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# PROCEDURES FOR THE EXTRACTION, SEPARATION AND ESTIMATION OF THE MAJOR FAT-SOLUBLE PIGMENTS OF HAY

By J. DAVIDSON

New methods are described for the extraction, chromatographic separation and spectrophotometric identification and estimation of chlorophyll-*a*, chlorophyll-*b*, phaeophytin-*a*, phaeophytin-*b*, carotene and xanthophyll in hay. The most suitable solvent for extraction was 85% acetone. The chlorophylls were separated chromatographically from the phaeophytins, carotene and xanthophyll on a sucrose–sodium sulphate mixture. The chlorophyll-*a* and chlorophyll-*b* were estimated simultaneously in the green solution, and phaeophytin-*a* and phaeophytin-*b* were estimated simultaneously in the yellow solution, by spectrophotometry. Carotene and, subsequently, xanthophyll were separated chromatographically from the yellow solution on magnesium oxide, and were estimated separately.

## Introduction

The chief fat-soluble pigments of dried plant material are chlorophyll-*a* and chlorophyll-*b*, the chlorophyll-degradation products phaeophytin-*a* and phaeophytin-*b*, and carotene and xanthophyll.

Although methods are described in the literature for the estimation of chlorophylls in fresh herbage,[1, 2] and of carotene and xanthophyll, no procedures have been described for the estimation of chlorophylls, carotene and xanthophyll in dried herbage, which contains phaeophytins and other degradation products.

The following procedures were developed for the extraction and separation of the chief fat-soluble pigments of hay, and were designed for small quantities of dry matter of the order 0·5–1·0 g., because in an investigation, to be described in a later paper, of the distribution of organic pigments in the rumen contents of sheep these quantities of dry matter were obtained from sedimented fractions separated from the contents of the sheep's rumen.

## Experimental

### *Extraction*

Hay samples, ground to pass through a sieve having circular holes of 0·4 mm. diameter, were used for developing the extraction techniques.

Preliminary trials showed that mechanical blending under solvent was more efficient than hand grinding with quartz under solvent in a mortar and pestle. Although in the first experiments pigment extraction was carried out in the micro-cup of an 'Ato-Mix' blender, this assembly was not entirely satisfactory because a bag of freezing mixture had to be held round the base of the cup during blending, to prevent the temperature of the extract rising above 30°. A 'Nelco 10' homogenizer with overhead drive, used later, gave equally efficient extraction without complications arising from heating effects.

Since the pigments were more difficult to extract from some hay samples than from others, it was decided to develop a pretreatment that would render the test material more susceptible to solvent action. Chemical methods of rupturing the cell walls had to be avoided because of the labile nature of the pigments, and so a physical method was sought.

Schertz & Van Sant[3] extracted chloroplast pigments from fresh vegetable material after freezing at '— 10° C or lower', and thawing. This type of treatment, which involved the rupture of cell walls, was used in the present study. As will be shown later, in Table IV, moistening the ground hay with water, quick-freezing and thawing, led to over 99% extraction of the fat-soluble pigments in all hay samples.

*Choice of solvent*

Many methods have been described for the extraction of pigments from both fresh and dried plant material, but most of these have been developed with a view to isolating one pigment or group of pigments only.

For the isolation of carotene, light petroleum,[4] mixtures of acetone and light petroleum,[5, 6] and ethanol[7] have been used.

For the isolation of chlorophylls, aqueous acetone[1, 2, 8–11] has been used, and for extraction of all pigments both aqueous acetone[12–14] and aqueous ethanol[13] have been used.

The choice for present purposes appeared to lie between aqueous acetone and a mixture of equal parts by volume of acetone and light petroleum, b.p. 40–60°.

**Table I**

*A comparison of the extractive powers of 85% aqueous acetone and 50% acetone in light petroleum (b.p. 40–60°)*

| Solvent | No. | Optical density of 100 ml. of extract 1-cm. light path 660 mμ | 450 mμ | 410 mμ |
|---|---|---|---|---|
| 85% aqueous acetone | 1 | 0·356 | 0·471 | 0·651 |
| | 2 | 0·335 | 0·444 | 0·615 |
| 50% acetone in light petroleum (b.p. 40–60°) | 3 | 0·063 | 0·093 | 0·124 |
| | 4 | 0·063 | 0·093 | 0·123 |

A comparison was therefore made of the extractive powers of these two solvents. The results in Table I show that the amounts of pigment extracted by 85% acetone were much higher than the amounts extracted by the mixture of acetone and light petroleum. Aqueous acetone was therefore chosen for the extraction of the pigments.

*Precautions*

Many workers have emphasized the need for taking precautions during the extraction of pigments to prevent chemical changes due to light, oxygen, heat, acid[8, 9, 15, 16] and enzymes.[17]

Accordingly all operations were carried out in dim light. Blending and all operations at reduced pressure were carried out under nitrogen to minimize oxidative changes. The temperature was kept below 30°. Magnesium carbonate[9] was added before pigment extraction to neutralize any plant acids that might be present and cause chlorophyll degradation. Immediately after extraction, the pigments were transferred from acetone solution to a less polar solvent to minimize changes due to enzyme action.[8, 17]

*Procedure adopted*

Weigh into small metal moisture dishes sufficient test material to contain 0·5–1·0 g. of dry matter. At the same time weigh out test material for dry-matter estimation. Add water to the test material to form a thin paste containing 7–8 ml. of water. Float the moisture dish containing the thin paste on an acetone/solid carbon dioxide freezing mixture at approx. − 80°, prepared by dropping crushed solid carbon dioxide into acetone until effervescence ceases. When freezing is complete remove the dish and allow the contents to thaw. Freeze the melted sludge again quickly, then allow it to thaw.

Transfer the sludge quantitatively with 43 ml. of acetone to the cup of a high-speed blender containing 0·2 g. of magnesium carbonate. Blend at full speed for four 1½-minute periods, washing down the solid particles on the sides of the cup with 10-ml. quantities of 85% acetone between each period. After this total of 6 minutes' blending transfer the contents quantitatively through a filter funnel into two 50-ml. centrifuge tubes.

Centrifuge for 5 minutes at 3500 r.p.m. On the centrifuge used the relative centrifugal force developed was 2500 ***g***. Filter the supernatant liquid through Whatman No. 40 filter paper into a 250-ml. graduated cylinder. Keep the filter funnel covered with a watch-glass during filtration to reduce the evaporation and concentration effect at the upper edge of the filter paper.

Transfer the residues back to the blender cup with 50 ml. of 85% aqueous acetone, and extract as before for two 1½-minute periods. Again centrifuge the mixture and filter the supernatant liquid into the 250-ml. cylinder.

Wash and centrifuge the residue three times with 20 ml. of solvent. Filter each supernatant liquid into the 250-ml. cylinder. The last wash should be colourless. Wash the filter

paper at least twice, cut off the outside strip of filter paper, which may be light green in colour, place it in the bottom of the filter funnel and wash it thoroughly. Make the combined extract and washings up to a convenient volume with solvent. Transfer the extracted residues to a Soxhlet thimble, allow to dry overnight at room temperature, and re-extract the residue with diethyl ether for 8 hours in a Soxhlet continuous-extraction apparatus from which light has been excluded by means of a black cloth. Reduce this diethyl ether extract to small volume, transfer to a 25-ml. graduated cylinder, and make up to a convenient volume. The colour value of this extract is used to assess the completeness of the acetone extraction.

*Chromatographic separation*

In the following chromatographic procedure chlorophyll-*a* and -*b* are separated from the other pigments comprising phaeophytin-*a* and -*b*, carotene and xanthophyll. Spectrophotometric measurements are made on the resultant green and yellow solutions, at the peaks in the red region of the spectrum, of chlorophyll-*a* and -*b* and phaeophytin-*a* and -*b* respectively. Carotene and xanthophyll are then separated from the yellow solution and estimated separately. It is realized that the separated carotene fraction may contain small amounts of carotene other than $\beta$-carotene, and that the xanthophyll fraction may contain small amounts of other carotenols or xanthophyll epoxide,[18] but for the writer's purpose further separation of pigments was unnecessary.

*The separation of chlorophyll on sucrose–sodium sulphate*

In chromatography, the green chlorophylls show strong adsorption characteristics owing to the complex system of double bonds in the molecule and the covalent magnesium ion.[19] A weak adsorbent has therefore to be used for separations if the chlorophylls have to be eluted afterwards, and sucrose has been widely used for this purpose. In the present work it was found that a 50% mixture by weight of sucrose and anhydrous sodium sulphate had the advantage that traces of moisture on the glassware and in the reagents did not affect the separation.

For the separation of chlorophylls from the other pigments on a weak adsorbent such as sucrose the colouring matter must be dissolved in a non-polar or only slightly polar solvent. Separation is then carried out with solvent mixtures of increasing polarity.

Owing to the low solubility of chlorophylls in light petroleum the pigments could not be transferred directly from the aqueous acetone extract to light petroleum without incurring serious losses during the removal of the acetone by water washing. The pigments were therefore transferred to diethyl ether, in which they are completely soluble. The solvent from a measured portion of this diethyl ether solution was removed by evaporation at a low temperature under reduced pressure in an atmosphere of nitrogen, and the residue transferred, with the aid of light petroleum (b.p. 40–60°) for the subsequent chromatographic separation on sucrose–sodium sulphate. Any traces of pigment remaining undissolved after the vacuum flask was rinsed with light petroleum were transferred by rinsing the flask with the mixed solvent used for subsequent development of the chromatogram.

Preliminary experiments showed that better separations of chlorophyll from the other pigments were obtained with diethyl ether/light petroleum (b.p. 40–60°) than with acetone/light petroleum mixtures, and a pressure attachment similar to that described by Williams[20] was the most convenient way of accelerating development.

*Separation of the phaeophytins and carotenoids on magnesium oxide*

Recently Zscheile *et al.*[21] have observed that in diethyl ether solution carotenols, but not carotenes, are adsorbed on magnesium oxide. Ethanol/diethyl ether solution eluted the xanthophylls. In the present study, magnesium oxide was found suitable for the separation of carotene and xanthophyll from the phaeophytins.

From diethyl ether solution the xanthophyll and phaeophytins were adsorbed on magnesium oxide while the carotene passed through the column. A 2% solution of ethanol in diethyl ether eluted the xanthophyll. The phaeophytins were strongly adsorbed at the top of the column and could not be eluted with 50% ethanol in diethyl ether.

*Procedure adopted*

Pour distilled water, saturated with magnesium carbonate, into a 500-ml. conical separating funnel fitted with an **S** tube, as described by Booth.[22] Run off the water until the **S** tube is full of water and the separating funnel is empty. Close the tap and add 50 ml. of diethyl ether to the funnel, followed by a suitable measured volume of the pigment extract in acetone

(usually between 100 and 200 ml.). Wash down the neck of the funnel with a further 70 ml. of diethyl ether. To prevent emulsification at the interface introduce slowly about 200 ml. of distilled water through the **S** tube from an inverted wash-bottle. Close the tap, disconnect the wash-bottle, open the tap wide and allow water from a reservoir to drop through the ether layer at approximately 120 drops per minute. After the passage of 2–3 litres of water, separate the ether layer and run it through a 1 cm. × 15 cm. column of anhydrous sodium sulphate into a glass stoppered graduated cylinder. Wash the column with diethyl ether and make up the solution to a known volume (usually 100 ml.).

Pipette a volume of this solution, sufficient to give accurate optical density readings on the separated pigment solutions, into a 150-ml. round-bottomed flask, and evaporate it under reduced pressure in an atmosphere of nitrogen, with constant swirling of the flask, on a water bath at $< 30°$. When the solution is evaporated almost to dryness, remove the flask from the water bath, dry and disconnect. Wash the end of the nitrogen leak tube with a few ml. of diethyl ether and blow off the diethyl ether by a stream of nitrogen. Dissolve the residue in the flask in approximately 10 ml. of light petroleum (b.p. 40–60°). The solution is now ready for the separation of pigments on the sucrose–sodium sulphate mixture.

Prepare a well-packed 1 cm. × 12 cm. column of an equal mixture by weight of anhydrous sodium sulphate and sucrose. This mixture should consist of particle sizes that pass through 50 mesh but are retained on a 200-mesh standard test sieve. The mixture should have been dried for four hours at 90° under a reduced pressure of 50–100 mm. Place a 1-cm. depth of anhydrous sodium sulphate on top of the column to prevent the solvent used in developing from disturbing the sucrose mixture and affecting the chromatogram. Run light petroleum through the column under pressure; this results in rapid expulsion of air bubbles and also compression of the column so that it does not shrink during subsequent use.

When the solvent level has dropped to within 5 mm. of the top of the column of adsorbent, pour and wash in the pigment mixture with 5–10 ml. of light petroleum. Develop the chromatogram with 7·5% and then 15% of diethyl ether in light petroleum and observe the downward progress of the green chlorophyll band behind the yellow and grey carotenoid and phaeophytin bands, which are collected as they are eluted. By the time the bottom of the green band is about 3 cm. from the bottom of the column the eluate should be colourless. Now elute all the chlorophylls with 20 ml. of 50% diethyl ether in light petroleum. Transfer the combined yellow eluates and the combined green eluates quantitatively to 150-ml. round-bottomed flasks, evaporate almost to dryness under reduced pressure in an atmosphere of nitrogen, dissolve in a little diethyl ether and transfer quantitatively to 10-ml. or other suitable glass stoppered graduated flasks. After spectrophotometric measurements the green solution can be discarded, but the yellow solution can be further separated into carotene, xanthophyll, and phaeophytins; the phaeophytins remain on the column.

Prepare a well-packed 1 cm. × 10 cm. column of magnesium oxide and cover with a 1-cm. layer of sodium sulphate. Run light petroleum through the column under pressure; when the solvent level is about 5 mm. above the magnesium oxide column pour on the yellow pigment solution and give three washes, of approximately 10 ml. each, with diethyl ether. Collect fractions of approximately 10 ml. until the eluate is colourless, denoting that all carotene has been eluted. Combine the yellow carotene eluates. Now elute the yellow xanthophyll with three 10-ml. washes of 2% ethanol in diethyl ether. The final eluate should be colourless. The phaeophytins remain on the column. Transfer the carotene eluate and the xanthophyll eluate quantitatively to 150-ml. round-bottomed flasks, evaporate almost to dryness under reduced pressure in an atmosphere of nitrogen, and blow off the last traces of solvent under nitrogen. Dissolve the residues in spectroscopically pure *n*-hexane, then transfer the solution quantitatively to 5- or 10-ml. graduated flasks and make up to the mark. Spectrophotometric measurements are then made.

### *The quantitative estimation of the separated pigments*

By separating the pigments as described, quantitative estimates can be made by spectrophotometry of the six chief pigments. Chlorophyll-*a* and -*b* can be estimated in the green solution by taking measurements in the red region of the spectrum. Phaeophytin-*a* and -*b* can be estimated in the yellow solution, which also contains carotene and xanthophyll, if measurements are taken in the red region, in which the carotenoids show no absorption. Both carotene and xanthophyll can be estimated individually after the separation on magnesium oxide.

Table II shows the absorption coefficients used in the present study. Chlorophylls and phaeophytins have such sharply defined peaks that small alterations in the conditions of

**Table II**

*Absorption coefficients used in the quantitative estimation of pigments*

| | Wavelength, mμ | Pigment and specific absorption coefficients, α | | Source |
|---|---|---|---|---|
| | | Carotene in hexane | | |
| | 450 | 257·7 | | Zscheile *et al.*[21] |
| | | Xanthophyll in hexane | | |
| | 444 | 398 | | Computed from Karrer & Würgler[23] |
| Binary mixtures | | Chlorophyll-*a* in ether | Chlorophyll-*b* in ether | |
| | 661 | 102 | 6·1 | Present study |
| | 642·5 | 15·0 | 64·5 | |
| | | Phaeophytin-*a* in ether | Phaeophytin-*b* in ether | |
| | 667 | 65 | 9·3 | Present study |
| | 655 | 20·1 | 41·8 | |

measurement might considerably affect the coefficients of absorption. Coefficients for those pigments used throughout this study were therefore determined on the spectrophotometer after preparing pure chlorophyll-*a* and -*b* and phaeophytin-*a* and -*b* according to the method of Zscheile & Comar.[8] The carotene and xanthophyll coefficients have been taken from the literature.

*Carotene and xanthophyll*

These were readily determined by the following equations incorporating the absorption coefficients given in Table II:

$$\text{Carotene, mg./100 g. of dried sample} = \frac{100(DV/\text{g. at } 450 \text{ m}\mu)}{257{\cdot}7} \quad . \quad . \quad (1)$$

and

$$\text{xanthophyll, mg./100 g. of dried sample} = \frac{100(DV/\text{g. at } 444 \text{ m}\mu)}{398} \quad . \quad . \quad (2)$$

where $D$ = optical density of a 1-cm. layer of solution
and $V$ = volume of the solution.

*Chlorophyll*-a *and chlorophyll*-b

In a binary mixture the concentration of each component can be calculated from optical density measurements at two wavelengths as follows:

At wavelength $\lambda'$, $D' = \alpha'_a c_a + \alpha'_b c_b$ . . . . . (3)
and at wavelength $\lambda''$, $D'' = \alpha''_a c_a + \alpha''_b c_b$ . . . . . (4)

where $D'$ and $D''$ are the optical densities of 1-cm. layers of solution read at $\lambda'$ and $\lambda''$ respectively.
$\alpha'_a$ and $\alpha'_b$ are the specific absorption coefficients of $a$ and $b$ respectively at $\lambda'$,
$\alpha''_a$ and $\alpha''_b$ are the specific absorption coefficients of $a$ and $b$ respectively at $\lambda''$,
and $c_a$ and $c_b$ are concentrations respectively of $a$ and $b$ in g./litre.

Equations (3) and (4) may be solved simultaneously to obtain $c_a$ and $c_b$ provided the absorption coefficients are known. The following equations apply to chlorophyll mixtures when the absorption coefficients at wavelengths of 661 and 642·5 mμ are substituted in equations (3) and (4).

$$\text{Chlorophyll-}a \text{ (mg./l.)} = 9{\cdot}95D^{661}_{1\text{ cm.}} - 0{\cdot}95D^{642{\cdot}5}_{1\text{ cm.}} \quad . \quad . \quad . \quad (5)$$
$$\text{Chlorophyll-}b \text{ (mg./l.)} = 15{\cdot}7D^{642{\cdot}5}_{1\text{ cm.}} - 2{\cdot}31D^{661}_{1\text{ cm.}} \quad . \quad . \quad . \quad (6)$$

Substituting the $DV$/g. values for $D$, a direct assessment of each pigment in 100 g. of dried test-material can be made.

Chlorophyll-*a* in mg./100 g. of dried sample
$= 0{\cdot}995(DV/\text{g. at } 661 \text{ m}\mu) - 0{\cdot}095(DV/\text{g. at } 642{\cdot}5 \text{ m}\mu) \quad . \quad . \quad (7)$
and chlorophyll-*b* in mg./100 g. of dried sample
$= 1{\cdot}57(DV/\text{g. at } 642{\cdot}5 \text{ m}\mu) - 0{\cdot}231(DV/\text{g. at } 661 \text{ m}\mu) \quad . \quad . \quad . \quad (8)$

*Phaeophytin*-a *and phaeophytin*-b

In a similar way:

Phaeophytin-*a* in mg./100 g. of dried sample
$$= 1{\cdot}65(DV/\text{g. at } 667\ \text{m}\mu) - 0{\cdot}366(DV/\text{g. at } 655\ \text{m}\mu) \quad . \quad . \quad . \quad (9)$$
and phaeophytin-*b* in mg./100 g. of dried sample
$$= 2{\cdot}57(DV/\text{g. at } 655\ \text{m}\mu) - 0{\cdot}794(DV/\text{g. at } 667\ \text{m}\mu) \quad . \quad . \quad . \quad (10)$$

*Method of assessing losses*

To assess losses throughout the separation procedure the optical density of a given coloured solution measured in 1-cm. cells was multiplied by the volume. The *DV* value thus obtained for any particular wavelength could be compared with the *DV* value of subsequent solutions, or could be referred back to the dry matter of the original sample extracted, thus deriving a quantitative colour value which could be compared directly with values derived from other samples.

For assessment of losses, *DV* values were calculated at wavelengths of 660, 450 and 410 m$\mu$ because these wavelengths were near main peaks of chlorophyll-*a*, carotenoids and phaeophytin-*a* respectively.

*Example*

A 50-ml. solution of mixed green and yellow pigments before separation on sucrose might give, in a 1-cm. cell, an optical density reading at 450 m$\mu$ ($D^{450}_{1\,\text{cm.}}$) of 0·200. The *DV* value would, at 450 m$\mu$, be $0{\cdot}200 \times 50 = 10$.

