have succeeded. For example, Ingram & Robinson[5] record the isolation of *Cl. botulinum* type A from experimental canned bread; Soloski & Cryns,[6] Kadavy & Dack[7] and Bever & Halvorson[8] also showed that canned bread from doughs inoculated with *Cl. botulinum* sometimes developed toxin on storage, unless the bread had a pH below 5·0 and a moisture content below 34%, and viable spores but no toxin were found in bread even with these properties.

It seemed to us from a review of the literature that further study of the temperature distribution in a loaf was needed, as apparently there must be occasions when, in parts at least, lethal temperatures are not attained.

Thus we have found: (i) that when cloth contaminated with *Staphylococcus aureus* and *Salmonella paratyphi* was inserted in doughs, although the salmonellas were all killed the staphylococci sometimes were not, and other non-sporing bacteria survived too, though they were not identified; (ii) that living baking-yeast can occasionally be recovered from the crumb, suggesting (cf. Lund[9]) that the localized temperature cannot have exceeded about 65°; and (iii) that moulds, especially *Aspergillus* spp., can sometimes be isolated from the loaf centre after baking, in conditions where a heavy infection might have occurred (e.g. from a contaminated proving-bag). Such experiences are by no means new: Roussel[10] found that tubercle bacilli in dough retained their virulence after being baked, and Gajoux[11] stated that they can survive in pastry (where the greater dryness perhaps offsets the higher temperatures); La Rosa[12] remarks that the local temperature attained in the loaf during baking decides whether pathogens will survive.

Accordingly we have infected doughs experimentally with known numbers of *Bacillus* spores, and enumerated these at different stages of the baking process in order to compare their rate of destruction with their heat resistance observed in buffered aqueous suspensions; we have also compared the temperatures reached during experimental bakes with those attained during the normal baking of a typical range of bread products.

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(2) Methods

(*a*) *Bacteriological technique*

To avoid any confusion due to natural infection we used a strain of *Bacillus subtilis* that produces a specific kind of ropiness different from the normal. This strain had been isolated from hay by Dr. D. D. Woods (Department of Biochemistry, Oxford University), and its specific identity was confirmed for us by Dr. T. Gibson (E. Scotland College of Agriculture).

Study of heat resistance in buffered suspensions.—We have been concerned with treatments short of complete sterilization, for which the normal technique is unsuitable. We have therefore compared the numbers of viable spores, before and after heating, by orthodox plating methods. This is basically the method of Williams, Merrill & Cameron,[13] but we heated the spores in the usual glass tubes described by Williams.[14]

Table I

The effect of starch on the proportions of heated Bacillus subtilis *spores that germinated in nutrient agar*

Time of heating at 100°, min.	Viable counts per ml. on replicate samples of heated suspensions plated on	
	(*a*) Nutrient agar	(*b*) Nutrient agar + 0·1% of soluble starch
0	$3{\cdot}0, 2{\cdot}0 \times 10^7$; $8{\cdot}5 \times 10^5$	$9{\cdot}3, 9{\cdot}1, 9{\cdot}2 \times 10^5$
15	$8{\cdot}5, 7{\cdot}2, 8{\cdot}0 \times 10^3$	$5{\cdot}9, 5{\cdot}2, 5{\cdot}5 \times 10^3$
30	59, 45, 55	77, 67, 105
45	24, 26, 23	27, 25, 26

As it is known that the heat resistance of spores depends on the conditions of their cultivation, etc.,[15] we followed a standard procedure. Cells were incubated at 37° for 5 days in nutrient digest broth with 0·3% of soluble starch. The cultures were then well shaken with sterile washed sand, and the cells centrifuged down and washed twice with sterile phosphate buffer of pH 6·5. After re-suspending in buffer, the suspension was shaken again for 5 min. at the top speed in a Mickle shaker, and then filtered through a layer of cotton wool in gauze. Viewed microscopically, the distribution of cells seemed satisfactorily uniform.

Finally, the suspension was given the heat treatment (30 min. at 80°) usual for killing vegetative cells. No further ' heat activation ' (see Evans & Curran[16]) was carried out, because our aim was not to get ideal heat-destruction curves, but to compare the effects of heating in buffer and in bread in which there is no preheating.

Samples (1·5 ml.) of suspension were placed in resistance-glass tubes (120 mm. × 7 mm., 1 mm. thick) containing two glass beads for stirring, and the tubes sealed and stored for not more than 5 days at 0°. There was no appreciable change in the numbers viable after the first day, nor was there any sign that the heat resistance of the spores changed during storage; this agrees with the experiences of Curran[17] and Williams.[18]

The tubes of spore suspension were heated by immersion in thermostatically controlled water baths at temperatures of 90 ± 0·5° and 100 ± 0·5°. Immersion of the tubes did not disturb the temperature appreciably and the suspension in the tubes attained the external temperature in 0·5 min. The coming-up time of 0·5 min. at 90° and 100° has been deducted from the actual times to give the values quoted in our results.

After being heated at 90° or 100° the tubes were transferred at once to ice-water for cooling. As soon as the tubes had been cooled, shaken and dried, they were scratched with a glass-cutter, flamed and opened. Four tubes were used at a time, and the contents of each were tipped into a small dry sterile bottle. From two of these 1·0-ml. samples were plated directly, and the remaining two were both diluted appropriately with quarter-strength Ringer solution. Plating was in triplicate on Macartney's tryptic digest (' nutrient ') agar, or on this agar with starch,[19] as indicated below. All plates were over-poured with 2% agar to discourage ' spreaders '.