If now the green and yellow pigments contained in this 50-ml. solution are separated, and the eluates reduced in volume to 10 ml. each in diethyl ether solution, and if the $D^{450}_{1\,\text{cm.}}$ values are 0·400 for the green solution and 0·500 for the yellow solution, then *DV* green = 4, and *DV* yellow = 5.

The loss during separation would then be taken as:
$$\frac{100[10 - (4 + 5)]}{10} = 10\%$$

By this method an assessment can be made of losses in the transfer from acetone to diethyl ether, and losses during transfer to, and separation on, the sucrose column. Extraction efficiency can be assessed from the *DV* values of the acetone extract and the diethyl ether extract of the residues.

It is realized that this method of assessing losses can only be approximate because, even if Beer's law holds for all pigments separated, the position and magnitude of the peaks will differ slightly according to whether acetone or diethyl ether is the solvent. However, the method is simple and was adequate for the writer's purpose. Losses directly attributable to the sucrose column were estimated after each separation by dissolving the column in water and extracting the solution with diethyl ether. The relative purity of separated pigment fractions was judged by inspection of the absorption curves, which were prepared from the spectrophotometric measurements made after each step in the separation procedure.

## Results

**Table III**

*Pigment estimates: mg./100 g. of dry matter of the test sample*

| Hay sample | Chlorophyll-*a* | Chlorophyll-*b* | Phaeophytin-*a* | Phaeophytin-*b* | Total tetrapyrroles | Carotene | Xanthophyll |
|---|---|---|---|---|---|---|---|
| 1 | 60 | 40 | 12 | 3 | 115 | 1·1 | 3·6 |
| | 64 | 42 | 11 | 2 | 119 | 1·1 | 3·9 |
| 2 | 65 | 38 | 9 | 1 | 113 | 0·9 | 4·1 |
| | 65 | 40 | 8 | 3 | 116 | 1·0 | 4·2 |
| 3 | 59 | 36 | 6 | 2 | 103 | 0·7 | 3·6 |
| | 56 | 34 | 8 | 1 | 99 | 0·7 | 3·4 |
| 4 | 61 | 40 | 7 | 1 | 109 | 0·7 | 3·6 |
| | 61 | 39 | 7 | 1 | 108 | 0·7 | 3·7 |
| 5 | 55 | 34 | 6 | 1 | 96 | 0·4 | 2·6 |
| | 56 | 36 | 8 | 1 | 101 | 0·5 | 2·6 |

The procedures described have been applied to five samples of hay. Good agreement was obtained between duplicate estimations. Table III shows the pigment estimates calculated from the results found and the equations already derived.

**Table IV**

*Summary of the extraction and separation results*

| | Pigments extracted by acetone treatment, % | | | Pigments transferred to diethyl ether, % | | | Pigments lost during the sucrose column separation, % | | | Pigments recovered from the sucrose column, % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wavelength, mμ | 660 | 450 | 410 | 660 | 450 | 410 | 660 | 450 | 410 | 660 | 450 | 410 |
| Sample | | | | | | | | | | | | |
| 1 | 99·9 | 99·7 | 99·6 | 97 | 97 | 77 | 12 | 6 | 5 | 1 | 1 | 2 |
| | 99·9 | 99·6 | 99·4 | 98 | 100 | 79 | 8 | 3 | 3 | 1 | 1 | 1 |
| 2 | 99·9 | 99·8 | 99·6 | 96 | 98 | 79 | 7 | 3 | 4 | 1 | 1 | 1 |
| | 99·9 | 99·9 | 99·8 | 93 | 89 | 73 | 7 | 4 | 4 | 1 | 1 | 1 |
| 3 | 99·8 | 99·8 | 99·6 | 97 | 94 | 72 | 4 | 1 | 3 | 1 | 1 | 2 |
| | 99·8 | 99·9 | 99·7 | 100 | 100 | 75 | 3 | 0 | 1 | 1 | 1 | 2 |
| 4 | 99·8 | 99·8 | 99·6 | 102 | 104 | 81 | 5 | 4 | 7 | 1 | 1 | 2 |
| | 99·8 | 99·9 | 99·7 | 100 | 101 | 80 | 5 | 1 | 4 | 1 | 1 | 2 |
| 5 | 99·6 | 99·6 | 99·5 | 97 | 101 | 78 | 5 | 6 | 7 | 1 | 2 | 3 |
| | 99·8 | 99·7 | 99·7 | 97 | 99 | 78 | 5 | 4 | 5 | 1 | 2 | 2 |
| Average | 99·8 | 99·8 | 99·6 | 98 | 98 | 77 | 6 | 3 | 4 | 1 | 1 | 2 |

The assessment of losses summarized in Table IV shows that over 99% of the fat-soluble pigments were extracted from each sample of hay by the procedure described, and indicates that, on the average, the results in Table III might be low by 5%, of which only 1% is recoverable from the sucrose–sodium sulphate column. The considerable loss of compounds absorbing light at 410 mμ, which occurs during the transfer of pigments from 85% acetone to diethyl ether, is caused by removal, during the water washing, of compounds more soluble in dilute aqueous acetone than in diethyl ether. These interfering compounds show a rapid increase in absorption below 450 mμ without showing characteristic peaks, but showing a small inflexion at about 270 mμ.

A later paper describes the use of these procedures in a study of the pigments in microbial fractions prepared from rumen contents taken from the sheep.

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# THE ROLE OF GLYCERIDES IN BAKING*

By J. B. M. COPPOCK, M. A. COOKSON, D. H. LANEY and (in part) D. W. E. AXFORD

**Part I**

The historical development of the use of glycerinated fats in baking is traced and some essential differences between American and British practice are discussed. Glycerinated fat comprises mixtures of mono-, di- and tri-esters; it is commonly but incorrectly called 'glyceryl monostearate' and the commercial material in this country, known as GMS, usually contains about 30–40% of glyceryl monostearate or about 20% of glyceryl monooleate. The effect of GMS products of varying composition on selected properties of bread, sponges and Madeira cakes is described.

Specifications for the most suitable types of GMS for use in these products are discussed and the effect that various flour improvers have on the quantities of GMS showing optimum improving effect is described.

Various theories that have been propounded to explain the mechanism of the action of fats, oils and GMS as crumb-softening and anti-staling agents are critically examined; the view is put forward that greater attention should be paid to the distribution of labile water between the coagulated gluten network and starch gel which comprise the system referred to as bread.

**Part II**

The effect of flour oils in bread making and the influence these substances have on the improving action of the glycerinated fats are discussed. Experiments are described which indicate the presence of monoglycerides in (*a*) oils extracted from 81%-extraction flour treated in various ways, (*b*) oils extracted from bread baked from the severally treated flours and by the aeration process and (*c*) fats normally used in baking and breads containing these fats. The periodic acid method of estimating monoglycerides in fatty materials is critically examined. The presence of small quantities of monoglycerides in certain of the above materials has been established by applying countercurrent extraction methods and by preparing the 2 : 4-dinitrophenylhydrazine derivatives of the reaction products in the chloroform layer after periodic acid assay, and by applying other methods of characterization. The influence the various findings have on the pharmacological considerations involved in the inclusion of glycerinated fats (GMS) in baked products is discussed, and in particular the relationship of this information to the concept of 'hundredfold' acceptability advocated by Frazer as one of the main criteria in assessing the absence of risk in the use of food additives. As there is also no direct indication of cumulation of glyceryl monostearate in the body, or of any significant nutritional defect caused by the use of GMS in the manner recommended by the authors, it is concluded that the use of glycerinated fats, within reasonable limits, in baked goods involves no risk of harm to the consumer.

## Part I: The Effects of Added Glycerinated Fats in Bread and Flour Confectionery

(M. A. Cookson and J. B. M. Coppock)

### Introduction

The development of the use of glycerinated fats in bread and cakes has evolved from a desire to improve the functional value of the various types of fats and oils used in the production of these two classes of baked goods.

The use of these surface-active agents was first suggested in the U.S.A. for incorporation in cakes of a special type involving the use of more sugar and liquid than is normal. These so-called high-ratio cakes often included about 150 parts of sugar to 100 parts of flour, or approaching 1½ to 2 times the sugar content of an ordinary cake, and a correspondingly higher proportion of liquid. It was found that the use of this additional sugar and liquid weakened the structure of a cake unless sufficient monoglyceride was present. The so-called high-ratio shortenings containing glycerinated fat were therefore evolved and from this starting point the incorporation of the glycerinated fats in all types of baked products has developed.

Because of differences in national taste and also in the scale and method of war-time rationing between the U.S.A. and this country, the way in which the use of these substances has been developed shows marked differences. For example, the leaner formulae of British bread, compared with American bread, and the differing extraction rates of flour existing in

* Read, in a modified form, before a joint meeting of the Oils and Fats Group and the Food Group on 5 December, 1952

recent years in the two countries lead to significantly different quantities of glycerinated fat being required for the purpose of crumb softening.[1] Such differences emphasize the importance in cereal research of clearly indicating all the relevant factors that influence the quantities involved when additives of this type are under investigation for use in baked products. At present the differences brought about by varying tastes and dissimilar qualities and quantities of ingredients are insufficiently appreciated by many workers on both sides of the Atlantic. Illustrations of this view will be found in subsequent sections of this paper.

It has long been known that the incorporation of triglycerides in bread doughs can, according to their nature, effect an increase in the softness and tenderness of bread crumb and in loaf volume.[2, 3] As a general rule, it has been found that the plastic or solid fats have a greater improving action in these respects than oils, although castor oil and, to a less extent, rapeseed oil, are exceptions to this generalization. Thus it can be seen that bread-improving action is to some extent dependent on the physical state of the triglycerides incorporated, i.e. whether it is a fat or an oil. Again, in flour confectionery, the various uses to which butter, cake and pastry margarine, compound cooking fat and oils are put depend on their physical and chemical characteristics. Similar considerations apply to the use of glycerinated fats in baking processes.

Before some of the work we have carried out, which contributes to this conclusion, is described, an explanation of the term glycerinated fat is desirable. About 25 years ago in America special shortenings were produced by adding glycerol to fat during the refining process, so that partial conversion of the tri-glyceride into mono- and di-glycerides occurred. According to the quantity of glycerol added, varying amounts of mono- and di-glycerides were present in the final product. Such products were termed superglycerinated fats. In this country, mixtures of the three glycerides, usually produced by the interaction of glycerol and a fatty acid, e.g. commercial stearic acid, became referred to as ' glyceryl monostearate ' or GMS, although it is clear that the name is usually a misnomer, as the mixture rarely contains more than 30–40% of the monoglyceride.

According to the method of manufacture, GMS may contain varying proportions of mono-, di- and tri-glycerides of fatty acids (usually stearic acid), some unchanged raw materials, and usually a little sodium stearate, resulting from either the use of an alkaline catalyst or treatment of some of the unchanged fatty acid with alkali; the sodium stearate (soap) serves to make the product self-emulsifying. Recently, molecular distillation has resulted in products containing quantities exceeding 90% of the monoglyceride, but these are not yet generally available in this country. Some typical compositions are shown in Table I, the final column indicating the figures specified in the B.P.C. for Monostearin Emulsificans.[25]

**Table I**

*Properties of some typical commercial GMS products*

| | | | | | | B.P.C. |
|---|---|---|---|---|---|---|
| Monoglyceride, % | 97·8 | 91·2 | 35·0 | 33·3 | 13·9 | > 32·5 |
| Sodium stearate, % | 0 | 0 | 2·6 | 0 | 4·0 | 2·5–7 |
| Free glycerol, % | — | — | 6·4 | 6·4 | 2·7 | 4–7 |
| Acid value | 1·1 | 1·1 | 2·2 | 6·5 | 5·6 | < 18 |
| Iodine value | 89·5 | 2·3 | 1·3 | 2·3 | 47·8 | < 8 |
| M.p., ° C | 45·5 | 72·5 | 56·5 | 55·7 | 39·5 | 54–57 |

## Experimental

### *Bread*

In the study of the functional value of glycerinated fats, instruments have been constructed that can measure the effect of various quantities added to bread in terms of such properties as loaf volume, firmness of the crumb, crumb toughness and stickiness. In our initial studies, treated flours of 85% extraction rate were used. (Unless specifically stated to the contrary the treated flours were flours improved with either agene or chlorine dioxide, with or without small additions of potassium bromate or ammonium persulphate, and prepared by the miller to yield a flour approximately suitable for a 3–4-hour bulk-dough fermentation process.) Doughs of basic formula were prepared from 2000 g. of flour, 28 g. of salt, 36 g. of yeast and a total of about 1140 g. of water. They were mixed in a laboratory mixer at 80° F and fermented at this temperature for 3 hours, with a knock back after 2 hours. They were then scaled at 1 lb., handed up, allowed 10 minutes' recovery and finally moulded into tins and proved under constant temperature and humidity conditions for 40 minutes, the loaves being baked for 30 minutes at 450° F. Where possible the GMS was added at the

dough stage as a 17% (w/v) emulsion in water, prepared by melting the GMS in five times its weight of very hot water and beating until cool. The actual water added to the dough was corrected for the quantity of water in the emulsion. A comparison of loaves containing 0, 0·05, 0·1, 0·2, 0·3, 0·5 and 0·7% of glycerinated fat, expressed on the flour weight, was made and showed that the best type of loaf, and one not exhibiting the initial signs of over-treatment indicated by a ragged break in the crust, contained about 0·3% of GMS (14 oz./sack of 280 lb. of flour) (see Fig. 1).

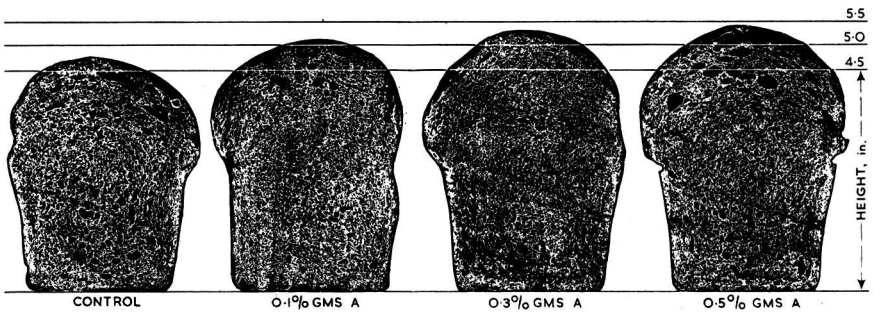


FIG. 1.—*The effect of GMS A, at levels of* 0·, 01, 0·3 *and* 0·5% *of the flour weight, on breads prepared from* 85%*-extraction treated flours*

*Note.* The reproductions of crumb structure and loaf girth given in Figs. 1 and 6 have been obtained by preparing ink prints and reversing, in the blockmaking process, the black outline obtained to give the normal impression of a white crumb

It should be noted, in examining Fig. 1, that increased volume manifests itself only in increased height, as the width and length of the loaf are determined by the size of tin. This has some bearing on nutritional arguments[4, 5] and indicates that a slice of given thickness possesses the same weight whatever the loaf height, provided the loaf weights are equal, as they are.

The glycerinated fat A used in these experiments was preponderantly saturated and contained 34% of monoglyceride and 5% of sodium stearate, i.e. it was of the self-emulsifying type.

In the assessment of the improvement in loaf quality in these experiments the most sensitive criterion is crumb softness, which is capable, within certain limits, of reasonably exact measurement in terms of firmness. This we achieved[6] by determining the weight in grams required to compress a disc of crumb about 1 cm. thick, 3·2 cm. in diameter and 8·02 sq. cm. in area to half its original thickness. Loaf volume is the next most sensitive criterion, and determinations of crumb toughness and tenderness are the least sensitive criteria.

The optimum improving effect of the GMS A having been established in terms of these four criteria, it was then compared with the effect of a preponderantly saturated sample B containing 90% of monoglyceride and 5% of sodium stearate. This also possessed an optimum effect at 0·3% based on the flour weight, and yielded a slightly softer loaf than that made with A and remained softer over a period of five days (Fig. 2).

The toughness of the crumbs was identical but sample B gave a crumb slightly more sticky than that given by sample A. The volumes of the loaves were (*a*) control without GMS, 1358 c.c., (*b*) GMS A, 1490 c.c. and (*c*) GMS B, 1462 c.c.

In a similar way a series of glycerinated fats of varying composition, of differing emulsification values, of different degrees of unsaturation and of free fatty acid content were examined. For example a GMS C was selected which had been prepared from an unsaturated oil or fat. It had an iodine value of 44·6 and melting point 41° C. The monoglyceride content was 14·3% and the sodium stearate content 4·5%. This was compared with a GMS D of monoglyceride content 28%, sodium stearate content 3%, melting point of the fatty acid 56° C and a low iodine value of 4·5 (i.e. preponderantly saturated). The optimum effect of each was 0·3% of the flour weight. From Fig. 3 it can be seen that sample D produced a less firm or more soft bread than sample C. The respective loaf volumes were : (*a*) control, 1250 c.c., (*b*) GMS C, 1360 c.c. and (*c*) GMS D, 1480 c.c. The results indicate that unsaturation is adverse to the production of bread possessing the necessary desirable criteria as defined above.

The main conclusions reached from these investigations were :

(1) Self-emulsifying glycerinated fats were superior in their effects to the non-self-

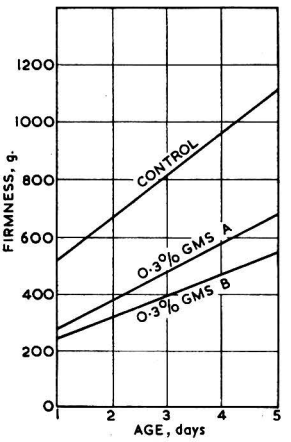


FIG. 2.—*The effect of two saturated GMS products of varying monoglyceride content on crumb softness*

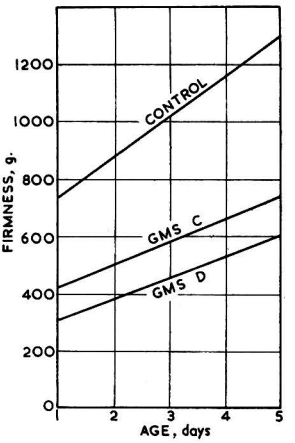


FIG. 3.—*The effect of a saturated GMS and an unsaturated GMS of similar monoglyceride content on crumb softness, added at levels of* 0·3%

emulsifying types unless the latter glycerinated fats were dispersed, e.g. by melting in fat before adding to the dough.

(2) With 85%-extraction treated flour in tin bread the optimum effect of the self-emulsifying GMS containing 25–40% of GMS was reached at a level of 0·3% expressed on the flour weight in lean-formula British-type bread.

(3) Increased monoglyceride content enhances the improving effect but was not noticeably apparent until high (about 90%) monoglyceride contents were reached.

(4) The glyceryl stearates were apparently more efficient in bread than glyceryl oleates or other unsaturated fatty-acid derivatives. Free fatty acids in reasonable quantity had no adverse effect, but undue amounts, about 50% of stearic acid, produced crumbliness in the bread.

When these experiments were repeated with 81%-extraction treated flour it was found that the same general conclusions could be reached, although, as the use of lower-extraction flour itself gives an improved loaf, the range over which the improvements occur on the addition of glycerinated fats was narrower. In further experiments with 81%-extraction treated flour it became apparent that the use of fat together with glycerinated fat in bread making permitted proportionately smaller quantities of each to be used than is necessary for producing the optimum effect of either alone. Thus although 14 oz./sack of GMS or 2 lb./sack of fat (0·3% and 0·7% respectively, expressed on the flour weight) had been found to be the optimum quantities when used separately, approximately the same effect was produced with a mixture of $2\frac{1}{2}$ oz. of self-emulsifying, glycerinated fat and 14 oz. of fat/sack (0·3% of fat and 0·05% of GMS expressed on the flour weight) (Fig. 4).

This is equivalent to the addition of about 5% of real monoglyceride to the shortening or fat.

In the recently proposed American bread standards[7] the maximum inclusion of real monoglyceride in the shortening used in the U.S.A. was fixed at 8%. As American bread often contains up to 6% of fat, American bread might contain as much as 0·5% of real monoglyceride (equivalent to about 1·5% of a typical British product) expressed on the flour weight. The greater rate of usage[8–10] in American bread is due to two factors: the minor one is the widespread use of 72%-extraction flour[2] in America, which requires a little more glycerinated fat to produce the same effective functional effects as are found in

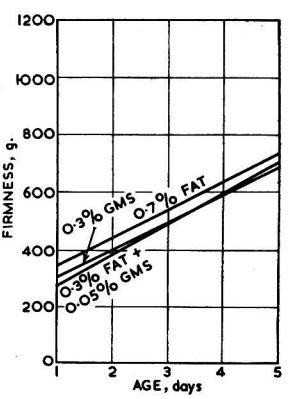


FIG. 4.—*The quantities of* (1) *GMS,* (2) *fat and* (3) *GMS and fat, required to produce approximately equal crumb softness*

flours of greater extraction rate; the major factor is concerned with the use of richer formulae for bread production in America, some containing as much as 6% of non-fat dried-milk solids, which have a considerable depressing effect on loaf volume and crumb softness—this effect is counteracted by the use of more GMS or fat or both.

The results so far described refer to tin bread prepared from treated flours of two different extraction rates. It is not generally appreciated that both the nature of flour treatment and the type of bread being produced have pronounced effects on the effective quantities of glycerinated fat required as a bread improver. Thus in experiments with 81%-extraction unbleached and untreated flour, if potassium bromate was the sole improver used, the average quantity required to give optimum flour-improving effects would be about 20 p.p.m. We have found that for a flour requiring this amount of potassium bromate (or ascorbic acid, which gives parallel effects[1]) the amount can be reduced to 10 p.p.m. in the presence of fat or glycerinated fat. Similarly if small additions of bromate are made to a flour partially treated with a gaseous improving agent (e.g. agene or chlorine dioxide) the optimum amount of GMS, 14 oz./sack (0·3%), can be considerably reduced and as little as 3–4 oz./sack of glycerinated fat prove effective,[11] i.e. there is evidence of synergism. This is also true when the flour improvement has been effected by physicochemical methods, such as in the aeration process.[11, 12] Thus as little as 0·1% of GMS, expressed on the flour weight, produces enhanced crumb-softening effects, and therefore keeping properties. As will be shown below, the smallness of this quantity of GMS has a pronounced influence on the physiological arguments in favour of the harmlessness of such an addition to bread, and the effect on nutritional value will be extremely small. This quantity is adequate for the production of softness in the larger type of tin loaf made commercially and commonly called the 2-lb. loaf (weighing at present 1 lb. 12 oz.). It appears to be slightly more effective in a 1-lb. loaf (weight at present 14 oz.) and still more effective in pup loaves weighing 2 oz. Thus in addition to the influence of flour-extraction rate and flour treatment, loaf size[10] also has an effect on the efficiency of GMS as a crumb-softening agent.

Our studies on tin or pan bread have been extended to include Scottish batch bread. This type of bread, prepared from a flour stronger than that commonly used in England and Wales for the production of tin bread, is set as dough pieces, often directly on the baking plate of the oven, each dough piece touching its neighbour. The final loaf, except those from the ends, has, on separation from its neighbours, a crust only at the top and bottom, the four sides being exposed crumb. The baking time is from 2 to 2½ times longer than for tin bread. Two-pound batch loaves exhibit little or no crumb-softening effects when the quantities of glycerinated fat effective in tin bread are used. There may, however, be a slight improvement in volume and in colour, the colour improvement being due to finer crumb vesiculation. The difficulties associated with research work in this field, and referred to earlier, are clearly indicated by this finding.

*Discussion on the action of fats, oils and glycerinated fats in bread*

Many workers have suggested that shortenings function as improving agents in bread by acting as a lubricant between the starch granules and by lubricating the gluten filaments, thus allowing greater extensibility in the dough. This theory does not explain why fats are usually more effective than oils. It has also been reported[3] that hard fats were superior to semi-solid fats, both being superior to oils in improving bread quality, but that doughs containing oils or no fat had the property on baking of exhibiting an abrupt cessation of both oven spring and gas retention, although oven spring commenced at the same rate in all cases. It was also found that breads containing hard or semi-solid fats possessed a texture highly permeable to air, a property found to be characteristic of tenderness in the crumb, indicating that the cell structure was largely disrupted. As hard and semi-solid fats would not blend so completely with the flour oil in gluten as the liquid fats would, it was suggested that they remained in masses, which weakened the dough film at many points, so causing the change in dough consistency. It was considered that their ultimate effect was to plug the pores produced by gas leakage in the cell walls, thus retaining gas and producing larger volume.

This theory has been criticized[13] on the grounds that, as oven spring starts to be rapid only above 32° C (90° F), at which temperature semi-solid fats are usually liquid, there should be very little increase in volume. With castor oil, which was found to give as good an effect as the solid fats, it was suggested[3] that the high viscosity of this oil so modified the character of the natural flour fat as to reduce the passing of gases through the cell walls during the baking of the dough.

Viscosity measurements carried out by us on liquefied fats and oils (temperature range

25–75° C), indicate that many solid or semi-solid fats have, on melting, lower viscosities than castor oil. It would not appear, therefore, that viscosity is the true explanation of the similarity in improving effect of castor oil and the solid and semi-solid fats.

The difference usually found in the extent of the effect between solid and liquid fats is not confined solely to the edible materials. Hard Russian paraffin wax, carnauba wax and Chinese insect wax have all been reported[3] to give an improvement in bread volume and texture similar to that of the solid, completely hydrogenated cottonseed oil. Mineral oils, however, possess no improving effect.

Alcock & King[14] found that paraffin wax at a level of 0·56% of the flour weight maintained bread fresh, as shown in terms of lower moisture loss, for at least 14 days, provided the wax was of setting point 50–60° C; less efficiency was obtained with waxes of other melting ranges. It is interesting to note that fat and sugar appeared to reverse the effect of the wax. It was suggested that the paraffin wax functions by strengthening the gluten, and, because of its non-polarity, prevents the movement of moisture between starch and gluten.

It should be made clear that work with some of these materials, and also castor oil, is recorded here solely for scientific information; it is undesirable—and, for mineral oils and waxes, also illegal[15]—to use them as ingredients in bread intended for human consumption.

Some interesting results have been obtained[16] in the U.S.A. with different types of solid shortening in rich doughs containing sugar and milk-powder in addition to flour, yeast, salt and water. It was found that compound fats were slightly superior to vegetable fats, both being superior to animal fats in volume improvement, although the animal fats were best in improving grain and texture. There were negligible differences in the melting points of the animal or compound fats, both of which were higher melting than the vegetable fats.

Thus it would appear that the differences in the crumb-softening effect of triglycerides in bread cannot be attributed directly to the physical state of the material, e.g. whether it is solid or liquid, or to some property such as viscosity.

There is still much confusion between substances that inhibit staling in bread and those that cause crumb softness. It can be seen from Fig. 2 that the main effect of such substances as glycerinated fat is to make the bread containing it more soft initially. The rate of hardening is such that the bread also remains softer over the period of test than a control loaf containing no additive. It will be seen, however, from the differences in slope of the various results plotted that the rates of hardening are also affected, so that superimposed on the crumb-softening effect is also a true anti-staling effect, but this is not the chief effect. It should be emphasized that compressibility or firmness measurements, as commonly carried out, are not sufficiently accurate to enable the interpretation of rates of hardening to be made with precision. Technique and the temperature at which determinations are made probably play a much larger part in the exactness of the tests than has hitherto been appreciated. For instance, Scottish batch-bread prepared from strong Manitoban flour has, on occasion, been found as soft initially as English tin-bread prepared from a somewhat softer flour and containing GMS. There have been indications, however, that though glycerinated fat has no influence on the softness of Scottish batch-bread prepared from strong Manitoban flour, it still produces a softening effect in batch bread prepared from weaker English flour, but the 'Manitoban' bread may also retain its small anti-staling effect, which is, however, difficult to prove because of the deficiencies in firmness measurements as indicated above. It is known that the *X*-ray-diffraction pattern of bread crumb changes during ageing, and certain American work[8] indicates that monoglycerides delay the staling rate as measured by this technique.

It should be remembered, in connexion with staling as opposed to crumb softening, that the linear amylose component (A-fraction) of the starch is regarded by Schoch & French[17] as being completely retrograded after baking, whereas they believe that the branched amylopectin (B-fraction) aggregates during ageing of the bread, and is the starch fraction that causes staling. This was shown by the water-soluble starch leached from fresh bread crumb being predominantly amylopectin, whereas amylose is the more soluble component before baking. When stale bread is heated at 50° C the percentage of soluble amylopectin is restored and amylose remains insoluble; this rehydration process is comparable to the re-freshening of bread by heating.

It is well known that many chemicals affect the gelatinization of starch. Compounds with two hydrophilic groups, such as the monoglycerides, have been found to reduce the swelling of starch more than those compounds containing only one hydrophilic group,[18] although it should be remembered that on this theory polyoxyethylene monostearate should not be as efficient as glyceryl monostearate, whereas the reverse appears true.

The ability of fats to disperse through doughs is probably related to their shortening

action, and the more hydrophilic compounds, such as glycerinated fats, might be expected to be better shortening agents than the triglycerides.[13] Crumb-softening effects would be parallel.

Fats appear to have negligible action upon the swelling power and solubility of starch.[13] Glycerinated fats have been found[19] to decrease the swelling or hydration of starch granules and inhibit the release of water-soluble starch (mainly amylose). Microscopical examination of doughs containing glycerinated fats showed these fats to be distributed during mixing into small globules, which are dispersed between the starch granules. Part of the monoglycerides may become chemically attached to the starch, so retarding the amylopectin aggregation associated with true staling; part, however, may coat the starch granules and so reduce their capacity to absorb water, thus making more of the dough-water available for hydrating the gluten. The increased hydration of the gluten may partially account for an increase in the softness of bread, in the same way that the addition of hydrated gluten to doughs produces this effect in commercial high-protein breads; this is also shown in the softness obtained in Scottish batch-bread, where, owing to the stronger flour used, there is an increase in the hydrating capacity of the protein. The gluten strands in bread made with monoglyceride shortenings have been shown to be finer in texture than in other breads. The action of glycerinated fats in reducing the release of water-soluble starch, referred to above, has also been thought to affect bread softness. Soluble starch, chiefly amylose, is known to increase the firmness of bread if it is added during dough making, and it has been suggested that the soluble starch holds together the starch granules and gluten strands that comprise the walls of the air cells in bread. Thus, if the soluble starch is retained within the granules, the rigidity of the air cell is decreased, and the softness of the bread increased.

It has also been found that oleic acid and polyoxyethylene stearate considerably reduce the amount of soluble extract and swelling power of starch (similar to monoglycerides), giving the effect of a gel already stale.[13, 17] The swelling power of starch from a fresh gel containing polyoxyethylene stearate was approximately equal to that from a standard gel which was about seven days old. It has been suggested that this action, which causes gelatinized starch to be apparently partially stale, results in a less rapid change with age of the bread.

However, as the order of effectiveness of chemical substances producing this action cannot be correlated with their improving action in bread (e.g. oleic acid is more effective than polyoxyethylene monostearate as an amylose precipitant, yet is detrimental to bread quality), this theory would appear inadequate.

It is of interest to note some recent research in the U.S.A. where it was found[20] that, when incorporated in a bread dough, lard is partially hydrolysed during baking, with the formation of monoglycerides. The amount of monoglycerides formed in the bread depended on the quantity of fat used in dough making, but the two levels of fat used were equivalent to about $7\frac{1}{2}$ lb. and 18 lb. of fat to 280 lb. of flour, compared with levels of 1–2 lb. of fat in this country. However, we have been unable to confirm the high conversions reported either with the high or low levels of fatty materials used in this country of similar or different type.

To summarize, suggested theories for the improving action of glycerinated fats are:

(1) they are more efficiently dispersed in the dough than the triglycerides and would therefore exhibit greater effect than the triglycerides;

(2) they maintain the soluble starch, which increases the firmness of bread crumb, within the starch granule;

(3) they depress the swelling and swelling power of starch gels, causing the starch to be apparently partially stale, so that the bread does not change (i.e. stale) so rapidly with age and

(4) in depressing the swelling of starch, they permit an increase in the moisture available for the hydration of gluten, so affecting softness.

In our view none of these theories explains more than a part of the many factors involved when a substance added during dough making produces in the resulting bread a softer and longer-keeping crumb. Apart from the nature of the additive, flour quality and particularly the nature of the flour oil (see Part II), correct dough fermentation, mode of baking and subsequent cooling all play a part in the final properties the bread possesses.

Moisture content plays a significant part in the determination of these properties, according to its distribution between the starch and the gluten and the tenacity with which it is bound to each. We have found, with Mr. F. J. H. Ottaway, that when American-type bread of low pH is sealed in a closed container under vacuum and stored at room temperature (about 60° F) that, even after 96 days' storage, equilibrium is not reached in the moisture distribution throughout the loaf. When such hermetically sealed bread was transferred to a room

maintained at 96° F, subsequent examination showed the presence of free moisture which had condensed on the sides of the bread and on the walls of the container with which the crust was in contact. At this temperature the bread, which at room temperature was noticeably stale, had become softer after the condensation had occurred. It is suggested that further attention should be given to the views of Alcock & King,[14] for we believe that labile water has a considerable influence on crumb properties. Additional evidence of the existence of such labile water has also been obtained from a quite different type of experiment involving studies on the survival and subsequent growth of *Bacillus subtilis* spores in bread.[21] The surprising result was obtained that, of the viable spores remaining after baking, only about 1% grow, and this was ascribed to changes in the distribution of moisture within the bread which is known to be near the critical level for bacterial growth.

The effectiveness of many crumb-softening agents under given conditions may be due to the influence they have, because of their hydrophilic nature, on the partition of this labile moisture between the starch and gluten in bread. Such agents may well possess the property of facilitating the initial retention of moisture in the gluten coagulated during baking, thus being responsible for the greater softness initially observed, and thereafter controlling the rate and method of moisture transference between the gluten network and starch gel comprising the system referred to as bread. We have designed a simple test to illustrate this. It is well known that stale bread may be re-freshened by heating. During staling, water is thought to be freed from the retrograding starch and absorbed by the gluten, from which it may be released for rehydrating the starch when the bread is re-freshened. The presence in the bread of a surface-active agent, such as GMS, which will be dispersed in the flour oil primarily contained in the gluten, might be expected to increase the retention of labile water by gluten and so retard the rate of re-freshening by heating. By compressibility determinations on breads containing (*a*) GMS at the level of 8 oz./sack and (*b*) a control containing no additive, the comparisons being carried out under identical conditions of temperature and humidity, we showed that the rates of re-freshening were greater for the control bread in which the movement of labile water was not restricted by the presence of the surface-active agent. This experiment was repeated three times; typical compressibility curves are given in Fig. 5. The loaves were re-freshened by heating in an oven at 100° C until the temperature of the centre of the loaves had reached 40° C, compressibility measurements being taken when the bread was one and seven days old and finally three hours after removal from the oven.

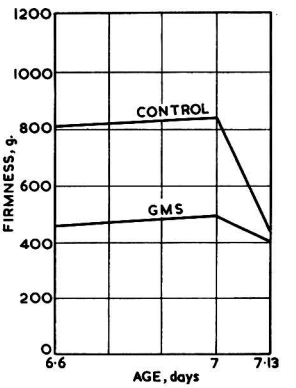


FIG. 5.—*The retarding effect of GMS on the re-freshening of bread as indicated by crumb softness*

### *Flour confectionery*

It will be appreciated, from the experimental work on the effect of the glycerinated fats in bread and the theories put forward to explain these effects, that the action of these substances on the starch–gluten–water system is highly complex, but that their main property is that of crumb softening. In cakes, and even more so in biscuits and shortbread, the part played by the fats is progressively very different from their role in bread. Thus in studying the effects of glycerinated fats in cakes there are two main features: (1) the direct effects on the quantity of fat used, its dispersion and its shortening properties, and (2) the effects on the physical properties of the crumb. Not surprisingly, we have found that the usefulness of the glycerinated fats in biscuits appears to be much more restricted than in bread and cakes. In order to obtain basic information on (2) sponge cakes were chosen for the initial investigation, because, except in special cases, they do not contain fats as ingredients. The properties in which improvement might be obtained, and which were looked for in this experimental work, were (*a*) increased batter-stability and improved sponge-texture, obtained from the emulsifying power of these esters, and (*b*) increased initial softness and longer-keeping life of the sponge, since the action of the partial glycerides on flour should be similar to their action in bread.

The types of glycerinated fats used for the experimental work on sponges and Madeira cakes are shown in Table II. The analytical figures are typical of these types, although slight variations were found in the different batches of each type used.

**Table II**

*Glycerinated fats used in flour confectionery*

| | GMS A | GMS B | GMS C | GMS D |
|---|---|---|---|---|
| Monoglyceride, % | 30·3 | 91·2 | 22·5 | 97·8 |
| *Sodium stearate, % | 1·7 | 2·5 | 1·6 | 2·5 |
| Acid value | 3·4 | 1·1 | 2·8 | 1·1 |
| Iodine value | 3·8 | 2·3 | 35·3 | 87·5 |
| M.p., °c | 57 | 71 | 47 | 46 |
| Physical state | powder | powder | fatty | fatty |

* Non-self-emulsifying forms, i.e. without sodium stearate, were used in certain experiments

Treated flours, 81%-extraction, were used in all the confectionery bakings. Unless otherwise stated bread flour and not chlorinated (cake) flour was used in these experiments.

*Sponges*

Sponges were more difficult to investigate than bread, as a large range of qualities exist (depending basically on the flour : egg ratio), and also the properties of sponges, particularly if made from frozen or shell egg, are sensitive to small changes in baking procedure (such as temperature, mixing times and handling).

The methods of batter mixing for dried-egg sponges and for frozen- or shell-egg sponges are different, causing variations in the results obtained when GMS is incorporated in the batters. In the preparation of frozen- or shell-egg sponges, the egg and sugar are whisked for about 10 minutes and the flour is then blended into the mixture. With dried egg, however, the egg, sugar, flour and liquid are beaten together with cream powder (the acid ingredient of baking powder) for about 20 minutes, then the baking powder, dissolved in a small quantity of liquid, is worked through the batter.

*Frozen- or shell-egg sponges.*—The formulae of sponges of this type investigated are shown in Table III.

**Table III**

*Formulae of frozen- or shell-egg sponges*

| | Full egg | Three-quarter egg | Half-egg |
|---|---|---|---|
| Whole egg, oz. | 20 | 15 | 10 |
| Water or milk, oz. | — | 5 | 10 |
| Sugar, oz. | 16 | 16 | 16 |
| Flour, oz. | 16 | 16 | 16 |
| Baking powder, oz. | — | $\frac{1}{8}$ | $\frac{1}{4}$ |

It was found that an improved texture, larger volume, and softer sponge with better keeping-qualities could be obtained with either GMS A or GMS B. Either self-emulsifying or non-self-emulsifying forms of the GMS were suitable, the non-self-emulsifying type possibly being preferable, provided the GMS was added as a fine powder. Emulsions sometimes caused the collapse of the delicate foam produced on whisking the sugar and frozen or shell egg.

About 0·25–0·5% of GMS, expressed on the total batter-weight added at any stage of the batter mixing, produced improvement in all three qualities of sponge. Quantities progressively greater than 0·5% of the batter-weight tended to produce increasingly either woolly or cake-like textures, and/or smaller volume. The increase in volume obtained when the smaller quantities of GMS were used was not maintained on ageing, for after keeping for several days the volume decreased to that of a sponge without GMS, although the improved texture and softness remained.

GMS B, with a high monoglyceride content, produced greater improvement than GMS A, the improvement being more pronounced than in bread, so that smaller quantities can be used. The unsaturated types, GMS C and D, prevented foaming during whisking, and produced, with increasing quantities of the additive, a sponge with a progressive decrease in volume and coarseness in texture, without increase in softness.

*Dried-egg sponges.*—The formulae of the dried-egg sponges studied are given in Table IV. This work was originally carried out on sponge sandwiches and later extended to Swiss rolls, but the results apply equally to both types.

**Table IV**

*Formulae of dried-egg sponges*

| | Full egg | Three-quarter egg | Half-egg |
|---|---|---|---|
| Dried egg, oz. | 5 | $3\frac{3}{4}$ | $2\frac{1}{2}$ |
| Water, oz. | 12 | $13\frac{1}{4}$ | $14\frac{1}{2}$ |
| Sugar, oz. | 16 | 16 | 16 |
| Flour, oz. | 16 | 16 | 16 |
| Cream powder, oz. | $\frac{1}{3}$ | $\frac{1}{3}$ | $\frac{1}{3}$ |
| Milk, oz. | 3 | 3 | 3 |
| Baking powder, oz. | $1\frac{1}{8}$ | $1\frac{1}{8}$ | $1\frac{1}{8}$ |

It was found that either GMS A or GMS B, added as an aqueous emulsion (3 parts of water : 1 part of GMS) at the beginning of batter mixing, in a quantity of 0·5–1·0% of the total batter-weight, produced a softer, longer-keeping sponge with an improved texture and brighter-coloured crumb, although volume was not usually increased.

An additional advantage found with dried-egg sponges was that the mixing time of the ingredients could be reduced by about 75%, 5 minutes' mixing in the presence of GMS achieving what would normally require a 20-minute period. Similar results were obtained with a laboratory-model pressure-mixer, when a time reduction from 3 minutes to 30 seconds was possible.

Improving action was obtained with all qualities of sponges, although the smaller quantity of GMS (about 0·5% of the batter-weight) was adequate with the better-quality products. When larger quantities of the GMS emulsions were used in dried-egg sponges containing quantities of GMS of the order of 1% or greater of the batter-weight, a tendency to cakiness was noted. The products were of small volume, fine cake-like texture, and were much shorter in eating-quality.