The sometimes slow and uncertain germination of heated spores in artificial media is said to be improved by addition of starch.[20] Hence we made two experiments to determine whether starch was necessary, and what period of incubation was suitable. In the first, samples of a suspension, in which the number of survivors had been reduced in varying degrees up to 10^{-5} by heating at 100°, were plated on nutrient agar and on the same with 0·1% of starch; in the second, bread samples, in which the spores surviving baking were 10^{-3} or less of the initial count, were plated on the nutrient agar plus 0·1% of starch. There was no significant difference between counts with and without starch (cf. Table I, which tabulates the results for the first

experiment). In both experiments, the plates were incubated for 48 h. at 37° and counted and then re-incubated at 28·5° for a further three weeks. This second incubation was performed as the work of Williams & Reed[21] suggested that a slightly sub-normal temperature favours germination of heated spores. In no plate was there a significant difference between the first and second counts; hence in the rest of our experiments we incubated for 48 h. at 37°.

The spore suspensions used for inoculating the dough (see below) were prepared thus: The organisms were grown for five days on nutrient broth containing 0·3% of starch. Sterile sand was added and the whole shaken; after settling, the supernatant fluid was poured into centrifuge tubes. After being spun down, the organisms were washed with buffer (50 ml. of 0·2M-potassium dihydrogen phosphate solution + 5·64 ml. of 0·2M-sodium hydroxide solution made up to 200 ml. and autoclaved, pH = 6·5). They were again spun down, suspended in buffer, shaken in a Mickle shaker and filtered through sterile cotton wool in gauze to remove clumps. The suspension was then heated for 30 min. at 80° and stored at 0° until used. The spores were counted by plating, and diluted for use with sterile buffer.

(b) Baking tests

These tests, denoted (I), (II) and (III), were designed to give normal baking treatments; the first was in a commercial electrically heated oven, and the last two in domestic ovens. Details of these tests are given in Tables IV and VI.

Doughs were prepared in the usual manner to a recipe commonly used in test baking: 1000 g. of flour (National, 81% extraction, agene-treated), 18 g. of yeast and 14 g. of salt, with the requisite amount of water (about 550 ml.) to which 25 ml. of spore suspension had been added. After being mixed by hand, the dough was fermented for 3 h. at 28–30°, with a 'knock-back' after 2 h. It was then scaled at 16 oz. (454 g.), moulded and placed in standard 1-lb. tins, silicone-treated. Finally, it was 'proved,' for 40 min. at 28°.

After proving, pieces of dough were removed for bacteriological analysis, to check their bacterial content on going into the oven. The material withdrawn was divided into two lots of samples: one was plated directly to give the total of viable cells, and the other was plated after heating for 30 min. at 80° to enumerate still-ungerminated spores. (Sampling details are given in Table IV.)

The rest of the dough was then baked for 30 min., which is normal for a 1-lb. piece. The oven temperatures in the three tests were about 215°, 240° and 235° respectively. In each test (i) a loaf was wrapped, immediately after baking, in sterile greaseproof paper, placed in a can which was then closed, and incubated at 37°; (ii) temperatures were measured in a second loaf by means of thermocouples which were not disturbed when the loaf was removed from the oven until its temperature had fallen to 80°. The loaves were then tipped from the tins into sterile paper, in which they were taken to the laboratory for bacteriological sampling.

To take the samples, the loaf was opened with a sterile knife to give access to the thermocouple positions, from which samples were taken with sterile instruments and divided for total and spore counts (details are in Table VI). In test (III) the position of each thermocouple was marked by a trace of cochineal with which it had been painted before insertion so that, when the loaf was opened, the bacteriological samples could be taken as near as possible to the point where the temperature had actually been measured.

All the samples, of dough or bread, were weighed into quarter-strength Ringer solution, and disintegrated by shaking in a bottle with glass beads.

(c) Measurement of temperature in bread

Temperatures were measured with specially constructed thermocouples of chromel–alumel, a material of low heat conductivity, selected to minimize errors due to conduction of heat along the wires. These were about 30 cm. long, of 33-S.W.G. chromel or alumel wire, insulated with a special varnish prepared by adding two drops of Teepol to 50 ml. of Sterling's S110 high-temperature varnish. They were enclosed in a glass sheath 8 cm. long, except at the actual junction, with the object of giving rigidity for pushing the wire into the loaf. The couple was joined to about 6 ft. of 19-S.W.G. chromel–alumel lead and both leads were taken to a cold junction in ice. Copper leads were taken *via* suitable switches and resistances to a galvanometer. The resistance network ensured (*a*) a large swamping resistance in series with each couple (which had been adjusted to have a resistance of 10 ohms), (*b*) a suitable scale reading and (*c*) critical damping of the galvanometer.

The thermocouples were calibrated in the range of 80–110° against an N.P.L. standard mercury thermometer.

(3) Results

(*a*) *Heat resistance of spores in buffer solutions*

The preliminary experiments were intended to indicate a suitable technique and to decide whether special procedures would be necessary to overcome germination inhibitors in the media.

The treatment of the plating medium with starch in order to obtain full germination of heated *Bacillus* spores has already been briefly described. Charcoal has a similar effect but, as it must be removed from media if it is not to interfere with counting of colonies, starch was preferred. As stated above, starch had no effect, even on spores heated for as long as 60 min. at 100° ; thus although the sensitivity of different *Bacillus* strains to the germination inhibitors that starch antagonizes is known to vary greatly,[20] our strain was little affected by them. Nevertheless, the starch was retained in the subsequent experiments to make the conditions more comparable with those in bread.

The resistance to different temperatures was investigated, two experiments being performed at 90° (A) and 100° (B), the temperatures of the water baths in which replicate tubes of spore suspension were immersed. At intervals, three tubes were removed and their contents mixed, the mixture was diluted, and from the final dilutions three replicate samples were withdrawn to be plated in triplicate. Fig. 1, A and B, gives the results. Although at 90° the destruction of the spores proceeded more or less exponentially with time (the classical picture), at 100° there was a relatively enormous mortality during the first few minutes, after which the number of spores remaining viable changed comparatively little for periods up to 60 min.

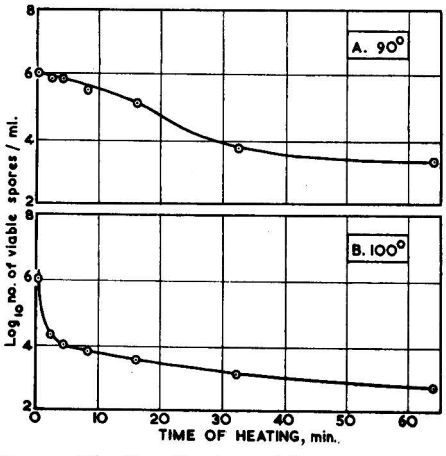

FIG. 1.—*The effect of heating at different temperatures in phosphate buffer, pH 6·5, on the survival of spores of* B. subtilis

Because the preliminary experiments had shown plainly that the heat resistances of suspensions prepared on different occasions might not be the same, it was essential in the baking tests to use the same suspension as had been used for the measurements of heat resistance in buffer [test (I)] or suspensions found on trial to have an equal resistance [tests (II) and (III)].

As in this work the accuracy of the data for 100° is of particular importance, the actual observations in buffer solution have been set out in full in Table II. The statistical analysis suggests that they are satisfactorily reliable.

(*b*) *Baking tests*

The procedures in these tests have already been described, in general terms, in section (2) (*b*) above.

In trying to follow the behaviour of spores through the baking process, a complication arose. After a dough has been infected and mixed (infection must be done first to ensure reasonable uniformity as well as to imitate actuality) the mix is kept under warm conditions for a considerable time during fermentation and proving. Since it seemed likely that many of the spores

might germinate during this treatment, we counted the spores and total viable cells in the doughs after proving. These counts are given in full in Table III, and summarized with other relevant data in Table IV.

The artificial infection was so large as to make negligible any natural infection likely to arise from the ingredients of the dough, and no natural infection was in fact observed. The kind of flour used contains only about 10 rope spores/g. (cf. also Barton-Wright[1]), and the yeast only about 2 spores/g. (much less than reported by Hoffman *et al.*[2]).

Table II

Actual plate counts, at various dilutions, in the heating of a suspension of Bacillus subtilis *spores at* 100°, *together with a statistical analysis of the results*

Time, min.	Actual plate counts									Statistical analysis			
										Mean count,* $\hat{m}$	Variance of $\hat{m}$	$\log_{10}\hat{m}$	Standard error (as $\log_{10}\hat{m}$)
	× 10^4			× 10^5									
0	126	142	148	15	10	12				149 × 10^4	$10^4\hat{m}/9{\cdot}9$	6·18	0·011
	183	165	160	24	20	18							
	161	137	132	13	12	16							
	× 10^3			× 10^4			× 10^5						
2	10	14	11	1	1	1	0	0	1	150 × 10^2	$10^3\hat{m}/9{\cdot}99$	4·18	0·035
	14	19	16	3	2	2	1	1	0				
	11	19	17	2	0	4	0	0	0				
	× 10^3			× 10^4			× 10^5						
4	7	7	9	3	1	1	0	0	1	91 × 10^2	$10^3\hat{m}/9{\cdot}99$	3·96	0·046
	6	11	7	3	0	1	0	0	0				
	10	10	9	1	3	1	0	0	0				
	× 10^2			× 10^3			× 10^4						
8	67	66	76	6	10	8	0	2	0	68 × 10^2	$10^2\hat{m}/9{\cdot}96$	3·83	0·017
	62	71	62	4	8	9	1	0	0				
	69	65	69	6	8	9	—	—	—				
	× 10^1			× 10^2			× 10^3						
16	390	418	380	41	43	49	5	3	2	39 × 10^2	$10\hat{m}/8{\cdot}99$	3·59	0·0073
	374	388	—	44	43	47	4	7	7				
	384	380	399	42	36	46	3	5	3				
	× 1			× 10^1			× 10^2						
32		—		124	152	135	15	11	14	13 × 10^2	$10\hat{m}/9{\cdot}9$	3·13	0·012
				125	139	137	11	12	15				
				134	141	128	14	14	17				
	× 1			× 10^1			× 10^2						
64		—		64	66	74	8	10	10	68 × 10^1	$10\hat{m}/9{\cdot}9$	2·83	0·017
				64	72	67	8	5	6				
				65	62	68	7	8	5				