GMS B was again found preferable to GMS A in dried-egg sponges. Use of the GMS powder alone has little or no beneficial effect, and may even be slightly detrimental, as a tendency to a sticky crust on the sponge surface has been noticed; however, this may also occur to a less extent with GMS emulsions. The unsaturated fatty types, GMS C and D, produced poor-quality products, particularly when the mixing time was reduced, when the sponges had smaller volume, coarse texture and firmer crumb. Emulsions of these types were less drastic in effect, improvement sometimes being obtained, but never as efficiently as when GMS A or B was used.

Chlorinated cake-flour has not been found to give significantly better results than bread-making flour in sponges, although a finer vesiculation to the crumb is imparted; an even finer vesiculation is given to such products by the inclusion of GMS A or B. The general impression obtained by the use of cake-flour is that GMS does not permit such large reductions of mixing times, except possibly with the best qualities, and that the degree of improvement obtained is less than with bread-making flour.

*Madeira cakes*

The use of glycerinated fats in Madeira cakes is closely related to the original application in high-ratio cakes. The essential difference between these two types of cake is that the high-ratio products contain a greater proportion of sugar and liquid than Madeira cakes, which are made from balanced recipes that give batters not normally requiring emulsifying agents to promote stability. Nevertheless, under certain conditions it becomes possible to use glycerinated fats in Madeira-cake mixings to improve quality without altering the recipe balance.

Table V shows the formulae of the Madeira cakes studied in this work

Some differences in results were found, depending on whether the cakes were prepared by the sugar-batter or flour-batter methods. This was probably because the latter method itself produces cakes of superior volume and texture (i.e. in the absence of GMS). In the sugar-batter method, the fats and sugar are creamed together, the egg is then beaten in, and finally the flour containing the baking powder is blended into the mixture. In the flour-batter method, the fats are creamed with about an equal quantity of flour, the whisked eggs and sugar beaten in, followed by milk if used, and finally the flour containing the baking powder is blended into the mixture.

In these experiments the glycerinated fats were added in quantities varying between 0·5 and 1·5% of the total batter-weight, either with the fat before creaming, or after the egg was

**Table V**

*Formulae of Madeira cakes*

| | Best quality | Medium quality |
|---|---|---|
| Cake margarine, oz. | 7 | 3 |
| Compound cooking fat, oz. | 1 | 1·5 |
| Sugar, oz. | 8 | 7 |
| Flour, oz. | 10 | 12 |
| Egg, oz. | 10 | 7 |
| Milk, oz. | — | 3·5 |
| Baking powder, oz. | $\frac{1}{14}$ | $\frac{1}{8}$ |

incorporated in the creamed mixture. There did not appear to be any significant advantage in the latter method or in dispersing the additives by melting them with part of the fat before mixing.

It was found with both best- and medium-quality recipes, with either method of mixing, that improvement in volume, texture, crumb brightness and softness (which is related to keeping-quality) of the cakes, was obtained with GMS C or D at a level of about $\frac{1}{2}$ to 1% of the total batter-weight. Similar improvement, usually to a less degree, particularly with respect to softness, was obtained with GMS A or B used as a fine powder. GMS C or D exhibited greatest improvement in cakes prepared by the sugar-batter method, and improved crumb-softness was most apparent with medium-quality cakes; GMS A or B was more efficient in cakes prepared by the flour-batter method. Although the high monoglyceride content of GMS B appeared to be no more effective in these experiments than GMS A containing less monoglyceride, the unsaturated GMS D gave greater improvements than GMS C except to some extent in cakes made by the flour-batter method, when a batter with an oily consistency was formed after the egg and sugar were mixed into the creamed fat and flour. In some cases, GMS D produced an equivalent effect in a quarter of the quantity of GMS C required for optimum improvement. Quantities of the order of about $1\frac{1}{2}$% of the batter-weight, of all additives, tended to be detrimental in one or more of the properties of volume, texture and softness. A sample of glyceryl mono-oleate was found to be slightly more effective than the relatively more saturated GMS C or D in some cakes prepared by the sugar-batter method, but the improvement was not thought to be sufficiently great to justify its use in preference to GMS C or D.

The effect in Madeira cakes of emulsions of the glycerinated fats (1 part : 3 parts water) was very critical. Generally, emulsions of GMS A or B were detrimental to volume and texture, whereas emulsions of GMS C or D produced in some cases greater improvement, particularly with respect to softness, than did the same product when not used as an emulsion. Emulsions of GMS C or D invariably gave lighter batters on mixing, producing cakes of remarkably large volume in the oven, particularly with best-quality cake prepared by the flour-batter method; however, this increase in volume was not maintained when the cakes were cooled, and there were varying degrees of shrinking. In some cases, as for example when cake-flour was used in the flour-batter method, the products were still of better final quality than when emulsions were not used. The additional improvement was most apparent in softness and therefore keeping-quality. Attempts were made to stabilize the light batters produced with GMS C or D emulsions so that the large volumes produced in baking were maintained on cooling. An emulsion of 1 part of water to 1 part of GMS C or D appeared to be more successful than the more aqueous emulsions. With the more aqueous emulsions the addition of wheat starch, dried gluten, or albumen, in quantities of about 1% of the total batter-weight, was of some value; gelatin and methyl cellulose were unsuccessful.

An important point arising from the use of unsaturated esters (GMS C or D) is the occasional offensive odour and flavour of these products, from either the use of poor-quality fatty acid reactant, or decomposition during reaction, or rancidity development on storage. In addition to the undesirability of using such materials, objectionable odour and flavour are transmitted to the cake.

The improvement obtained with unsaturated rather than saturated glycerinated fats has also been found in high-ratio cakes by American workers,[22] who have stated in addition that monoglycerides were much more effective than the mixed glycerides because of the absence of di-esters. Earlier American work[23] showed that improvement, particularly in increased volume, could be obtained with a saturated glycerinated fat.

*Creams*

Glycerinated fats may be used in filling-creams for cakes as one of the emulsifying agents

added to produce stable emulsions. Usually the quantity added is about 1% and they are therefore omitted in Part II of this paper in the toxicological considerations, as weight-for-weight a portion of cream-filled cake will contain the same amount of monoglyceride as when such creams are absent.

### Conclusions and specifications

It has been shown that glycerinated fats possess improving actions in bread and flour confectionery, the extent of their improving action for a given flour quality and product depending on composition and method of incorporation. The following specifications are proposed in the light of the results discussed in this paper:

Table VI consists of analytical standards, based on analyses carried out in this Laboratory by Mr. W. H. Templeton of over 100 samples of commercial GMS, and indicates their suitability for use in bread, sponges and Madeira cakes.

**Table VI**

*Suggested standards for GMS in baking*

| Use | Bread | Sponges | | Madeira cakes | |
|---|---|---|---|---|---|
| | | Frozen or whole egg | Dried egg | | |
| Physical state | powder or flake | powder | powder or flake | powder | fatty |
| *Monoglyceride,[24] % | as high as possible, but not less than 30 for powders or flakes and not less than 20 for waxes | | | | |
| Sodium stearate,[25] % | 1·5 to 3·0 | 0 | ←1·5 to 3·0→ | | |
| Free glycerol,[25] % | ←less than 5→ | | | | |
| †Acid value[26] | ←less than 5→ | | | | |
| †Iodine value[26] | ←less than 5→ | | | | 30 to 100 |
| M.p., °C[26] | ←not less than 55→ | | | | 30 to 50 |

Powders or flakes should be colourless, and practically odourless; waxes should be not deeper in colour than amber, and their odour and taste should not be objectionable

* The washing procedure, which is essential in the determination, was carried out by the method given in the British Pharmaceutical Codex[25]

† The acid and iodine values were determined on the washed material, which is then melted, filtered and dried

## Part II: The Influence of Flour Oils on the Behaviour of Glycerinated Fats in Baking, and the Effect of Natural Monoglycerides present in Flour Oils and Baking Fats on the Pharmacological Desirability of using Glycerinated Fats in Baked Products

[J. B. M. Coppock, M. A. Cookson, D. H. Laney and (in part) D. W. E. Axford]

### Some properties of flour oil

A factor frequently overlooked in considering the role of oils, fats and related products in baking is the oil naturally contained in flour. This oil, which may be extracted by digesting flour with solvents such as carbon tetrachloride or ethyl ether, is a complex mixture, and its removal considerably alters the properties of the residual flour. In the first place coloured pigments are removed together with the oil, to leave a whiter flour which has also become finer in particle size. A loaf baked from this flour, and yeast, salt and water only, possesses a far brighter crumb; moreover it is greater in volume and more even in texture than a loaf prepared under identical conditions from non-solvent-extracted flour. In experiments we have

made with flour milled to 81% extraction, this result is obtained whatever the treatment the flour has received, although the effect is greater with unbleached and untreated flour. Such loaves are usually not as soft as when the natural flour-oil is present. It might be thought that the addition of a normal bakery fat or glycerinated fat, both of which are good crumb-softening agents, to such flour in bread making would produce a loaf as soft or softer, depending on the quantities used, than the original non-extracted flour; however, this is not so, and a poorer loaf is obtained.

Conflicting results have been reported,[27] however, some workers stating that the bread prepared from defatted flour was tougher and smaller in volume. Unfortunately, in some of this work it is difficult or impossible to trace the degree of extraction to which the flours used were milled, their subsequent treatment and the formulae of the breads prepared. It would appear, therefore, that there may be a number of different substances present in flour oils which, according to their relative quantity and possibly the origin of the flour, influence such properties as loaf volume, crumb texture and softness.

Large quantities (70 lb.) of flours were extracted with carbon tetrachloride for about 24 hours at room temperature, filtered, and the residual flours washed with ethyl ether and air-dried. The mixed solvents were removed by distillation and the remaining oils stored in the dark in glass-stoppered bottles. It was observed that after a few weeks' storage a precipitate began to form; after about eight months it was filtered off free of the oil. This precipitate was produced when the flour extracted was either unbleached and untreated, or when the flour was agenized, and it continued to be deposited after the filtration mentioned above. Unlike the material described by Moran *et al.*,[28] obtained from flour treated with chlorine dioxide (10 times normal level), it appeared to be particulate. About a third of this substance was ether-insoluble and from the ether-soluble part approximately half was precipitable with acetone; a further fraction was obtained from the acetone-soluble portion by concentration. These materials are being further investigated.

The fraction precipitated by acetone was found to have a remarkable effect on loaf volume and crumb softness. Whereas GMS depresses loaf volume and increases crumb firmness when added to the solvent-extracted flour at a level of 8 oz./280 lb. flour (0·18%), the addition of 0·003% (expressed on the flour weight) of the fraction precipitated by acetone improved loaf volume and crumb softness to a degree beyond that obtained when the flour oil as a whole was returned to the defatted flour. The level of the addition was estimated to be the quantity naturally occurring in the precipitate formed from the flour oil. Fig. 6 shows the changes in loaf volume found; it also shows that GMS exhibits its specific improving effect only in the presence of flour oil, and even one-quarter of the natural quantity present of this oil is sufficient for improving action to be restored.

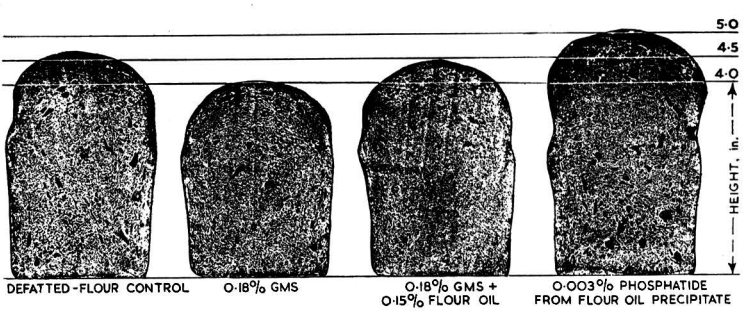


FIG. 6.—*The effect of* 0·18% *of the flour weight of GMS, alone and together with a quarter of the natural flour oil, and also the acetone-insoluble 'phosphatide' fraction separated from the precipitate formed on ageing of the flour oil (in quantity of* 0·003% *of the flour weight), on breads prepared from defatted untreated flour*

It has also been found that lecithin behaves in the same way as GMS when added to defatted flour. Thus it would appear that the phospholipids, and probably other substances in the acetone-insoluble fraction of the flour-oil precipitate, are different from those in lecithin. It would indeed seem that the theories described in Part I to account for the effect of glycerinated fat are in themselves incomplete in the light of the finding that this fraction contains a natural substance essential for the type of bread-improvement under discussion.

*The natural occurrence of monoglycerides in flour oil and fats used in baking*

Kuhrt *et al.*[20] have shown that when bread was baked containing 2·5% of lard (expressed on the flour weight), monoglycerides could be isolated from the bread in a pure form corresponding in quantity to 5·9% of the initial lard present. Although we believe this figure may be too high for reasons described below, nevertheless it would appear that whenever bread has been baked containing fat, the consumer has ingested monoglycerides without harmful effect. Our work has also indicated the presence of naturally occurring monoglycerides in flour oil, so that the consumption of any product containing flour has always involved the ingestion of monoglycerides.

The monoglyceride contents of (*a*) some bakery fats and of the oils obtained from breads containing these fats and (*b*) of the oils extracted from a number of flours both normally treated and overtreated, and of flour–yeast–salt–water breads prepared from them, have been determined by the well-known periodic acid method.[24] This method was initially applied by us on a semi-micro scale to the oils in group (*b*); most of the results quoted were obtained on small quantities of bread and flour oils (0·7–1·5 g.), but the accuracy of the method in the form in which it was applied was verified in certain instances by the use of larger quantities (about 5 g.), when comparable results were obtained. The results on any one oil have been found reproducible within narrow limits.

The apparent monoglyceride content of oils extracted from wholemeal flour, 81%- and 72%-extraction unbleached and untreated flours, ranged from 6·8 to 9·6%. Flours, 81%-extraction, treated with (1) chlorine dioxide at the normal level (30 p.p.m.) and at 10 times this level and (2) agene at the normal level (60 p.p.m.) × 10, showed apparent contents ranging from 6·1 to 8·5%. The apparent percentage of monoglyceride in bread oils extracted from breads prepared from these flours varied between 5·7 and 9·5; and from flours containing 20 p.p.m. of potassium bromate or 160 p.p.m. of ammonium persulphate the results were again within this range. Bread oils isolated from breads prepared by the aeration process[11, 12] showed an apparent monoglyceride content of 6·8 and 7·6%. It would therefore appear safe to conclude that both flour-extraction rate and treatment have little effect on the apparent monoglyceride content of flour and bread oils.

It was clearly of great importance to establish whether these apparent monoglyceride contents were in fact of the order of magnitude suggested by the above determinations. For this reason we have carried out an exhaustive investigation into the validity of the periodic acid method of estimating monoglyceride. This method is open to doubt because although it is generally applicable to compounds containing adjacent hydroxyl groups, other substances, e.g. hydroxy-acids and α-amino-alcohols, are known[29] to react quantitatively with periodic acid. Further, we have found that a large number of additional substances can interfere with this technique. Examples of reacting substances, with the apparent monoglyceride content shown in parentheses, include aliphatic unsaturated compounds such as oleic acid (about 2·5%) and octene-2 (16%); caprylic acid does not interfere, and although benzene and toluene do not react with periodic acid, phenol (about 10%) and the three isomeric cresols (*o*-, 140; *m*-, 126; *p*-, 270%) do. We have, therefore, ascertained to what extent substances interfering with this analytical technique occur in flour oil. The chief potentially interfering substances that might be present in about the same amount as monoglycerides are the sterols, free fatty acids and phospholipids, none of which contain adjacent hydroxyl groups. The interference from oleic acid has already been mentioned; it is known, and we have confirmed, that flour oil contains about 10% of free fatty acids, and this quantity increases with flour ageing.[30] The natural presence of these acids might itself indicate the existence of mono- and di-glycerides, probably formed by the action of wheat lipase on the triglycerides in the flour oil. Phytosterol (1%) and α-tocopherol (87%) also reacted with periodic acid, but the interference of each on the monoglyceride determination should, however, be negligible owing to the small quantities involved. Phosphatides, however, cannot be neglected; the fraction precipitated by acetone from flour oil (about 10% of the total oil) showed an apparent monoglyceride content of 30–40%, thus accounting for about 3% in the total oil. It is of interest to note that samples of commercial soya lecithin (25%) and egg lecithin (18%) differed in the extent of their reaction with periodic acid from the phosphatides of butter fat (4%) and lard (0%).

It is clear, therefore, that the determined (apparent) values for the quantities of monoglycerides in flour and bread oils are at least 3% higher than they should be. We therefore considered it important to establish the presence of monoglycerides by procedures additional to the periodic acid method of analysis.

This was found to be difficult because of the small quantities capable of isolation from these oils by the method of Kuhrt *et al.*[20] These workers isolated much larger quantities from lard and breads containing lard than we have found, and used such identification methods as periodic acid assay, saponification, infra-red spectroscopy and countercurrent distribution. One or more of the following procedures were used by us as aids to the identification of monoglycerides: (1) countercurrent distribution, by which monoglycerides are said to be concentrated in a characteristic manner and (2) periodic acid analysis, followed by (*a*) treatment of the products in the chloroform layer resulting from this assay with 2 : 4-dinitrophenylhydrazine, as it has been found that the oxidized monoglycerides formed reasonably characteristic 2 : 4-dinitrophenylhydrazones (2 : 4-DNPH derivatives), and/or (*b*) treatment of the aqueous layer from the periodic acid assay with chromotropic acid,[31] by which a characteristic violet colour is formed if formaldehyde derived from the glyceryl portion of the monoglyceride is present; in a number of cases the presence of formaldehyde in the aqueous layer from the assay was further confirmed by the preparation of the dimedone derivative by standard procedures.[32]

The method of Kuhrt *et al.* permits both saturated and unsaturated monoglyceride to be isolated from an oil or fat by solvent fractionation. This comprises the following stages: (1) The fat or oil is dissolved in acetone, any precipitate (of phosphatide etc.) removed, and the acetone distilled. (2) The residue is dissolved in ethyl ether, and the solution is well washed with water until emulsions resulting from this process no longer form. With bread and flour oils particularly this stage is difficult, since the centrifuging to break emulsions (in a laboratory Sharples centrifuge at about 25,000 r.p.m.) may frequently take up to 3–4 hours for many of the initial water-washes. The ethereal solution is dried over anhydrous sodium sulphate and the ether removed. (3) The residue is digested three times with warm methanol, the methanol-soluble fractions are separated, and the solvent is removed. As the material treated with acetone in Stage 1 is sometimes itself a methanol extract of an oil or fat, its partial solubility in this solvent at this present stage is of interest. (4) The residue after methanol treatment is dissolved in light petroleum (40–60° C) and the solution cooled to 5° C. Saturated monoglycerides should crystallize out at this stage and if so are removed by filtration. We believe, however, that in the presence of relatively large quantities of other substances, e.g. unsaturated glycerides, this crystallization may be inhibited. The light petroleum is distilled off. (5) The residue is then dissolved in methanol (where, as in Stage 3, complete solution is not always obtained) and water added until the methanol concentration is 70% by volume. The 70% methanol fraction is separated, the process repeated, and the aqueous methanol removed by distillation. This residue is said to be unsaturated monoglycerides.

The procedure described above has been modified in certain essential details for the treatment of flour and bread oil. Thus, free fatty acids tend to contaminate the monoglyceride fractions throughout the separation, and therefore at Stage 2 the earlier water-washes are replaced by washing with 5% aqueous sodium bicarbonate. It has also been found an advantage to overcome apparent modification causing abnormal partial solubility of the residues at various stages by carrying out all operations involving heat in an atmosphere of nitrogen.

Table VII summarizes the information obtained by us on a variety of oils and fats by the procedure described above.

The results shown in Table VII differ in several ways from those found by Kuhrt *et al.*[20] for lard and breads containing lard, notably in the smaller quantity of monoglycerides recovered and the lower purity of the unsaturated monoglycerides (70%-methanol-soluble fractions), as indicated by periodic acid analysis. Kuhrt's procedures were followed closely, including the use of his formulae for preparing the breads containing lard, the only real difference being in the samples of lard that were used, although they were both ' prime steam rendered '. In addition, we did not find any substantial increase in the quantity of monoglyceride formed on baking when we examined the oil from breads made either without added fat, or with hardened palm-kernel oil at an even higher level of usage than for lard breads.

The sample of flour oil, washed with alkali as described above to remove free fatty acid, did not contain saturated monoglyceride, but appeared to contain the equivalent of about 0·25% of unsaturated monoglyceride. Although this percentage is probably low, for it can be seen that even with an authentic monoglyceride mixture recovery of the individual monoglycerides was no greater than about 80%, it is considerably less than the apparent percentage of monoglyceride determined by direct periodic acid assay of flour oil.