* The best mean estimate (m_x) of the count, referred to the lowest dilution counted (10^{-x}), is calculated from all the counts by the formula

$$m_x = \frac{\Sigma c_x + \Sigma c_{x+1} + \Sigma c_{x+2}}{n_x + 10^{-1}n_{x+1} + 10^{-2}n_{x+2}}$$

where c_x and n_x are the counts and the number of plates at the lowest dilution, and c_{x+1} and n_{x+1} etc. refer to successively higher dilutions. The best estimate of the mean count in the initial suspension is then

$$\hat{m} = m_x \times 10^x$$

In test (I) (Table IV), out of $1{\cdot}06 \times 10^5$ spores present initially, only $3{\cdot}7 \times 10^4$ finally remained; hence $6{\cdot}9 \times 10^4$ must have germinated. The total viable cells, including spores, were $1{\cdot}3 \times 10^5$ finally; hence there must have been $9{\cdot}3 \times 10^4$ vegetative cells, i.e. 6·9 spores present initially were represented by 9·3 vegetative cells after fermentation and proving. In tests (II) and (III) similar relations were found although, in these tests, the differences may have been partly due to variation between samples of dough. Thus, besides the presence of vegetative cells, the number of spores entering the oven was always less than half of that in the original

Table III

Total viable cell and viable spore counts after fermentation and proving of dough infected during mixing with about 10^7 B. subtilis *spores per g.*

	Sample	$\log_{10}$ (subsequent dilution)	Test (I) Wt. of sample, g.	Test (I) Vol. of suspending fluid, ml.	Test (I) Plate counts	Test (II) Wt. of sample, g.	Test (II) Vol. of suspending fluid, ml.	Test (II) Plate counts	Test (III) Wt. of sample, g.	Test (III) Vol. of suspending fluid, ml.	Test (III) Plate counts
Total counts	A	0	2·0	38		1·0	19		1·41	19	
		− 2						86 107 108			(~500 × 8)
		− 3			4 2 4			15 14 19			420 398 445
		− 4			0 1 5						
		Average count			10×10^4			2×10^5			6×10^6
	B	0	2·3	38		1·0	19		1·3	19	
		− 2						136 126 125			(~400 × 8)
		− 3			12 5 6			12 26 16			310 326 338
		− 4			5 2 0						
		Average count			2×10^5			3×10^5			5×10^6
	C	0				1·0	19				
		− 2						102 88 86			
		− 3						13 21 20			
		Average count						2×10^5			
	D	0				1·0	19				
		− 2						126 115 120			
		− 3						36 19 22			
		Average count						3×10^5			
Spore counts	A	0	2·0	38		1·0	19		1·3	19	
		− 1						300 368 521			269 257 276
		− 2			16 21 17			39 45 49			25 22 36
		− 3			2 2 3						
		− 4			1 2 0						
		Average count			4×10^4			8×10^4			4×10^5
	B	0	2·3	38		1·0	19		1·34	19	
		− 1			193 198 —			321 359 415			270 266 270
		− 2			26 25 28			48 49 57			30 22 29
		− 3			3 3 2						
		− 4			1 0 2						
		Average count			4×10^4			8×10^4			4×10^5
	C	0				1·0	19				
		− 1						308 447 459			
		− 2						56 46 38			
		Average count						8×10^4			
	D	0				1·25	9				
		− 1						793 432 721			
		− 2						117 99 110			
		Average count						6×10^4			

dough, the proportions indicated as remaining being 0·35, 0·46 and 0·28 in the three tests respectively. This means that spore survivals as a result of oven heating must be calculated on the reduced number remaining in the dough after proving, i.e. before heating, and, further, that the question of the survival of vegetative cells has to be considered.

Moreover, in computing the cell content of a sample after baking, allowance must be made for the appreciable loss in weight during baking. Accordingly the proportions surviving after

Table IV

Germination of Bacillus *spores in bread dough as a result of fermentation and proving (total time 3 h. 50 min. at 28–30°)*

Notes

(*a*) The number of spores added is calculated from the proportion of suspension included in mixing the dough, taking no account of the weight loss during fermentation and proving, which is negligible here

(*b*) The procedure, in preparing the dough samples, was somewhat different in the three tests: (I) Two samples were prepared, aggregate weight about 2 g. each, composed of some half-dozen pieces from randomly selected points in the dough. The suspensions made from these samples were first used for the total viable-cell count, then heated at 80° for 30 min. to get the viable-spore count. (II) Four 1-g. samples were taken at random from each of two pieces of dough; two from either piece were used to prepare a suspension for total counts, and the remainder for spore counts. (III) Four 1-g. samples were from a single piece of dough; two were used for total and two for spore counts

Test	Number of viable cells, per g. (I)	(II)	(III)
Initial spore suspension	6·4, 6·8, 6·4, 7·1 average $6·7 \times 10^6$	1·04, 1·00, 1·02 average $1·02 \times 10^7$	8·2, 11·3, 8·3 average $9·27 \times 10^7$
Calculated no. of spores added	$1·06 \times 10^5$	$1·60 \times 10^5$	$1·48 \times 10^6$
Total viable cells	0·97, 1·60 average $1·28 \times 10^5$	2·1, 2·7, 2·0, 2·7 average $2·4 \times 10^5$	5·8, 5·0 average $5·4 \times 10^6$
Viable spores	3·8, 3·6 average $3·7 \times 10^4$	8·0, 7·6, 8·2, 5·6 average $7·35 \times 10^4$	4·2, 4·1 average $4·15 \times 10^5$