In order to learn more of the constitution of the apparent monoglyceride fraction soluble in 70% methanol the procedure for countercurrent distribution, referred to earlier, was introduced here.

## Table VII

*The materials used, and the quantities recovered by countercurrent separation, for certain oils and fats, expressed (a) by weight, g., and (b) by periodic acid analysis, %*

| Product examined | Quantity extracted with methanol (if applicable) | Material extracted with acetone | | Recovery of saturated monoglycerides (light-petroleum fraction insoluble at 5° c) | | Recovery of unsaturated monoglycerides (70%-methanol-soluble fraction) | |
|---|---|---|---|---|---|---|---|
| | | (a) | (b) | (a) | (b) | (a) | (b) |
| Known mixture of saturated and unsaturated monoglycerides | — | 3·57 | 27·5 | 0·46 | 84·0 | 0·30 | 58·0 |
| Hardened palm-kernel oil | 1000 | 8·34 | 0·2 | 0·06* | 88·0 | 2·52 | 4·5 |
| Bread made with 12% (based on flour) hardened palm-kernel oil† | — | 20 | 0·92 | 0·01 | — | 1·51 | 2·1 |
| Lard, prime steam-rendered | 2000 | 5·51 | 0·14 | 0·02 | — | 0·28 | 12·0 |
| Bread made with 6% (based on flour) of lard | — | 83 | 0·35 | trace | — | 1·61 | 7·7 |
| Oil (obtained by carbon tetrachloride extraction) from untreated and unbleached flour | 45·0 | 13·98 | 9·3 | 0 | — | 0·48 | 28·0 |
| Oil (obtained by carbon tetrachloride extraction) from a simple flour–yeast–salt–water bread prepared from the above-mentioned flour | 50·0 | 12·26 | 12·7 | 0 | — | 0·96 | 22·6 |

* M.p. 63° c after recrystallization 4 times with light petroleum; m.p. glyceryl monolaurate, 63° c

† This bread contained twice as much shortening as a suggested U.S.A. formula for canned bread.[44] 98 g. of fat was extracted from a bread containing about 125 g. of hardened palm-kernel oil

Fig. 7 shows the distribution curve obtained by partitioning between *n*-hexane and 85% methanol in 25 tubes. It will be observed that peaks occur at tubes 1, 5, 19 and 23 and possibly also at tubes 9 and 15; that at tube 5 is said by various workers[20, 33] to be characteristic of monoglycerides. The materials in the tubes at some of the peaks, together with the substances in the tubes immediately adjacent to the peak values, were subjected to periodic acid assay followed by the preparation of the 2 : 4-DNPH derivatives from the material in the chloroform layers. Formaldehyde in the aqueous layer from the assay was characterized with chromotropic acid. The 2 : 4-DNPH derivatives were obtained by drying the chloroform layer with anhydrous sodium sulphate, removing the solvent, and treating the residue with 2 : 4-dinitrophenylhydrazine in ethanol containing a trace of hydrochloric acid. The results are shown in Table VIII; the melting points of the 2 : 4-DNPH derivatives of the periodic acid reaction products should be compared with those of authentic samples obtained

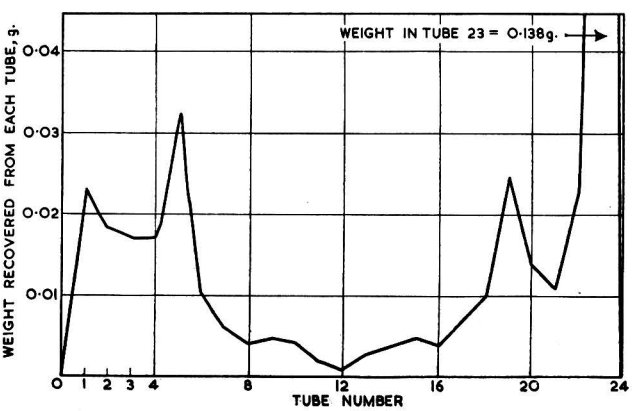


FIG. 7.—*Flour-oil fraction (from unbleached and untreated flour) distributed between 85% methanol and n-hexane. Weight taken = 0·36 g.*

from glyceryl monostearate and mono-oleate. According to Pohle *et al.*[29] these esters, on oxidation, should yield aldehydes of the form $R{\cdot}O{\cdot}CH_2{\cdot}CHO$, where R is stearyl or oleyl. The oleyl compound may, however, further react at the double bond to give aldehydes of the types $CH_3{\cdot}[CH_2]_6{\cdot}CHO$ and $CHO{\cdot}CH_2{\cdot}O{\cdot}CO{\cdot}[CH_2]_8{\cdot}CHO$, which would account for some of the mixed crystals observed, although we are aware that complex systems of crystals have been reported for 2 : 4-DNPH derivatives for other reasons.[34]

Various samples of glyceryl monostearate and mono-oleate have yielded 2 : 4-DNPH derivatives of their oxidation products possessing colours ranging from yellow to red, and melting (with decomposition) between 310° and 330° C. Other materials that gave a reaction with periodic acid did not yield 2 : 4-DNPH derivatives within this range of melting, e.g. phytosterol (peach-coloured plates, 134° C); oleic acid (red oil); butter-fat phosphatides (fawn-coloured solid, 46° C); soya lecithin (dark-red solid, 50° C). The phosphatidic fraction precipitated by acetone from flour oil gave mixed crystals, mainly red, melting (with decomposition) between 250° and 270° C. This may indicate the existence of a phosphatide–monoglyceride complex in flour oil and explain the peculiar crumb-softening effect, referred to earlier, that this material possesses.

**Table VIII**

*Properties of fractions obtained by countercurrent separation of the unsaturated monoglyceride concentrate of the oil from an untreated flour*

| Tube No. | Apparent monoglyceride (periodic acid assay), % | 2 : 4-DNPH derivative, m.p. ° C | Reaction with chromotropic acid |
|---|---|---|---|
| 0–3 | 63 | 275–300 | + |
| 4–7 | 62 | 260–290 | + |
| 8–16 | 31 | 250–280 | – |

The results given in Table VIII for tubes 4–7, which are equivalent to the second peak in Fig. 7 with a maximum at tube 5, can reasonably be accepted as a confirmation of the presence of monoglyceride in flour oil. Nevertheless, some explanation is necessary for the peak at tube 1, which, with the adjacent tubes, yields a material of 63% monoglyceride content, and gives periodic acid reaction products virtually identical with those of tubes 4–7. The most probable explanations are:

(i) That there is a mixture of two or more substances in tubes 0–3, one of which is capable of reacting with periodic acid and which shows an apparent monoglyceride content of 63% although present in smaller amount than, say, glyceryl mono-oleate (which would be true if the substance had a lower molecular weight). One would also expect to find some of the same monoglyceride as in tubes 4–7, for the substances under a given peak will be spread over a number of tubes, and this would account for the similar melting point and colour of the 2 : 4-DNPH derivatives of the two fractions, these being comparable to those obtained with authentic samples of glyceryl monostearate or mono-oleate.

(ii) That it is an isomeric form of the monoglyceride, possibly $\beta$-mono-olein, with a somewhat different value for its partition coefficient, but which, under the acid conditions of the assay and the production of the 2 : 4-DNPH derivatives, gives apparently identical reaction products.

The results on the tubes 8–16 might again be explained by the continued distribution of residual monoglycerides, particularly as the weights of material involved were small. This in turn would lead to the formation of very small quantities of the periodic acid-assay reaction products; the chromotropic acid colour reaction is possibly too insensitive to detect any formaldehyde.

Fig. 7 also indicates the presence of two other main peaks at tubes 19 and 23. The material in tubes 17–21 was in the form of white crystals, and that in tubes 22–24 was a yellow oil. Although it was thought that the yellow oil might prove to contain di-olein, since di- and tri-glycerides of fatty acids have been reported to be distributed in this position in similar countercurrent experiments, it did not possess the characteristics of di-olein, having an acid value of 33·6, an acetyl value of 17·0 and an ester value of 222·4.

On the basis of the above results the quantities of monoglycerides naturally present as such in flour oils are considerably less than those found by direct periodic acid assay of the oils, and probably do not exceed 0·25% of the oil. This is equivalent to an amount of about 25 p.p.m. of monoglyceride in the flour itself.

*Pharmacological considerations*

Although we have been unable to find the quantities of monoglyceride in the lard and breads containing lard that Kuhrt has reported,[20] nevertheless it can be concluded, both from his work and from that now described on hardened palm-kernel oil and the flour oils, that ever since bread has been eaten some monoglyceride has been consumed from one or more of these sources. Further, in experiments on two human subjects, Kuhrt *et al.*[35] have shown that the ingestion of lipids leads to 37·6% and 50% conversion into monoglycerides in the intestinal tract. Similar results have been obtained by Mattson *et al.*[36] on the lipids recovered from the lumen of the intestines of rats. These results substantiate the views of Frazer,[37] that triglyceride absorption through the intestine requires an emulsion involving monoglycerides, and the work of Reiser *et al.*,[38] who concluded that 55–75% of triglycerides labelled with $^{14}C$ and fed to rabbits were hydrolysed to, and absorbed as, monoglycerides. Further evidence obtained by Reiser & Williams[39] has indicated that 73% of monoglycerides, when fed as such through a cannula to rats, was hydrolysed, and subsequently converted into triglycerides, probably in the intestinal mucosa.

Thus, in adding glycerinated fat to any food there are substantial reasons for believing that no abnormal substance foreign to the body is being introduced. Nevertheless, because bread, and to a somewhat less extent cake, are essential dietary constituents it is necessary to examine the quantities of glycerinated fat that might be used in their preparation and consequently the scale of their ingestion.

In his consideration of substances that might be added to food, Frazer[40–42] believes that indications of the levels safe for human consumption might be obtained by analogy with drug dosages. Thus approximately ten-fold increases in amount might be reasonably assumed when progressing in the following stages: (1) food additive level, or acceptable dose, which is functionally satisfactory, (2) ineffective level toxicologically, which is still harmless, (3) effective therapeutic level which could safely be used medicinally, (4) toxic level and (5) lethal level. These views are generally in agreement with those expressed by Lehmann.[43] Thus a criterion for food additives is that a hundred-fold increase in dose over the functional level (1) should still be non-toxic, i.e. below the toxic E.D.50. This criterion of ' hundred-fold acceptability ', which itself excludes the risk of direct toxic action, requires that there shall be no significant difference noticeable in animal tests carried out in several different species when the test substance is administered at (chronic) dosage levels 100 times the standard dietary dose. Frazer[41] has shown in acceptability tests designed to meet this criterion, and including life-span and multi-generation tests, that the acceptability level for glyceryl monostearate is approximately 2000 mg./kg. of body weight. No direct evidence of cumulation of glyceryl monostearate could be found in the life-span studies.

If we assume an addition of 4 oz. of glycerinated fat (commercial GMS as commonly used in this country contains about 30% of real glyceryl monostearate) per sack (280 lb.) of flour, this means in effect that if a man (weighing about 80 kg.) consumes 1 lb. of bread per day he will ingest 1·2 mg./kg. of body weight of real monostearate derived from the additive. Should the person also eat per day ¼ lb. of cake in which glycerinated fat is added at an average amount of 0·75%, expressed on the batter-weight, he would consume a further 3·5 mg./kg. of body weight, or a total per day of about 5 mg./kg. of body weight in the form of added glyceryl monostearate. This is approximately 400 times less than the experimentally determined acceptability level. Even if bread contains 8 oz./sack of glycerinated fat the safety factor would be 300.

In addition, however, the effect of the natural monoglycerides in flour oil and in fats that may be added to baked goods, including the decomposition of such fats into monoglycerides during baking, must be taken into account. The contribution from the flour oils would not appear to exceed about 0·1 mg./kg. of body weight. Further, on the basis of our own work, the contribution from fat contained in 1 lb. of bread at the rate of 2 lb./280 lb. of flour, and also from ¼ lb. of cake containing 25% of fat, would be less than 0·01 mg. and 0·07 mg./kg. of body weight respectively. Thus, our work suggests that even if all these monoglyceride contributions are added together, and are regarded as having nearly the same acceptable dosage level as glyceryl monostearate, the safety factor would be at least 250.

It must be pointed out, however, that, if Kuhrt's results on monoglycerides in lard, and the increased quantities of monoglycerides formed when breads containing lard are baked, were regarded as generally applicable to other fats and baked products, then the contribution of monoglycerides from the fats in 1 lb. of bread (containing 2 lb. of fat/280 lb. of flour) and ¼ lb. of cake (containing 25% of fat), would be nearly twice as great as Frazer's acceptability level for glyceryl monostearate, i.e. the safety factor is reduced to 50. If we assume the

conversion of fat into monoglyceride is about 5% in bread making and 10% in cake making, there would be about 2 mg. and 35 mg. respectively of monoglycerides per kg. of body weight ingested from these products. Our work leads us, however, to doubt whether Kuhrt's findings on the lards he examined are generally applicable to oils and fats and breads containing them. If this view is accepted it is clear that the use of glycerinated fat as a food additive in the ways described in this paper is well within the criterion of ' hundred-fold acceptability ' advocated by Frazer & Lehmann.

In addition, Mattson *et al.*[45] have shown that, except for differences in caloric value, the mono-, di- and tri-glycerides of corresponding fatty-acid composition are nutritionally equivalent in rat-feeding experiments. However, even if glyceryl monostearate in no way contributed to the caloric intake, the use in bread of glycerinated fat at the rate of 4 oz./sack of flour would not reduce the nutritive value in terms of bread weight by as much as one-tenth of one per cent.,[5] so that the incorporation of glycerinated fat in the manner suggested would appear neither harmful to man nor likely to impair his nutrition. It should always be remembered in this connexion that, in common with flour improvers, bread improvers, of which GMS is one, are self-limiting in quantity and excessive use leads to overtreatment and makes the bread unsuitable for sale. It may be concluded that the use of glycerinated fats in baking in the manner described in Part I of this paper holds no risk to the consumer, nor even at somewhat greater levels of inclusion than those discussed.

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Herts.

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# STUDIES OF LACTIC ACID BACTERIA ASSOCIATED WITH BREWERY PRODUCTS. I.—Identification of Types Isolated from Beer and from Yeast

**By R. R. BHANDARI, C. RUSSELL* and T. K. WALKER**

A survey has been made of a selection of the lactic acid bacteria occurring in 12 samples of yeast and 9 samples of beer from British breweries. Strains of 8 known species were identified and these included *Lactobacillus buchneri, Lb. bifidus, Lb. leichmannii, Lb. plantarum* and *Streptococcus cremoris*, none of which has hitherto been detected in beer. In addition, three rod-shaped organisms which were isolated proved to be the type cultures of three new species of *Lactobacillus*. Five cocci which were isolated were found to be related to *Pediococcus damnosus* var. *salicinaceus* Mees and have been classified as strains of this species. These organisms had very small cells, those of *Ped. damnosus* var. *salicinaceus* itself being relatively much greater in diameter.

In 1943, Walker & Parker[1] described the isolation of more than 30 cultures of lactic acid-forming bacteria from nine different ' top-fermentation ' beers and one pitching yeast. Subsequently, a selection was made of those organisms which appeared of sufficient interest to justify further study. These were A1(12), C1(2), D2(4), D4(6), D6(13), D7(1), E3(11), G1(15), G2(20), G3(22), G4(23) and G5(14). The letter followed by a number is the provisional designation taken from the original paper of Walker & Parker ;[1] the number shown in parentheses is that by which reference will be made to the particular organism in this and later communications. In addition to these organisms, others were isolated from brewery yeasts during 1946 and were designated respectively, $\alpha$, $\beta$, C, D, K, L, W1, W4, W5, W7 and W10. Finally, in 1949, a third series of lactic acid-producing bacteria were isolated from a selection of eight yeasts from different breweries. These last organisms were coded as follows: AA1–AA7, BB1–BB5, CC1–CC8, DD1, DD2, DD4, DD5, EE1–EE6, FF1–FF3, FF6, GG1–GG6 and HH1–HH6. Thus a total of 69 cultures was made available as a basis for a survey of the lactic acid-producing bacteria of top-fermentation breweries in this country.

Shimwell[2, 3] has described in detail the *Lactobacillus pastorianus* infection in British beers. He has also made contributions to our knowledge of the beer cocci and Shimwell & Kirkpatrick[4] have assigned this group of organisms to the genus *Streptococcus*. Apart from these studies the only recent observations in this field have been those of Kulka, Cosbie & Walker,[5] who reported the occurrence in beer of a new ' rope-forming ' coccus, *Streptococcus mucilaginosus*, and of Andrews & Gilliland,[6] who have described a new variety of *Lb. pastorianus* and one of *Pediococcus damnosus*, both of which have the ability to hydrolyse dextrin.

## Experimental

All the organisms included in the present study were shown by preliminary examination to be members of the family Lactobacteriaceae. Thus, both the rod forms and the cocci were Gram-positive, heterotrophic, facultative anaerobes. They were all non-sporing, non-motile, catalase-negative and unable to reduce nitrate. They all fermented sugars with formation of lactic acid.

The organisms were then divided into groups in accordance with their morphological features, their homo- or hetero-fermentative activity towards glucose, and their behaviour at different temperatures and pH values. In carrying out these tests the medium employed was unhopped beer with addition of glucose (1% w/v), except when fermentation was studied, where a double digest of casein[7] was used. Scheme I shows the grouping of the rod-shaped organisms on this basis.

**Scheme I**

*Rod-shaped organisms*

Homofermentative

| Growth at 48°, not 50° | Growth at 43°, not 46° | Growth at 38°, not 40° | Growth at 35°, not 38° | Growth at 30°, not 33° |
|---|---|---|---|---|
| AA1, AA3, AA5, AA6, AA7 | AA2, AA4, EE4, EE5 | — | CC2, CC7, CC8, GG2, GG4, GG6 | — |
| P1 | P1 | | P6 | |

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**Scheme I** (*contd.*)

*Rod-shaped organisms*

Heterofermentative

Growth at

| 48°, not 50° | 43°, not 46° | 38°, not 40° | 35°, not 38° | 30°, not 33° |
|---|---|---|---|---|
| CC3, HH6, α, β, C, K, 1, 2 | DD1, DD5, EE1, EE2, EE3, EE6, FF3 | BB1–BB5, CC1, DD2, DD4, HH4, W1, W4, W5, D, L, 4, 6, 12, 13, 14, 20, 22, 23 | CC4, CC5, W7, W10 | HH1, HH2, HH3, HH5 |
| P2 | P3 | | P4 | P7 |

| Growth below pH 4·1 : all except 4, 6, 23 | No growth below pH 4·1 : 4, 6, 23 |
|---|---|
| P4 | P5 |

The groups thus differentiated were labelled P1–P7. The organisms within each of these groups, except those within P5, were similar to each other in their morphological, physiological and biochemical characters. Comparisons were made of the organisms of each group with named species of *Lactobacillus* described in the literature (particularly by Breed *et al.*,[8] Shimwell[9] and Pederson[10]). This enabled us in most instances to identify the organisms of a given group as strains of a known species. These identifications are shown in Scheme II.

**Scheme II**

*Rod-shaped organisms*

| Group | Identified as strains of: |
|---|---|
| P1 | *Lb. leichmannii* Bergey *et al.* |
| P2 | *Lb. buchneri* Henneberg |
| P3 | *Lb. bifidus* Tissier |
| P4 | *Lb. pastorianus* Van Laer |
| P6 | *Lb. plantarum* Orla-Jensen |

The organisms of group P7 were found to be strains of a new species, which we have named *Lactobacillus frigidus*, a description of which has recently appeared.[11]

Group P5 was subdivided according to the behaviour of its members, as shown in Scheme III. Organism 23 was identified as a pH-sensitive strain of *Lb. pastorianus*. Organisms 4 and 6 were entirely different from each other and from any named species of *Lactobacillus* cited in the literature. Organism 4 has been designated *Lactobacillus malefermentans* and organism 6 has been named *Lactobacillus parvus*. These new species have formed the subjects of separate communications.[12, 13]

**Scheme III**

Group P5

Organisms 4, 6, 23

| Growth in beer at 10° | No growth in beer at 10° |
|---|---|
| 4, 23 | 6 |
| | Length, 1–1·5 μ |

| 4 | 23 |
|---|---|
| Length, 2–6 μ<br>Does not ferment arabinose, xylose, galactose | Length, 7–9 μ<br>Ferments arabinose, xylose, galactose |

The spherical organisms were similarly divided into groups, as depicted in Scheme IV. They were compared with other lactic acid-forming cocci described in the literature and were identified with known species. This identification is shown in Scheme V.