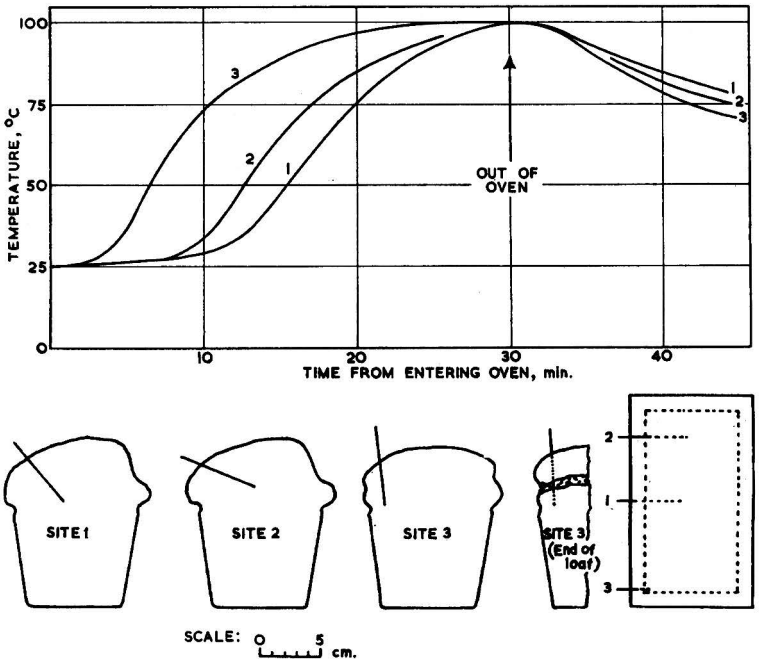

FIG. 2.—*Heating and cooling curves at different positions in the loaf; test (I), oven temperature 215–217°*

baking, presented below, are calculated on an initial number of spores adjusted by multiplying by 8/7, since 16 oz. (454 g.) of dough yield 14 oz. (397 g.) of bread. This correction is only approximate because, although it is easy to get an average for the whole loaf, more moisture must obviously be lost from the crust than from the middle, to an extent that cannot be precisely determined. This means that our calculated proportions of survivors will be over-estimates near the crust and under-estimates in the centre, but these errors are insignificant in comparison with the changes observed.

Table V

Total viable cell and viable spore counts in bread after baking of dough infected during mixing with about 10^7 *spores of* B. subtilis *per g.*

	Sample	$\log_{10}$ (subsequent dilution)	Test (I) Wt. of sample, g.	Vol. of suspending fluid, ml.	Plate counts	Test (II) Wt. of sample, g.	Vol. of suspending fluid, ml.	Plate counts	Test (III) Wt. of sample, g.	Vol. of suspending fluid, ml.	Plate counts
Total counts	Position (1)	0	1·35	38		1·0	19	16 19 —	0·68	19	15 17 —
		− 1			3 3 8			9 3 9			2 0 3
		− 2			2 1 3			3 3 2			
		− 3			0 0 1						
		Average count			2×10^3			5×10^2			5×10^2
	Position (2)	0	1·8	38		1·0	19	4 — 1	0·6	19	1 1 1
		− 1			4 3 4			1 — 0			0 0 0
		− 2			0 0 0			1 0 —			
		− 3			0 3 0						
		Average count			9×10^2			6×10			3×10
	Position (3)	0	0·4	19		1·0	19	2 1 —	0·66	19	2 0 1
		− 1			3 2 0			0 0 —			0 0 0
		− 2			2 0 1						
		− 3			1 0 2						
		Average count			2×10^3			3×10			3×10
Spore counts	Position (1)	0	1·35	38		1·0	19	8 7 10	0·95	19	12 11 8
		− 1			1 3 0			2 2 2			2 0 1
		− 2			0 0 0			1 0 0			
		Average count			4×10^2			2×10^2			2×10^2
		repeat 0				1·0	19	22 5 4			
		− 1						8 8 5			
		− 2						16 4 8			
		Average count						5×10^2			
	Position (2)	0	1·8	38		1·0	19	4 5 7	1·83	19	1 1 0
		− 1			1 1 3			2 0 0			0 0 0
		− 2			0 0 0			0 0 0			
		− 3			0 2 0						
		Average count			5×10^2			1×10^2			7
		repeat 0				1·0	19	10 7 15			
		− 1						2 1 1			
		− 2						4 2 2			
		Average count						3×10^2			
	Position (3)	0	0·4	19	3 3 3	1·0	19	3 0 0	1·1	19	1 1 1
		− 1			0 0 0			— — —			0 0 0
		− 2			0 0 0						
		− 3			0 2 0						
		Average count			2×10^2			2×10			2×10
		repeat 0				1·0	19	1 1 0			
		− 1						1 0 0			
		Average count						2×10			

In estimating survivals we concentrated on three positions, intended to represent a range of conditions within the loaf: in the centre, near the crust, and at a point midway between the two (see Fig. 2). Thermocouples were inserted there, and samples withdrawn at the same points as already described for total and spore counts.

The counts on the bread samples, after baking, are given in Table V, and reviewed in Table VI. The colonies of our strain of *B. subtilis*, all under agar, had a characteristic appearance and no other type appeared on the plates; so it is reasonably certain that no significant

Table VI

The total viable cell and viable spore counts per g. of bread, after baking dough infected with about 10^7 *spores of* B. subtilis *per g.*

Note

The taking of samples for the total and spore counts was different in the three tests:

(I) A composite sample of bread from near the thermocouple was homogenized, the suspension first used for the total count, and then heated 30 min. at 80° to obtain the spore count

(II) Three samples were taken at each position, and homogenized separately, one suspension being used for total and two for spore counts

(III) Two samples were taken at each position, one for total and one for spore counts

Position	Count	Test (I)	Test (II)	Test (III)
1	Total	2000	500	500
	Spore	400	200, 500	200
2	Total	900	60	30
	Spore	500	100, 300	7
3	Total	2000	30	30
	Spore	200	20, 20	20

proportion of the colonies represented here were those of contaminants accidentally introduced into the bread. The numbers of bacteria recovered were small and agreement between dilutions was not good, perhaps because the bread samples could not be perfectly homogenized. Some of the plates, too, were spoiled by ' spreaders ', despite the over-pouring with agar; hence the resulting counts can be regarded as only approximate. Nevertheless when the spore samples in test (II) were examined in duplicate, the counts agreed quite well, there being no statistical difference between the three pairs. This encouraged us to draw some tentative conclusions.