## Scheme IV

*Homofermentative cocci*

| Organism | Size and arrangement of cells | Temperature range of growth | Maximum pH value tolerated | Litmus milk | Nutrient broth | Acetyl-methyl-carbinol | Sugar fermentation |
|---|---|---|---|---|---|---|---|
| 11 | 0·8 μ, mainly tetrads | 15–37° | 6·9 | No action | No growth | Negative | Fructose, salicin |
| 15 | 0·8 μ, mainly pairs | 10–34° | 6·2 | Growth, with separation of clot and acid production | No growth | Positive | Salicin, but not fructose |
| GG3, GG5 | 0·5–0·6 μ, single cells, and pairs | 10–30° | 6·9 | No action | Strong growth (increased viscosity in presence of glucose) | Negative | Glucose, fructose, mannose and salicin only |
| FF2, GG1 | 0·6 μ, mostly pairs | 10–27° | 6·6 | No action | No growth | Positive | Salicin, but not sucrose nor galactose |
| FF1, FF6 | 0·6 μ, mostly pairs | 15–30° | 6·6 | No action | No growth | Positive | Sucrose, galactose and salicin |

NOTE: All organisms, except GG3 and GG5, ferment other sugars in addition to those indicated in the Scheme

## Scheme V

*Homofermentative cocci*

| Provisional designation | Identification or classification |
|---|---|
| 11 | A high-temperature strain of *Ped. damnosus* var. *salicinaceus* Mees |
| 15 | A strain of *Strep. cremoris* Orla-Jensen |
| FF1, FF2, FF6 and GG1 | Classified as strains of *Ped. damnosus* var. *salicinaceus* Mees |
| GG3, GG5 | Strains of *Strep. mucilaginosus* Kulka, Cosbie & Walker |

## Discussion

Lactic acid bacteria which, up to the present time, have been found as contaminants in brewery wort, beer or yeast, comprise strains of *Lb. delbrueckii*, *Lb. leichmannii*, *Lb. pastorianus* (and varieties of this), *Lb. plantarum*, *Ped. acidi lactici*, *Ped. damnosus* (and varieties), *Ped. lindneri*, and *Strep. mucilaginosus*. Of these, according to Shimwell,[14] only strains of *Lb. pastorianus* and of *Ped. damnosus* have been reported to grow in beer. Shimwell has stated his opinion that under systematic investigation beer might be found to possess a rich flora of lactic acid bacteria, both lactobacilli and streptococci, of interesting and perhaps hitherto undiscovered types. On this last point the present survey has confirmed his view, for it has revealed that strains of *Lb. leichmannii* and *Lb. plantarum* can grow in beer as well as in wort, and that, in addition to the organisms listed by Shimwell, strains of *Lb. bifidus*, *Lb. buchneri* and *Strep. cremoris* can also proliferate in beer. The ability of such well-known *Lactobacillus* species to adapt themselves to life in brewery products might have been anticipated in view of the observation of one of us (T. K. W.)[15] some years ago that *Lb. brassicae fermentatae*, *Lb. helveticus* ε, *Lb. casei* and *Lb. pentoaceticus* can be induced to accommodate themselves to cultivation in unhopped beer. Further, two new species, *Lb. malefermentans* and *Lb. parvus* have now been isolated from beer, and one other new species, *Lb. frigidus*, has been separated from brewery yeast.

The organisms described in the present communication probably form a fairly representative cross-section of the lactic acid bacteria of top-fermentation beers and yeasts in this country, inasmuch as these organisms were isolated from 12 different specimens of yeast and 9 different

beers, all of which came from different breweries. The distribution of the organisms was as follows:

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Lb. pastorianus* | 24 | strains | from | 14 | sources | *Lb. plantarum* | 6 | strains | from | 2 sources |
| *Lb. leichmannii* | 9 | ,, | ,, | 2 | ,, | *Ped. damnosus* | 3 | ,, | ,, | 2 ,, |
| *Lb. buchneri* | 8 | ,, | ,, | 6 | ,, | *Strep. mucilaginosus* | 2 | ,, | ,, | 1 ,, |
| *Lb. bifidus* | 7 | ,, | ,, | 3 | ,, | *Strep. cremoris* | 1 | ,, | ,, | 1 ,, |

From these figures it is evident that *Lb. pastorianus* predominates as a beer contaminant.

Some idea of the degrees of infection of the specimens of yeasts and of beers which were examined may be obtained from the following data:

| | | | |
|---|---|---|---|
| Yeast C | yielded | 3 species | *Lb. buchneri, Lb. pastorianus, Lb. plantarum* |
| Yeast D | ,, | 2 ,, | *Lb. bifidus, Lb. pastorianus* |
| Yeast E | ,, | 2 ,, | *Lb. bifidus, Lb. leichmannii* |
| Yeast F | ,, | 2 ,, | *Ped. damnosus, Lb. bifidus* |

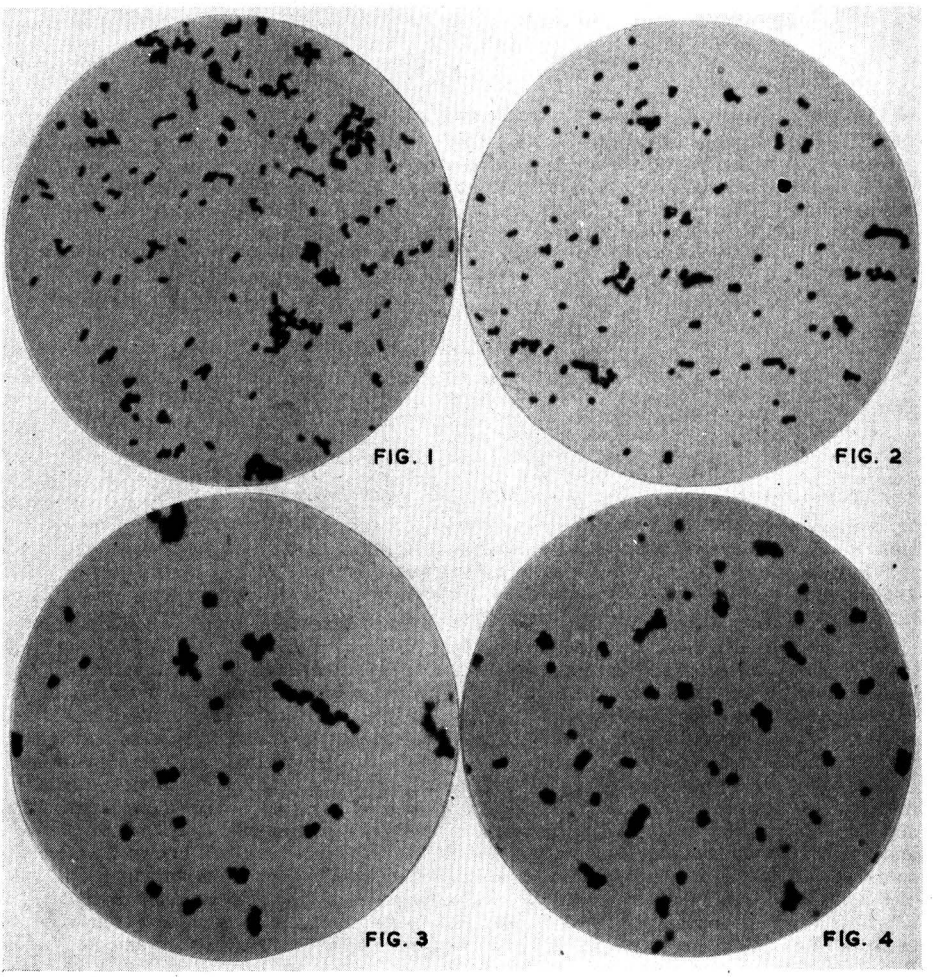


FIG. 1.—Lb. buchneri ×1200
FIG. 2.—Lb. parvus (*new species*) ×1200
FIG. 3.—Strep. cremoris ×1200
FIG. 4.—Ped. damnosus *var.* salicinaceus ×1200

| | | |
|---|---|---|
| Yeast G yielded | 3 species | *Lb. plantarum, Strep. mucilaginosus, Ped. damnosus* |
| Yeast H ,, | 3 ,, | *Lb. buchneri, Lb. pastorianus, Lb. frigidus* (new species) |
| Beer No. 4 ,, | 4 ,, | *Lb. buchneri, Lb. pastorianus, Lb. malefermentans* (new species), *Lb. parvus* (new species) |
| Beer No. 7 ,, | 2 ,, | *Lb. pastorianus, Strep. cremoris* |

This would seem a convenient point at which to refer to present views on the nomenclature of the beer cocci. For the past 68 years many cocci from beer have been classified as species of *Pediococcus*. This generic term came into use in 1884. In 1934 Mees[16] showed clearly that the beer cocci are non-sporulating, Gram-positive, catalase-negative, true lactic acid bacteria, but instead of classifying such cocci as species of *Streptococcus* he preferred the old generic designation *Pediococcus*. Shimwell & Kirkpatrick[4, 17] contend that the beer cocci fall naturally into the plant division of the genus *Streptococcus* and should be classed as such, and they urge that use of the term Pediococcus should be discontinued. Were this course to be followed it would certainly simplify classification in this field. Shimwell has described varieties of *Ped. damnosus* under the respective designations *Strep. damnosus* var. *viscosus* and *Strep. damnosus* var. *limosus*. In a grouping of lactic acid bacteria by Davis & Thiel,[18] *Pediococcus viscosus* and *Pediococcus perniciosus* are placed with *Strep. citrovorus* in *Streptococcus* type III. In a recent private communication to one of us (C. R.), Dr. R. S. Breed states that, although he and Dr. C. S. Pederson agree with Dr. Shimwell's opinion that the beer pediococci should be placed in the tribe with the streptococci, they would not unite the beer cocci in the genus *Streptococcus*, but prefer to keep the two groups separate, retaining the generic title *Pediococcus* for those beer cocci which produce inactive lactic acid. In view of this and of the observations of Pederson[19, 20] on this topic we have used the generic title *Pediococcus* with reference to two different cocci isolated during the present work, since these possessed characters qualifying them for admission to this genus.

Photomicrographs (magnification × 1200) were prepared from slides of four of the organisms isolated and examined in the course of this work. The slides were prepared with cells developed during 72 hours on unhopped beer agar and they were stained with carbofuchsin. The organisms photographed were a strain of *Lb. buchneri* (Fig. 1), the type culture of *Lb. parvus* (new species) (Fig. 2), a strain of *Strep. cremoris* (Fig. 3) and a high-temperature strain of *Ped. damnosus* var. *salicinaceus* (Fig. 4).

In order to obtain information which might be helpful as a means of throwing light on the incidence of brewery infections by lactic acid bacteria, nutritional studies have been carried out with 34 of the organisms mentioned in this communication, and it is hoped to report the results in further papers.

**Acknowledgments**

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# THE RELATIONSHIP BETWEEN THE CONSTITUTION AND THE EFFECT OF CHEMICAL COMPOUNDS ON PLANT GROWTH. IV.*—Derivatives and Analogues of 2-Benzoylbenzoic Acid

By R. L. JONES, T. P. METCALFE and W. A. SEXTON

Selective inhibition of the root growth of germinating seeds is shown by compounds in which a benzene or naphthalene ring is linked in the *ortho*-position to benzoic acid by CO, $CH_2$, NH or CO·NH. Rape is usually more susceptible than wheat. The effect of substitution in the benzene ring and of esterification has been examined. 2-(4-Phenylbenzoyl)benzoic acid and *N*-α-naphthylphthalamic acid were found to be the most active compounds.

Some compounds of these classes abolish the normal geotropic responses of rape and rye-grass roots at concentrations below those at which there is marked inhibition of root growth. High activity was found particularly in the benzoylbenzoic acid and phthalamic acid series. Examination of a few of the compounds reveals that they also affect the phototropic response of shoots of rape and rye-grass.

## Introduction

The effect of 2-benzoylbenzoic acid and a number of its substituted derivatives upon seed germination has already been reported in a brief communication.[1] It was shown that some of these compounds inhibited the germination of seeds of oats and charlock, the latter being much more susceptible than the former. It was found that substitution of chlorine in the benzoic acid nucleus depressed the activity but that substitution of chlorine in the benzoyl radical sometimes enhanced the activity, 2-(4-chlorobenzoyl)benzoic acid being more active than the unchlorinated compound. A further examination of compounds of this class and of certain related compounds, by the use of rape and wheat seeds, has now been made with the dual object of finding more active compounds and of correlating structure with activity. In the course of this work, effects of some of the compounds upon the geotropic and phototropic responses of seedlings were observed; routine test methods for these responses were devised and are described.

## Experimental

*Preparation of compounds*

With three exceptions, all the compounds used in this investigation are fully described in the literature. The exceptions are the ethyl, *n*-propyl, and *n*-butyl pseudo-esters [see below, formula (II)] of 2-(4-chlorobenzoyl)benzoic acid. They were made by the general method described by Meyer[2] and Egerer & Meyer.[3] Analyses and melting points are given below.

| | M.p., °c | Found, % | | Required, % | | Recrystallized from |
|---|---|---|---|---|---|---|
| | | C | H | C | H | |
| Ethyl pseudo-ester | 80–82 | 66·2 | 4·5 | 66·5 | 3·6 | Ethanol |
| *n*-Propyl ,, | 66–8 | 67·8 | 5·2 | 67·5 | 4·95 | 80–100° light petroleum |
| *n*-Butyl ,, | 95–6 | 68·5 | 5·5 | 68·2 | 5·4 | ,, ,, ,, |

*Seed germination test for inhibitory effect upon root growth*

The test method, employing seeds of rape (English Broad-leaved) and wheat (Red Pilot) germinating in agar, is described in the first paper of this series.[4] In the Tables referring to this test, two plus-signs signify the highest order of activity (over half the roots being less than 50% of the length of controls germinating on agar alone). The acids were used in the form of their soluble sodium salts. The neutral substances were dispersed by dissolving in polyethylene glycol (approximate mol. wt. 300) and pouring into water.

*Geotropic test with seedlings*

Rape seed as used in the germination test was quite suitable for the geotropic test on roots, but wheat was not a suitable monocotyledon, since it gives more than one root. For this reason rye-grass (English Leafy Italian), which gives a single root, and is much more sensitive than wheat, was chosen as the monocotyledon. The seeds of rape or rye-grass were allowed to germinate on agar until the roots were 1 cm. long. The seedlings were removed

* Part III: *Biochem. J.*, 1949, **45**, 143

by tweezers and transferred to the surface of agar containing the substance under test. The agar plate was placed in a vertical position in a dark room at a temperature of 68° F. In the controls, the root tip turned downwards and the shoot grew upwards, these changes of direction being apparent after 24 hours. With an antigeotropic compound present at appropriate concentration in the agar, the roots continued to grow horizontally (Fig. 1). In Table III a minus sign signifies no difference from the controls. A single plus-sign indicates that most of the roots were at an angle of about 45° to the vertical, i.e. there was partial activity. Two plus-signs indicate full activity, the root extending horizontally. About a dozen seedlings were used in each test and the result was available in 24 hours after the transference to test agar. It will be noted from Fig. 1 that both shoots and roots were affected, but it is our general experience that shoots are considerably less sensitive than roots.

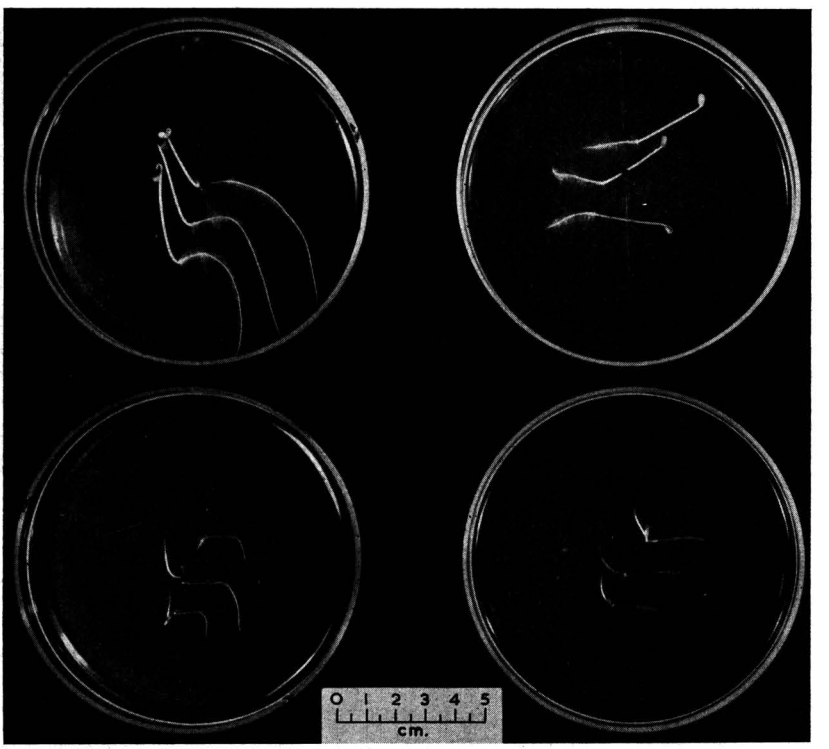



FIG. 1.—*Antigeotropic effects with rape seedlings (top) and rye-grass (bottom), showing controls (left) and the influence of an active compound (right)*

*Phototropic test with seedlings*

In this test the effect of the various substances on the positive phototropic response of shoots was examined. Waxed-paper cartons, 3 in. × 4 in. × 2 in., were filled to a depth of 1½ in. with coarse silver sand. Seeds of rape or rye-grass were sown in two parallel lines on the surface and covered by ¼ in. of silver sand. The cartons were watered with 80 ml. of the test solution (or water, for controls) and placed in a long box, painted mat black inside. Light from a 6-watt bulb was admitted through a slot 1 in. × 1½ in. cut in the side of the box at a level with the top of the cartons. Shoots of the control seedlings bent towards the light, whereas in the presence of an 'antiphototropic' compound the shoots grew in a random manner and were unaffected by the light (Fig. 2). As before, the degree of response is indicated by one or two plus-signs.

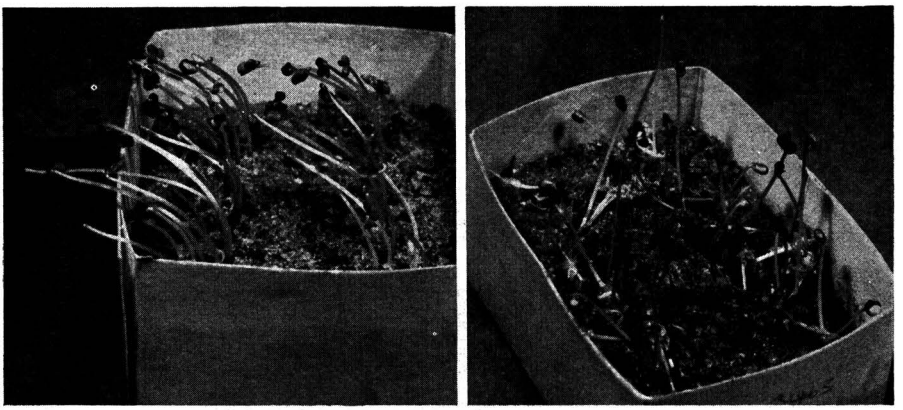


FIG. 2.—*Antiphototropic effect with rape seedlings. Normal untreated seeds (left) and seeds grown in the presence of an active compound (right). Both boxes illuminated from one side*

## Results and discussion

The methods employed were designed for the rapid examination of large numbers of chemical compounds, in order to select those individuals or groups that require more careful and detailed study. The quantitative aspects of the results must therefore be taken with some reserve, and no significance is attached to small differences in activity that may well be beyond the limits of error of the experimental technique.

*Toxicity (inhibition of root growth)*

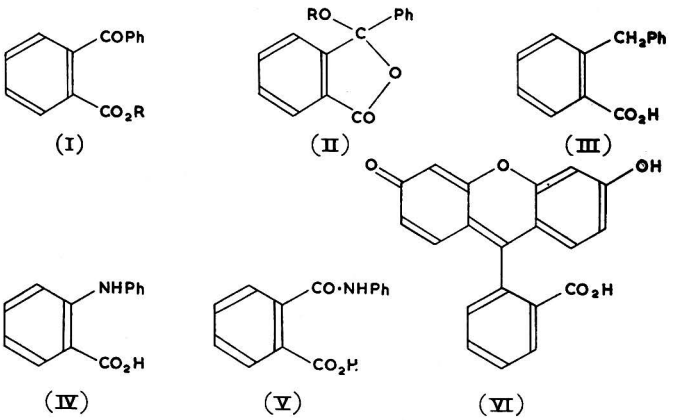
The results of seed-germination tests on derivatives of 2-benzoylbenzoic acid are recorded in Table I. The most active compound appeared to be the diphenyl derivative, 2-(4-phenylbenzoyl)benzoic acid, which at least equalled in potency the 4-chlorobenzoyl compound, the most active one of the series recorded by Sexton & Templeman.[1] Benzoylbenzoic acid gives rise to two series of esters, the normal esters (I) and the pseudo-esters (II). The esters were compared with the free acid for the 4-chlorobenzoyl compound.

### Table I

*Effect of 2-benzoylbenzoic acid derivatives on the root length of germinating rape and wheat*

| Compound | Concn., p.p.m. | Rape | Wheat |
|---|---|---|---|
| 2-Benzoylbenzoic acid | 50 | + | + |
| | 10 | + | − |
| 2-(*p*-Tolyl)benzoic acid | 50 | + | − |
| | 10 | + | |
| 2-(2 : 4-Dichlorobenzoyl)-benzoic acid | 50 | + | − |
| 2-(3 : 4-Dichlorobenzoyl)-benzoic acid | 50 | ++ | + |
| | 10 | + | − |
| 2-(α-Naphthoyl)benzoic acid | 50 | ++ | + |
| | 10 | + | − |
| 2-(4-Phenylbenzoyl)-benzoic acid | 50 | ++ | ++ |
| | 10 | ++ | + |
| | 1 | + | − |
| 2-(4-Chlorobenzoyl)-benzoic acid (A) | 10 | ++ | − |
| | 1 | + | − |
| Normal esters of (A) | | | |
| Methyl | 10 | ++ | − |
| | 1 | − | |
| Ethyl | 10 | + | ± |
| Propyl | 10 | − | − |
| Butyl | 10 | + | + |
| Pseudo-esters of (A) | | | |
| Methyl | 50 | ++ | − |
| | 10 | − | |
| Ethyl | 50 | ++ | − |
| | 10 | + | − |
| Propyl | 50 | ++ | + |
| | 10 | + | − |
| Butyl | 50 | − | − |

On the whole, the esters of both series were less active than the acid, and there was no obvious difference between the normal and pseudo- series. This perhaps would be expected if the activity of the esters was due to their hydrolysis to the free acid, which is of type (I) (R = H). That the pseudo-structure (II) plays no significant role in determining the activity




is further supported by tests with the 2-benzylbenzoic acids (Table II), where no such tautomeric structure is possible. Indeed, activity is shown by several types of compound in which benzoic acid is linked in the *ortho*-position through a linking group to another benzene ring. The results in Table II include 2-benzylbenzoic acids (III), 2-carboxydiphenylamines (IV) and *N*-arylphthalamic acids (V). The compounds as a whole were more active against rape than against wheat, though there are one or two exceptions. The effect of substituents on the benzene ring not bearing the $CO_2H$ group, however, varies with the nature of the linking group, CO, $CH_2$, NH or CO·NH. Thus, for example, the high activity conferred by a 4′-phenyl substituent in 2-benzoylbenzoic acid is not shown in 2-benzylbenzoic acid ; 4′-chloro increases the activity in benzoylbenzoic acid and carboxydiphenylamine, but not in benzylbenzoic acid or *N*-phenylphthalamic acid. The effect of linking the rings in positions other than *ortho* to the $CO_2H$ was not thoroughly examined, but *p*-benzoylbenzoic acid was practically inactive at 50 p.p.m.

*N*-Arylphthalamic acids were first applied to growing plants by Hoffman & Smith.[5] On

## Table II

*Effect of 2-benzylbenzoic acids, diphenylaminecarboxylicacids, and N-arylphthalamic acids on the root length of germinating rape and wheat*

| Compound | Concn., p.p.m. | Rape | Wheat |
|---|---|---|---|
| 2-Benzylbenzoic acid | 50 | ++ | + |
| | 10 | + | − |
| 2-(4-Methylbenzyl)benzoic acid | 50 | + | + |
| | 10 | − | ± |
| 2-(4-Chlorobenzyl)benzoic acid | 50 | ++ | + |
| | 10 | + | + |
| 2-(4-Phenylbenzyl)benzoic acid | 10 | + | ± |
| 2-(4-Methoxybenzyl)-benzoic acid | 50 | ++ | − |
| | 10 | ± | − |
| 2-Carboxydiphenylamine | 50 | ++ | + |
| | 10 | − | − |
| 2-Carboxy-4′-chloro-diphenylamine | 50 | ++ | ++ |
| | 10 | + | + |
| 2-Carboxy-4′-methoxy-diphenylamine | 50 | + | + |
| 2-Carboxyphenyl-α-naphthylamine | 50 | ++ | + |
| | 10 | + | |
| Phenylphthalamic acid | 50 | + | + |
| *o*-Chlorophenylphthalamic acid | 50 | ++ | − |
| | 10 | + | |
| *p*-Chlorophenylphthalamic acid | 50 | + | + |
| | 10 | − | − |
| 2 : 4-Dichlorophenyl-phthalamic acid | 50 | ++ | ++ |
| | 10 | + | + |
| 2 : 5-Dichlorophenyl-phthalamic acid | 50 | + | + |
| | 10 | + | − |
| 3 : 4-Dichlorophenyl-phthalamic acid | 50 | + | + |
| *o*-Tolylphthalamic acid | 50 | ++ | − |
| | 10 | ++ | |
| | 1 | − | |
| *p*-Tolylphthalamic acid | 50 | ++ | + |
| | 10 | + | |
| *p*-Nitrophenylphthalamic acid | 50 | + | + |
| α-Naphthylphthalamic acid | 50 | ++ | + |
| | 10 | ++ | + |
| | 1 | + | + |
| Fluorescein | 10 | ++ | + |
| | 1 | + | − |
| Eosin | 10 | ++ | + |
| | 1 | + | − |

tomatoes, these authors observed effects on fruit setting and also formative effects. Substituent groups in the aryl nucleus such as halogens, nitro and methyl often increased the activity, and α-naphthylphthalamic acid was unique among the series examined. This compound completely inhibited fruit-set at 20 p.p.m., the phenyl compound being inactive in this respect at a hundred times the concentration. Further, at less than 1 p.p.m. α-naphthylphthalamic acid produced epinastic responses. We have examined the arylphthalamic acids by the seed-germination technique because of their structural relationship to the 2-benzoylbenzoic acids. In our tests (Table II), the α-naphthyl compound was also the most active of those examined.

*Inhibition of geotropic and phototropic responses of seedlings*

A further important biological response of plants to α-naphthylphthalamic acid has been recorded by Mentzer *et al.*[6] The French workers observed an abolition of the normal geotropic responses of seedlings. We have also observed abolition of geotropic response and have extended the observation to the phototropic response with these and other compounds described in this paper. Our results are summarized in Table III. In addition to the compounds listed in Table III, several others which are known to exhibit auxin-like activity were also examined in the geotropic and phototropic tests. It frequently happened, however, that possible activity in these tests was masked by the toxicity of the compounds at the concentrations examined. This applied, for example, to 2 : 4-dichlorophenoxyacetic acid, β-naphthoxyacetic acid and α-naphthylacetic acid.

**Table III**

*Effect of various compounds on the geotropic and phototropic responses of seedlings*

| Compound | Concn., p.p.m. | Geotropism | | Phototropism | |
|---|---|---|---|---|---|
| | | Rape | Rye-grass | Rape | Rye-grass |
| Phenylphthalamic acid | 50 | | | − | − |
| | 10 | − | − | | |
| *o*-Tolylphthalamic acid | 10 | + | − | | |
| *p*-Tolylphthalamic acid | 10 | + | + | | |
| *o*-Chlorophenylphthalamic acid | 10 | ++ | ++ | | |
| | 1 | + | − | | |
| | 0·5 | + | | | |
| *p*-Chlorophenylphthalamic acid | 20 | | | − | + |
| | 10 | + | ++ | | |
| | 5 | − | ++ | | |
| | 1 | | − | | |
| *p*-Nitrophenylphthalamic acid | 10 | − | − | | |
| 2 : 4-Dichlorophenylphthalamic acid | 20 | | | + | − |
| | 10 | ++ | ++ | | |
| | 1 | ++ | − | | |
| | 0·5 | ++ | | | |
| | 0·1 | − | | | |
| 3 : 4-Dichlorophenylphthalamic acid | 10 | ++ | ++ | | |
| | 1 | − | | | |
| α-Naphthylphthalamic acid | 5 | | | ++ | + |
| | 1 | ++ | ++ | − | |
| | 0·1 | ++ | + | | |
| 2-Benzoylbenzoic acid | 10 | ++ | + | | |
| | 5 | − | | | |
| 2-(*p*-Tolyl)benzoic acid | 10 | ++ | ++ | | |
| | 1 | ++ | ++ | | |
| | 0·1 | − | | | |
| 2-(4-Chlorobenzoyl)benzoic acid | 50 | | | + | − |
| | 1 | ++ | ++ | | |
| | 0·1 | − | | | |
| 2-(4-Chlorobenzoyl)benzoic acid normal methyl ester | 10 | − | − | | |
| 2-(4-Chlorobenzoyl)benzoic acid pseudo methyl ester | 10 | ++ | + | | |
| | 1 | − | | | |
| 2-(4-Phenylbenzoyl)benzoic acid | 10 | ++ | ++ | | |
| | 1 | ++ | ++ | ++ | + |
| | 0·1 | ++ | + | | |

**Table III** (*contd.*)

| Compound | Concn., p.p.m. | Geotropism | | Phototropism | |
|---|---|---|---|---|---|
| | | Rape | Rye-grass | Rape | Rye-grass |
| 2-(α-Naphthoyl)benzoic acid | 10 | ++ | ++ | | |
| | 1 | ++ | + | | |
| | 0·5 | + | | | |
| 2-(3 : 4-Dichlorobenzoyl)benzoic acid | 10 | ++ | ++ | | |
| | 1 | ++ | + | | |
| 2-Benzylbenzoic acid | 10 | − | − | | |
| 2-(4-Methylbenzyl)benzoic acid | 10 | − | − | | |
| 2-(4-Phenylbenzyl)benzoic acid | 10 | − | − | | |
| 2-(4-Chlorobenzyl)benzoic acid | 10 | ++ | − | | |
| | 1 | − | | | |
| 2-Carboxydiphenylamine | 10 | + | − | | |
| 2 : 3 : 6-Trichlorobenzaldehyde | 10 | T* | T | + | ++ |
| | 1 | | − | | |
| | 0·1 | − | | | |
| 2 : 3 : 6-Trichlorobenzoic acid | 10 | T | + | | |
| | 5 | T | + | T | ++ |
| | 1 | + | + | | |
| 2 : 3 : 5-Tri-iodobenzoic acid | 50 | ++ | ++ | − | − |
| | 20 | | | − | − |
| | 10 | + | ++ | | |
| | 1 | − | + | | |
| Fluorescein | 10 | ++ | ++ | | |
| | 5 | | | − | − |
| | 1 | + | | | |
| | 0·5 | − | | | |
| Eosin | 10 | ++ | ++ | | |
| | 5 | | | − | + |
| | 1 | + | + | | |
| | 0·5 | − | | | |

* T = too toxic

Vaníček[7] has reported a destruction of the geotropic sensitivity of the roots of germinating seeds by 2 : 3 : 5-tri-iodobenzoic acid. We have confirmed this, though at the effective concentration there was marked reduction of root length. In the geotropic test on rape roots, our results show a clear effect at concentrations below those at which marked reduction of root length occurred with the several compounds in the benzoylbenzoic and phthalamic acid classes.

Only a few members of the 2-benzylbenzoic acid and 2-carboxydiphenylamine classes have so far been examined, but there was a marked response in the geotropic effect on rape roots at 10 p.p.m. with 2-(4-chlorobenzyl)benzoic acid. Two things are clear from these results: (i) the abolition of the geotropic response of rape roots is not necessarily due to a toxic action causing cessation of growth, (ii) the special significance of the –CO·NH– group in the *N*-arylphthalamic acids suggested by Mentzer *et al.*[6] is not valid.

*Phototropic activity*

In the phototropic tests, few compounds have so far been examined, but α-naphthylphthalamic acid and 2-(4-phenylbenzoyl)benzoic acid were both markedly active on rape. Further experiments are being carried out and a fuller discussion of both the phototropic and geotropic results is reserved for a subsequent paper.

It has already been mentioned that certain auxin-like substances failed to show activity in the geotropic test because of the complication of marked reduction in root length (toxicity). It will be seen from Table III, however, that 2 : 3 : 6-trichlorobenzaldehyde and 2 : 3 : 6-trichlorobenzoic acid, for which auxin-like activity has been reported,[8–10] both responded in the phototropic test on rye-grass. Bennet-Clark & Kefford[11] have recently referred to a modification of the geotropic response of rhizomes of *Aegopodium podagraria* induced by indolylacetic acid or 2 : 4-dichlorophenoxyacetic acid. The conventional auxin tests such as the pea test and the *Avena* curvature test have not yet been applied to most of the compounds described in the present paper. Professor R. L. Wain, however, has very kindly examined 2-(4-chlorobenzoyl)benzoic acid for us and found no activity on the *Avena* cylinder test and only a very

low degree of activity by the Went pea test. Further, our colleague Dr. W. G. Templeman, who has also examined the antigeotropic effect of many of these compounds, found no interference by α-naphthylphthalamic acid or eosin with the normal response of indolylacetic acid or 4-chloro-2-methylphenoxyacetic acid in the Went pea test. Antigeotropic activity is therefore not necessarily a manifestation of auxin or anti-auxin activity.

Finally, reference should be made to the other important chemical class which has been found to affect the geotropic and phototropic responses of plants, namely the fluoresceins, and in particular the tetrabromo- and tetraiodo-derivatives, eosin and erythrosin.[12–14] Although the action of these dyes may, as suggested by Galston[15] and Ferri,[16] be associated with a photochemical destruction of auxin, it is perhaps significant that the structure of fluorescein (VI) reveals some similarity to the colourless substances discussed in this paper, in the attachment of an *o*-carboxyphenyl residue to a weighty cyclic nucleus. In our experiments fluorescein and eosin showed little or no antiphototropic activity but both were active in the antigeotropic test, though less so than several compounds of simpler structure described here (Tables II and III).

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# THE RELATIONSHIP BETWEEN THE CONSTITUTION AND THE EFFECT OF CHEMICAL COMPOUNDS ON PLANT GROWTH. V.*—Aromatic Nitro-compounds and Nitramines

By R. L. JONES, T. P. METCALFE and W. A. SEXTON

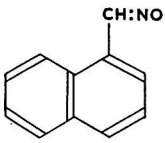
The root growth of germinating seeds of rape and wheat is markedly reduced when the seeds are grown in the presence of certain aromatic nitro-compounds. No correlation is apparent between the toxicity and the chemical reactivity, but within comparable groups of compounds water-solubility appears to be a limiting factor governing toxicity. Methylation of nitrophenols modifies their toxicity but does not destroy it.

Arylnitramines of the formula $Ar{\cdot}NH{\cdot}NO_2$ are selective inhibitors of the root growth of rape as compared with wheat. The activity is believed to be associated with the acidic nature of the nitramine group and is affected by the nature of the substituents in the benzene ring. The arylnitramines inhibit the geotropic response of roots of rape and rye-grass, and in four out of seven compounds examined the phototropic response of shoots of rape and rye-grass was also inhibited.

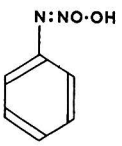
* Part IV: preceding paper

## Introduction

2 : 4-Dinitro-6-alkylphenols have been employed for some time as practical herbicides and there is a considerable literature on their general biochemical behaviour. Another example of the effect of a nitro group on the regulation of plant growth has been provided by Veldstra,[1] who found that α-naphthylnitromethane showed auxin-like activity, probably associated with the tautomeric structure (I).




(I)




(II)

Little study appears to have been made, however, of the phytocidal properties of simple nitro-compounds, and it is not known, for example, whether the hydroxyl group in dinitrophenols is essential for phytotoxicity. Many nitro-compounds have therefore been examined by using the seed-germination technique which is the basis of this series of papers. Though no exceptionally high activities have been observed, some of the results obtained are of interest in view of the relationships of structure and physicochemical properties to potency.

We also record observations on another type of nitro-compound, namely the aromatic nitramines, whose acidic tautomeric structure (II) resembles that of the *aci*-form of α-naphthylnitromethane. These compounds constitute a new chemical class showing activity in inhibiting phototropic and geotropic responses.

## Experimental

The simple aromatic nitro-compounds used in this investigation were all well known and readily available compounds. The aromatic nitramines $Ar{\cdot}NH{\cdot}NO_2$ are, as a rule, unstable substances, which readily undergo rearrangement with entry of the nitro group into the benzene ring. However, if appropriate positions in the benzene ring are occupied by substituents, the compounds are more stable. They give rise to soluble salts of structure (II). The compounds examined are all described in the literature and were prepared by the action of nitric acid on the aromatic amine. The less stable ones were isolated as barium derivatives, which are more stable than the free nitramines, and were tested as barium derivatives. The others were tested as free nitramines, their solubility in water being adequate for the purpose. In two instances tests were carried out with both forms, free nitramine and barium derivative, and no significant difference in activity was noted. Many of the simple aromatic nitro-compounds used in this investigation were only sparingly soluble in water, and, as a routine, polyethylene glycol (mol. wt. approximately 300) was used to assist solution. This material was found to be without effect on the germinating seeds at concentrations much higher than those employed in our general procedure, which was as follows: The compound under test (20 mg.) dissolved in the polyethylene glycol (3 ml.) was rapidly added to warm water (100 ml.) to yield either a solution or a fine dispersion. This was further diluted with warm water to a concentration of test substance of 100 p.p.m.; the solution was mixed with an equal volume of warm 3% agar and poured on plates to give a gel containing 50 p.p.m. of test substance. Lower concentrations were obtained by further dilution of the 100-p.p.m. preparation before mixing with agar.

The biological test methods, in which rape, wheat and rye-grass seeds are used, are the same as those described in an earlier paper[2] and the highest measure of activity is indicated in the Tables by two plus-signs. The term toxicity refers to reduction in root length.

## Results and discussion

### 1. *Toxicity of substituted nitrobenzenes*

As pointed out in Part IV, the experimental technique was designed for a rapid survey of large numbers of compounds, so that individuals requiring more precise quantitative study could be singled out. Small quantitative differences between compounds reported here are

therefore possibly not significant, but certain conclusions may be drawn with justification from gross differences.

In Table I are listed the results obtained with halogen- and alkyl-substituted nitrobenzenes. With the more active compounds, tests conducted at lower concentrations showed a rapid diminution of activity. From these results, and also from those recorded in Tables II and III, it is at once clear that aromatic nitro-compounds can exhibit growth-inhibiting action without the presence of a phenolic group. It is also seen that substituents other than hydroxyl can exert a profound effect on the activity of a nitro-compound.

**Table I**

*Toxicities of halogen- and alkyl-substituted nitro-compounds to germinating rape and wheat*

| Compound | Concn., p.p.m. | Rape | Wheat | Compound | Concn., p.p.m. | Rape | Wheat |
|---|---|---|---|---|---|---|---|
| Nitrobenzene | 50 | + | + | *m*-Fluoronitrobenzene | 50 | − | ++ |
| | 10 | − | − | | 10 | | + |
| *o*-Chloronitrobenzene | 10 | − | ++ | *p*-Iodonitrobenzene | 50 | − | − |
| *m*-Chloronitrobenzene | 10 | − | ++ | *o*-Nitrotoluene | 50 | + | + |
| *p*-Chloronitrobenzene | 10 | − | − | *m*-Nitrotoluene | 50 | + | ++ |
| *o*-Bromonitrobenzene | 50 | ++ | ++ | | 10 | | − |
| | 10 | + | ++ | *p*-Nitrotoluene | 50 | + | + |
| | 1 | − | | 3 : 5-Dichloronitrobenzene | 10 | − | ++ |
| *o*-Iodonitrobenzene | 50 | ++ | ++ | 2 : 5-Dichloronitrobenzene | 50 | + | ++ |
| | 10 | ++ | ++ | | 10 | − | + |
| *p*-Fluoronitrobenzene | 50 | − | − | 2 : 5-Difluoronitrobenzene | 50 | − | ++ |
| | | | | | 10 | | + |

It will be noted in the monohalogenonitrobenzenes that placing a halogen atom *para* to the nitro group destroys the activity. Activity (generally greater against wheat than rape) is thus not connected with the chemical reactivity of the halogen atom, for otherwise the *meta*-isomers would be distinguished from the *ortho*- and *para*-isomers. In further support of this finding, the following compounds, which contain halogen atoms *para* to the nitro group, were found to be inactive or almost so at 50 p.p.m.: 3 : 4-dichloronitrobenzene, 3 : 4-di-iodonitrobenzene, 3-bromo-4-iodonitrobenzene, 2 : 4 : 5-trichloronitrobenzene, 6-chloro-3-nitrotoluene. Alkyl substitution did not show the same effects as halogen substitution, for the three isomeric nitrotoluenes were all of comparable low activity.