The total, and spore, counts deduced from the observations in Table V are collected in Table VI. Although at first sight it would seem that there were more total viable cells than spores, the difference is not statistically significant (P about 0·15). Hence there is no good evidence that any vegetative cells survived the baking, and it is legitimate to combine the total and spore counts, regarding them all as spores. On this basis there was, statistically, a significantly greater kill at position 3 than at positions 1 and 2, as would be expected from the temperature curves presented in Fig. 2, which show that position 3 had much the highest average temperature. There was, in addition, a generally smaller destruction of spores in test (I); this is significantly different from destructions in tests (II) and (III) which do not differ significantly. It seems likely that the lower temperature of treatment in test (I) accounts for this difference.

To compare the observed destruction with that to be expected from the heat treatment, we must first calculate the total effect of the temperature cycle, which can be done only approximately. We assume that, for periods of the order in question, temperatures below 85° can be neglected and that, above this, the temperature curves of Fig. 2 can be regarded as stepped at 90° and 100°. This leads (Table VII B) to a series of times at these temperatures, which strictly relate only to test (I); the temperatures in tests (II) and (III) are not known with such precision, but they followed a similar pattern. From the time at each particular temperature, the proportions of spores destroyed can be calculated from Fig. 1, and the geometric sum of these indicates roughly the proportion of spores destroyed during the whole heating cycle (Table VII B). The values so calculated are compared with the observed values (Table VII, A and C).

If the survivals are considered as a whole, they agree tolerably well with those predicted by calculation. The actual results and the basis of the above calculation are too rough to justify closer comparisons. We conclude, at least, that the resistance of spores to heat is no less in bread than it is in buffer.

We have already mentioned that duplicates of the loaves used for bacteriological analysis were incubated to determine whether the surviving spores would develop. They did, producing the typical form of rope seen in Fig. 3, with a characteristic smell and only a moderate degree of ropiness.

Fig. 3 confirms that in the surface layer of the loaves the temperature treatment sufficed to destroy all the spores, but that elsewhere, and especially in the centre, some remained.

But a discrepancy is also indicated in Fig. 3A. Our bacterial counts revealed the presence of viable spores throughout the crumb in numbers of the order 10–100/g., whereas the foci from which rope-formation started were of the order of only 1/10 g.—a roughly 100-fold difference. This might conceivably have arisen from the following experimental causes although, in fact, none of them seems likely for the reasons given: (i) errors in the spore counts: these are

Table VII

Comparison of the number of spores of B. subtilis *surviving heat treatment in the oven with those expected from experiments in buffer*

A The observed proportions of spores surviving the oven treatments

Test	(I)	(II)	(III)
Initial numbers*/g. (throughout)	$4{\cdot}2 \times 10^{4}$	$8{\cdot}4 \times 10^{4}$	$4{\cdot}7 \times 10^{5}$
Finally at position			
1. Average no./g.†	1200	400	350
Proportion surviving	$2{\cdot}8 \times 10^{-2}$	$4{\cdot}8 \times 10^{-3}$	$7{\cdot}5 \times 10^{-4}$
$\log_{10}$ (proportion surviving)	$\bar{2}{\cdot}45$	$\bar{3}{\cdot}68$	$\bar{4}{\cdot}88$
2. Average no./g.†	700	150	18
Proportion surviving	$1{\cdot}7 \times 10^{-2}$	$1{\cdot}8 \times 10^{-3}$	$3{\cdot}8 \times 10^{-5}$
$\log_{10}$ (proportion surviving)	$\bar{2}{\cdot}23$	$\bar{2}{\cdot}26$	$\bar{5}{\cdot}58$
3. Average no./g.†	1100	23	25
Proportion surviving	$2{\cdot}6 \times 10^{-2}$	$2{\cdot}7 \times 10^{-4}$	$5{\cdot}3 \times 10^{-5}$
$\log_{10}$ (proportion surviving)	$\bar{2}{\cdot}42$	$\bar{4}{\cdot}43$	$\bar{5}{\cdot}73$

* From Table IV, corrected for weight lost in baking
† From Table VI

B Approximate integration of the time–temperature treatment and consequent expected spore kills (as change in $\log_{10}$ of numbers, from Fig. 1)

Position	85–90° (90°)		>95° (= 100°)		95–85° (90°)		$\log_{10}$ (total kill)
	Minutes	log decrement	Minutes	log decrement	Minutes	log decrement	
1	3·5	0·08	8	2·20	6	0·16	2·44
2	4	0·10	9·5	2·25	5	0·10	2·45
3	4·5	0·12	16	2·50	3·5	0·02	2·64

C The observed (Table VII A) and calculated (VII B) proportions of spores destroyed (expressed as log decrements to base 10)

Position	Observed in test				Calculated
	(I)	(II)	(III)	Average	
1	1·55	2·32	3·12	2·33	2·44
2	1·77	1·74	4·42	2·64	2·45
3	1·58	3·57	4·27	3·14	2·64

admittedly not accurate, but we cannot believe that they were all 100 times too high ; (ii) unevenness in mixing the spores in the dough : this is inconsistent with the uniformity of the bread as well as with the general agreement between counts on comparable samples of dough and bread ; (iii) large differences in infection between duplicate loaves : this seems improbable, partly for the reasons under (ii) and partly because all the tests indicated a similar discrepancy ; (iv) the rope might have arisen entirely from natural infection, the artificial infection not developing : against this there were the peculiar characteristics of the ropiness ; also we made isolations from ropy areas and found their cultural and biochemical properties to be identical with those of the original strain. We conclude, consequently, that the discrepancy was real, and that for some reason a high proportion of the viable spores present did not generate rope in the bread within the period of our observations.

The results suggest that baking reduces the survival of *Bacillus* spores by a factor of about 10^{-4} ; hence if, as seems probable, the heat resistance of rope spores is about the same as that of the spores used, an initial infection from flour of the order of 10/g. might lead to the survival of a focus for development in about 1 loaf (weight 1¾ lb.) in 2. However, the possibility of subsequent development is apparently of the order of 100 times less, i.e. rope would not be found in more than 1 in 200 loaves if conditions were favourable for it to develop. This gives an indication of the extent of the risk and why within normal periods of keeping it appears sporadic, but serves to emphasize the need for special precautions, e.g. by using acid doughs, once a substantial infection has occurred.

(c) *Temperatures during baking of different breads*

To show that Fig. 2 represents normal behaviour, thus giving wider validity to the conclusions, many observations on a variety of baked products have been summarized in Table VIII and are discussed below.

(4) Discussion

It is usually assumed (cf. Rahn,[22] Stumbo[23]) that the destruction of bacterial spores at a particular temperature is always exponential with time. This was plainly far from true in our

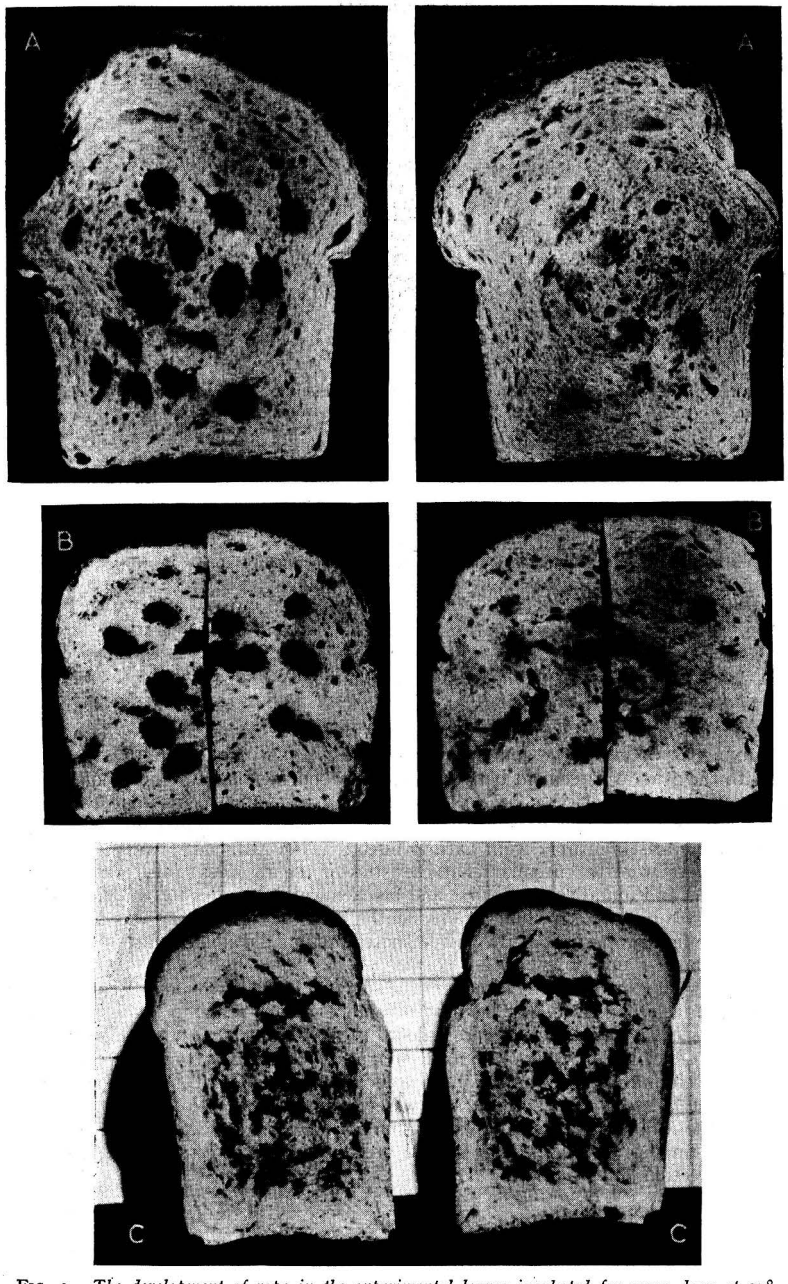

FIG. 3.—*The development of rope in the experimental loaves incubated for seven days at 37°, in a sealed tin to prevent drying out*

A and B: Loaves from test (I), A cut transversely, B cut longitudinally, the pairs of faces represented being mirror images; on the left, the ropy areas have been stained with very weak iodine solution to emphasize their distribution

C: A loaf from test (III), cut transversely, no treatment; the large black area one-quarter of the way down from the top crust is a crack, present when the loaf was opened, but accentuated by drying out before the photograph was taken

experiments at 100°, where an unduly large proportion of the spores was killed during the first few minutes, and the small number remaining declined rather slowly. Such behaviour is important in experiments like these because, unless rather large numbers of spores are present initially, the final numbers rapidly become too small to be enumerated properly by means of normal counting techniques. The thermal death/time curves are thus markedly convex downwards and, though such curves were in fact obtained by earlier workers,[24, 25] it has only recently been generally recognized that they occur often.[26, 28] Reed *et al.*[27] thought that such curves (obtained by them with anaerobes) were due to the presence of a proportion of spores less susceptible to heat.

If this is so, our results suggest the possibility of a troublesome consequence of heat activation, which is sometimes effected at temperatures not much below the temperature at which the heat resistance is measured. The preliminary heating, for activation, is then equivalent to an appreciable period at the experimental temperature, and hence might destroy some of the spores more susceptible at that temperature, leaving a relatively uniform population more likely to give an exponential decline, i.e. it would abolish the head of the curve. This effect would plainly increase greatly if the temperature used for activation were too near the experimental temperature; and it may reasonably be supposed that our treatment for removing vegetative cells (30 min. at 80°) sufficed to induce it considerably at 90°, but less at 100° and still less at higher temperatures. Apparently exponential thermal death/time curves could be spuriously produced in this way by appropriate heat-activation treatments.

Our observations suggest that, despite the high oven temperatures during baking, the temperature in the moist crumb of bread does not usually rise above 101°, although in rolls that are baked to a lower moisture content the maximum values may be slightly higher (Table VIII). Stout & Drosten,[29] too, state that at no time does the temperature exceed 101°, and Aubertin *et al.*[3] report temperatures of 100–101°. The temperature within the moist crumb is apparently limited because the cooling due to evaporation is sufficient to keep pace with the slow transfer of heat from outside, for 101° corresponds roughly with the boiling point, indicated from other properties of the bread (about 4% of sodium chloride in the aqueous phase after baking, and an equilibrium relative humidity about 96% at normal temperatures), the internal atmospheric pressures due to structural strength being negligible (about 2·5 mm. according to Baker & Mize[30]). Bailey & Munz[31] recorded maximum temperatures of only 90–95°, in positions far from central in the loaf, which was ascribed by Baker & Mize[30] to evaporation of alcohol. No doubt there are substantially higher temperatures in parts of the loaf that become dry during baking (Roussel[10] gives crust temperatures of 125–140°) and in drier products such as biscuits (see Table VIII).

Table VIII

Internal temperatures during baking of various breads and related products

Type of baked product	Baking time, min.	Time to reach 80° at centre, min.	Centre temp., °c	
			At half baking time	At end of baking time
1-lb. loaf of bread	30	15	78	101
1-lb. loaf of bread	30	19	65	97
2-lb. loaf of bread	35	22	59	99
2-oz. bread roll	20	8	98	102
Fermented buns	9	5	72	103
Swiss roll	9	3	94	111
Sponge	18	6	97	101
Madeira cake	55	35	53	102
Scone	22	6	97	102
Rock cake	17	10	70	98
Open jam puff	15	2	103	111
Short-pastry jam tart	15	6	94	108
Shortbread biscuit	18	3	114	136

According to our observations, temperatures near 100° may be barely attained in the middle of a normally baked loaf, and from our results it is evident that failure to reach them, even by a few degrees, must greatly increase the proportion of spores surviving; such conditions would obviously not be expected to exterminate bacterial spores if present in appreciable numbers. In the crust, where higher temperatures obtain for longer times, sterility might be expected under all practical conditions. This tallies, not only with the pattern in which infection developed in our incubated loaves (cf. Fig. 3), but with general experience of ropiness in bread.

Consideration of our heat-destruction curves shows how much more critical the initial number of cells is with an organism behaving in the manner described above than with one in

which the survival/time curve is exponential. It is easy to kill a few cells, since at 100° the initial decimal reduction time is about 1 min.; but for the complete extermination of a large number the decimal reduction time becomes very long (about an hour in our experiments), and an infection of 10^6 spores/g. might not be eliminated by any practical baking treatment. (Similar results were obtained by Bushnell,[25] decimal reduction times at 98° increasing from about 5 min. initially to 15 min. finally with *B. subtilis*, and from 5 to 25 min. with *B. mesentericus*.) In any event, the number of surviving spores is likely to be small and to depend greatly on the sampling position, which may explain occasions reported in the literature where no viable spores were recovered after baking.

The apparent inability of most of the spores remaining viable to develop promptly in bread has not been remarked hitherto. It may be related to the relatively large numbers used in our experiments. There seems no obvious reason why a high proportion of the spores that can develop on starch agar should not do so in bread, and one suspects that their failure to produce ropy spots (even if they germinate) may be due either to slight inequalities in moisture content, which is known to be near the critical level,[32] or to changes consequent on the development of the first ropy patches, e.g. the withdrawal of water into regions where starch is being hydrolysed. On either view, one must suppose that some of the water is 'labile' (i.e. can move freely) in the system known as bread.

The temperatures we observed in baking our bread would, on the other hand, be expected to kill vegetative cells, and there was statistically insufficient evidence to show that any survived, although this still remains a possibility at a level of significance of 1 in 7. Our experiments must be regarded as inconclusive on this point, but show that to prove it with sporing organisms requires a much more critical examination than any yet given.

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