Examination of a series of *m*-dinitrobenzene derivatives revealed that the introduction of a chlorine atom in the *ortho–para*-position to the nitro groups destroyed the activity, and to this extent there was a parallel with the inactivating influence of a *para*-halogen atom in the halogenonitrobenzenes. When a fourth substituent was introduced into the benzene ring, the results were different, for six 1 : 5-halogeno-2 : 4-dinitrobenzenes (not given in Table) all showed marked activity. Further, the placing of certain other groups *ortho–para* to the two nitro groups did not destroy the activity, though in some cases it was markedly reduced.

These results could provide no evidence for connecting the biological activity with the chemical reactivity of the molecules as modified by the various substituents, and attention was therefore directed towards the physicochemical properties of the different substances. It is, of course, well known that physicochemical properties can profoundly modify biological activity; there are examples of this, for seed germination, in the homologous-series effect with esters of phenoxyacetic acids, in arylcarbamic esters and in quaternary ammonium salts. In the homologous-series effect, biological activity usually rises to a maximum and afterwards falls. The point of 'cut off' is the point at which low solubility in water becomes a limiting factor (cf. Ferguson[3]).

The solubilities in water of many of the compounds examined is not known; nevertheless data on a sufficient number of key compounds have been recorded to enable solubility to be compared with activity, and certain tentative conclusions to be drawn. This comparison is made in Table II; the solubility figures given are approximate and are derived mainly from data in Seidell's compilation.[4] (We are indebted to Mr. J. M. Thorp for his determination of the solubilities of *o*-chloronitrobenzene, *p*-fluoronitrobenzene, 2 : 4-dinitroaniline and 2 : 4-dinitrodimethylaniline.)

Of the three chloronitrobenzenes, the one that showed no activity had a much reduced

## Table II

*Comparison of phytoxicity with water solubility*

| Compound | Approx. solubility, % | Concn., p.p.m. | Rape | Wheat |
|---|---|---|---|---|
| Nitrobenzene | 0·19 | 50 | + | + |
| | | 10 | − | − |
| *o*-Nitrotoluene | 0·065 | 50 | + | + |
| *m*-Nitrotoluene | 0·05 | 50 | + | ++ |
| | | 10 | − | − |
| *p*-Nitrotoluene | 0·04 | 50 | + | + |
| *o*-Chloronitrobenzene | 0·04 | 10 | − | ++ |
| *m*-Chloronitrobenzene | 0·65 | 10 | − | ++ |
| *p*-Chloronitrobenzene | 0·003 | 10 | − | − |
| *p*-Fluoronitrobenzene | 0·165 | 50 | − | − |
| *m*-Dinitrobenzene | 0·06 | 10 | ++ | ++ |
| | | 1 | + | − |
| 1-Chloro-2 : 4-dinitrobenzene | 0·001 | 50 | − | − |
| 2 : 4-Dinitrotoluene | 0·03 | 50 | ++ | ++ |
| | | 10 | − | − |
| 2 : 4-Dinitroaniline | 0·007 | 50 | + | + |
| 2 : 4-Dinitrodimethylaniline | 0·006 | 50 | + | + |
| 2 : 4-Dinitroanisole | 0·02 | 50 | ++ | ++ |
| | | 10 | ++ | + |

solubility. There is no such difference, either of solubility or activity, between the isomeric nitrotoluenes. A similar association of reduced solubility with loss of activity is apparent in the introduction of chloro, amino and dimethylamino groups into the *ortho–para*-position of *m*-dinitrobenzene. This solubility in water may be a factor limiting this type of biological activity, but more extensive and accurate data are required before firm conclusions can be drawn.

The effect of converting nitrophenols into their methyl ethers is of some interest (Table III). With the phenols themselves there is no distinction in the activities of compounds where the

## Table III

*Comparison of the phytotoxicities of nitrophenols with their methyl ethers*

| | Phenol | | | Methyl ether | | |
|---|---|---|---|---|---|---|
| | Concn., p.p.m. | Rape | Wheat | Concn., p.p.m. | Rape | Wheat |
| *o*-Nitrophenol | 50 | + | + | 50 | ++ | ++ |
| | | | | 10 | + | + |
| *m*-Nitrophenol | 50 | + | + | 50 | ++ | ++ |
| | | | | 10 | ++ | ++ |
| | | | | 1 | + | |
| *p*-Nitrophenol | 50 | ++ | + | | | |
| | 10 | + | | | | |
| 2 : 4-Dinitrophenol | 50 | ++ | − | 50 | ++ | ++ |
| | 10 | ++ | | 10 | ++ | + |
| | 1 | + | | 1 | − | |
| 3-Nitro-*o*-cresol | 50 | ++ | + | 50 | + | + |
| | 10 | + | | | | |
| 5-Nitro-*o*-cresol | 50 | ++ | − | 50 | ++ | ++ |
| | 10 | − | − | | | |
| 4-Nitro-*m*-cresol | 50 | ++ | − | 50 | + | ++ |
| | 10 | − | | 10 | | + |
| 2-Nitro-*p*-cresol | 50 | ++ | − | 50 | ++ | − |
| | 10 | − | | 10 | ++ | |
| | | | | 1 | + | |
| 4 : 6-Dinitro-*o*-cresol | 50 | ++ | + | 50 | ++ | ++ |
| | 10 | ++ | + | 10 | + | + |
| | 1 | − | | 1 | | |

## Table IV

*Results of toxicity, geotropic and phototropic tests on arylnitramines with seeds of rape, wheat and rye-grass*

| Nitramine | Form tested | Concn., p.p.m. | Toxicity | | Inhibition of geotropism of roots | | Inhibition of phototropism of shoots | |
|---|---|---|---|---|---|---|---|---|
| | | | Rape | Wheat | Rape | Rye-grass | Rape | Rye-grass |
| Phenyl | Ba deriv. | 50 | ++ | + | | | | |
| | | 10 | + | − | ++ | − | | |
| | | 5 | | | − | | | |
| *p*-Tolyl | ,, | 50 | ++ | + | | | | |
| | | 10 | + | − | ++ | + | | |
| | | 5 | | | + | | | |
| *o*-Chlorophenyl | ,, | 50 | ++ | + | ++ | | | |
| | | 10 | + | + | ++ | ++ | | |
| | | 1 | + | | + | | | |
| *p*-Chlorophenyl | ,, | 50 | ++ | + | ++ | + | | |
| | | 10 | ++ | − | + | − | | |
| | | 1 | + | | + | | | |
| 2 : 4-Dichlorophenyl | Ba deriv. and free | 50 | ++ | + | | | | |
| | | 20 | | | | | ++ | + |
| | | 10 | ++ | − | ++ | ++ | | |
| | | 5 | | | | | ++ | − |
| | | 2 | | | ++ | − | | |
| | | 1 | | | | | − | |
| 3 : 4-Dichlorophenyl | Free | 50 | ++ | + | ++ | − | | |
| | | 10 | ++ | + | ++ | − | | |
| | | 1 | + | − | − | | | |
| 2-Chloro-4 : 6-dimethyl-phenyl | ,, | 50 | ++ | + | | | | |
| | | 10 | ++ | − | ++ | ++ | | |
| | | 2 | + | | ++ | | | |
| | | 0·5 | | | + | | | |
| 4 : 6-Dichloro-2-tolyl | ,, | 50 | ++ | + | | | | |
| | | 10 | + | + | ++ | ++ | | |
| | | 2 | | | ++ | ++ | | |
| | | 0·5 | | | + | | | |
| 2-Bromo-4 : 6-dimethyl-phenyl | ,, | 50 | ++ | + | | | | |
| | | 10 | + | + | ++ | ++ | | |
| | | 1 | + | | ++ | ++ | − | − |
| | | 0·5 | | | ++ | ++ | | |
| 2 : 6-Dibromo-4-tolyl | ,, | 50 | ++ | + | | | | |
| | | 20 | | | | | + | + |
| | | 10 | + | − | ++ | ++ | | |
| | | 5 | | | | | + | + |
| | | 1 | | | ++ | − | − | − |
| 2 : 4 : 6-Tribromophenyl | Ba deriv. and free | 50 | ++ | + | | | | |
| | | 10 | + | − | ++ | ++ | | |
| | | 1 | | | ++ | − | − | − |
| | | 0·5 | | | + | | | |
| 2 : 4 : 6-Trichlorophenyl | Free | 20 | | | | | ++ | − |
| | | 10 | ++ | + | ++ | ++ | | |
| | | 1 | − | | | − | − | − |
| | | 0·5 | − | | − | | | |
| 2 : 6-Dibromo-4-nitrophenyl | ,, | 50 | − | + | | − | | |
| | | 10 | − | + | ++ | − | | |
| 2 : 4 : 6-Tribromo-*N*-methyl-phenyl | ,, | 50 | − | − | | | | |
| | | 10 | − | + | ++ | − | | |
| | | 2 | | | + | | | |
| α-Naphthyl | Ba deriv. | 50 | ++ | + | | | | |
| | | 10 | ++ | − | ++ | − | | |
| | | 5 | | | | | − | − |
| | | 1 | + | | − | | | |

nitro and hydroxyl groups are *meta* on the one hand or *ortho* or *para* on the other hand. The tautomeric possibilities of the *ortho*- and *para*-substituted compounds are therefore of no significance. Generally, but not always, the activity is greater against rape than against wheat. That the activity of the nitrophenols is not dependent on the phenolic function is indicated by two facts: (i) *o*- and *m*-nitrophenols are less active than the corresponding chloronitrobenzenes, and (ii) etherification does not destroy the activity—it may even enhance it or modify the species selectivity. No study has been made of the possibility of metabolic demethylation. Generally, the effect of methylation was to increase the susceptibility of wheat (though there are two exceptions). There was no regularity in the effect on the susceptibility of rape.

2. *Toxicity and effect of nitramines on tropic responses*

The results of the tests are summarized in Table IV. In the toxicity test, rape was more susceptible than wheat. In this the arylnitramines resemble other acidic substances such as the phenoxyacetic acids, chlorinated benzoic acids, 2-benzoylbenzoic acids and phthalamic acids. With 2 : 4 : 6-tribromophenylnitramine the toxicity to rape was destroyed, or at least markedly diminished, by *N*-methylation; here, of course, the acidic function is lost. This confirms the association of toxicity to rape with the acidic nature of the arylnitramines. Halogen and methyl substituents sometimes, but not always, increased the activity against rape.

None of the compounds examined had a toxicity comparable with that of such substances as 2 : 4-dichlorophenoxyacetic acid. In the one compound where a nitro group was introduced into the benzene ring no activity against rape was found at the highest strength tested. (Nitro groups do not enhance the activity of phenoxyacetic acid.[5])

In the geotropic test, activity was shown by almost every compound examined, and several trisubstituted nitramines were comparable in activity to *N*-α-naphthylphthalamic acid and the most potent of the derivatives of 2-benzoylbenzoic acid described in Part IV of this series.[2] In six compounds there was activity in the geotropic test against rape at concentrations well below the toxicity level. The most active compound of all appeared to be 2-bromo-4 : 6-dimethylphenylnitramine; generally, trisubstitution gave higher activities than those found with the less highly substituted compounds. Not all the compounds were examined by the phototropic test, but definite activity was shown by four out of seven compounds, the most active being 2 : 4-dichlorophenylnitramine. The most potent antigeotropic compound, 2-bromo-4 : 6-dimethylphenylnitramine, was at the highest strength tested inactive in the phototropic test. The activities in the two tropism tests are therefore not parallel. This is not surprising, since the phototropic test is done on shoots and the geotropic test on roots. A prerequisite for activity in the phototropic test is the ability of the compound concerned to be transported to the shoots. This may be the cause of the inactivity of the 2-bromo-4 : 6-dimethyl compound. No compound showing activity in the phototropic test fails to show activity in the geotropic test. This applies both to the present series of nitramines and to the phthalamic and 2-benzoylbenzoic acids reported previously.[2] There will be further discussion of the mode of action of the antigeotropic compounds in Part VI of this series.

Our colleague Dr. W. G. Templeman has examined 2 : 4 : 6-tribromophenylnitramine by the *Avena* coleoptile cylinder test and finds that it causes no modification of the normal response of the *Avena* coleoptile to indolylacetic acid. Its action in modifying the geotropic response does not therefore appear to be due to an interference with auxin function.

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# THE RELATIONSHIP BETWEEN THE CONSTITUTION AND THE EFFECT OF CHEMICAL COMPOUNDS ON PLANT GROWTH. VI.*—Some Derivatives of Fluorene

By R. L. JONES, T. P. METCALFE and W. A. SEXTON

Geotropic and phototropic responses of seedlings of rape, wheat and rye-grass are inhibited by 9-fluorenol-9-carboxylic acid and certain of its substituted derivatives. The antitropic effect of these and other acidic aromatic substances is discussed, and it is suggested that they may act through a competitive interference with the lateral transport of β-indolylacetic acid.

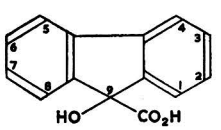
## Introduction

In Parts IV[1] and V[2] of this series it was shown that an inhibition of the geotropic response of roots and the phototropic response of shoots of seedlings of wheat, rape and rye-grass could be brought about by representatives of several classes of chemicals. These included certain *N*-arylphthalamic acids, in which the antigeotropic effect had already been independently observed by other workers, certain related *o*-carboxyphenyl derivatives of aromatic structures (e.g. 2-benzoylbenzoic acids) and the substituted aromatic nitramines. It is the purpose of this communication to record results with another chemical class that has been found active in a similar way, and to present a brief general discussion of the antitropic effects.

## Experimental

(*a*) *Preparation of compounds*

A specimen of 9-fluorenol-9-carboxylic acid (I) was already available in this Laboratory having been prepared some years ago for a different purpose by the action of alkali on phenanthraquinone.



(I)

It was first selected for examination by the seed-germination technique because of our general experience of the effects of aromatic carboxylic acids upon plant growth. It was found to be active, and, since it readily undergoes certain chemical changes, an examination of certain analogues and derivatives was made. Not all the compounds prepared and examined are reported upon in detail, since the biological test methods, designed originally for the rapid screening of large numbers of compounds, are not sufficiently delicate to detect other than fairly gross differences in activity. Of the compounds mentioned in Table I two are new. Their methods of preparation are as follows:

*2-Chloro-9-fluorenol-9-carboxylic acid.*—2-Chlorophenanthraquinone[3] (70 g.) was ground to a fine suspension in 10% aqueous sodium hydroxide (3 l.). The suspension was heated to 70–80° over 30 minutes and stirred at this temperature for 10 minutes. It was then cooled and acidified with hydrochloric acid until nearly all free sodium hydroxide was neutralized. Acetic acid was then added until the solution was acid to litmus. The precipitated impurities were separated and the 2-chloro-9-fluorenol-9-carboxylic acid was precipitated from the filtrates by addition of excess of hydrochloric acid. Yield, 53 g. The product is pale yellow-green in appearance, m.p. 194–195° (decomp.). The m.p. was not raised by recrystallization from ethanol–water (Found: C, 64·7; H, 3·25; Cl, 13·7. $C_{14}H_9O_3Cl$ requires C, 64·5; H, 3·45; Cl, 13·6%).

n-*Butyl-9-fluorenol-9-carboxylate.*—9-Fluorenol-9-carboxylic acid (5 g.) was heated in the steam bath for 5 hours with *n*-butanol (75 c.c.) and concentrated hydrochloric acid (25 c.c.). The reaction mixture was poured into water, the non-aqueous layer extracted with aqueous sodium carbonate and the excess of butanol removed under reduced pressure. The residual oil solidified on cooling and was recrystallized from 80–100° petroleum. Yield: 3 g., m.p. 68–69° (Found: C, 76·7; H, 6·5. $C_{18}H_{18}O_3$ requires Cl, 76·6; H, 6·4%).

* Part V: preceding paper

(*b*) *Biological tests*

The biological test methods are fully described in Part IV and the results are summarized in Table I. Those compounds that were acidic were tested in the form of their sodium derivatives, which were sufficiently soluble in water. The others were dispersed with the aid of polyethylene glycol as described in Part IV. In Table I, two plus-signs signify the highest activity and a minus-sign signifies no detectable activity. A single plus-sign indicates an intermediate degree of activity.

**Table I**

*Toxicity and antitropic activity of 9-fluorenol-9-carboxylic acids and analogous substances*

| Compound | Concn., p.p.m. | Toxicity | | Antigeotropism | | Antiphototropism | |
|---|---|---|---|---|---|---|---|
| | | Rape | Wheat | Rape | Rye-grass | Rape | Rye-grass |
| 9-Fluorenol-9-carboxylic acid | 50 | ++ | + | | | | |
| | 10 | ++ | | ++ | ++ | | |
| | 5 | | | | | ++ | ++ |
| | 1 | + | | ++ | − | + | ++ |
| | 0·1 | | | − | | | |
| 2-Nitro-derivative | 50 | − | + | ++ | ++ | | |
| | 10 | − | − | − | + | | |
| 4-Nitro-derivative | 50 | − | + | | | | |
| | 10 | | | − | − | | |
| 2-Chloro-derivative | 50 | ++ | ++ | | | | |
| | 10 | + | ++ | | | ++ | ++ |
| | 1 | + | + | ++ | ++ | ++ | ++ |
| | 0·1 | | | ++ | ++ | | |
| Methyl ester | 10 | + | + | ++ | ++ | | |
| | 1 | − | | + | − | ++ | ++ |
| Butyl ester | 50 | + | ++ | ++ | ++ | | |
| | 10 | + | + | ++ | ++ | | |
| | 1 | | | − | + | | |
| Fluorene-9-carboxylic acid | 50 | ++ | ++ | ++ | | | |
| | 10 | ++ | + | ++ | ++ | | |
| | 1 | | | − | + | | |

In the geotropic test, fluorene-9-carboxylic acid was less active than its 9-hydroxy-derivative, and in fact it can hardly be claimed that the antigeotropic activity is any more than a manifestation of toxicity. The hydroxyl group may therefore be essential for true antigeotropic activity. That the carboxyl group (either free or esterified) was essential was proved by tests on three 9-alkyl-9-hydroxyfluorenes and on fluorenone, all of which proved inactive at 50 p.p.m. It will be seen that the introduction of a nitro group reduced or destroyed the activity, but a 2-chloro substituent caused a marked elevation of activity. 2-Chloro-9-fluorenol-9-carboxylic acid is one of the most potent antigeotropic compounds that we have examined. Esterification of 9-fluorenol-9-carboxylic acid did not destroy its activity, but hydrolysis *in vivo* to the parent acid must be considered to be a possible complication. As with 2 : 4 : 6-tribromophenylnitramine and *N*-α-naphthylphthalamic acid, our colleague Dr. W. G. Templeman found no interference by 2-chloro-9-fluorenol-9-carboxylic acid with the normal behaviour of indolylacetic acid in the *Avena* coleoptile cylinder test for auxin activity.

Only three compounds were examined for phototropic activity. Marked activity in these, coupled with the previous findings with other chemical types, probably indicates a common biochemical or biophysical mechanism governing the antigeotropic and antiphototropic effects.

## General discussion

The abolition of the positive geotropic response of seedling roots by chemical treatment has now been observed in a number of chemical classes. It was first observed in the fluorescein dye, eosin (tetrabromofluorescein), by Boas & Merkenschlager,[4] and was further studied by Boysen-Jensen[5] with erythrosin (tetraiodofluorescein). Boysen-Jensen observed a decrease in the auxin content of roots of *Vicia faba* and *Pisum sativum* after treatment with erythrosin. Further investigations with fluorescein dyes have been more concerned with their effects on phototropic rather than geotropic responses,[6, 7] and dye-sensitized photochemical destruction of auxin may be implicated.[8, 9] The antigeotropic effect of eosin has also been confirmed in

this Laboratory, with rape and rye-grass.[1] The antigeotropic effect of *N*-α-naphthylphthalamic acid on the roots of certain species was first reported by Mentzer & Nétien[10] and extended to other *N*-arylphthalamic acids by Mentzer *et al.*[11] It has been further confirmed and extended to the structurally related 2-benzoylbenzoic acids, 2-benzylbenzoic acids and diphenyl-*o*-carboxylic acids by Jones *et al.*[1] Activity in arylnitramines was found by Jones *et al.*[2] The only other substance so far recorded as having antigeotropic activity is 2 : 3 : 5-tri-iodobenzoic acid.[1, 12]

The earlier literature on the mechanism of tropic responses in plants is reviewed by Thimann[13] and by Zimmerman & Hitchcock.[14] The tropic responses are undoubtedly concerned with auxin, since removal of root tips or coleoptile tips in *Avena* abolishes the geotropic and phototropic responses.[15] The modern view of the mechanism of tropic responses owes much to the work of Boysen-Jensen and Schrank. Boysen-Jensen[16, 17] showed that one-sided irradiation and the stimulus of gravity both caused a transverse displacement of growth substance. With the phototropic response the growth substance had a higher concentration on the shaded side, and under the influence of gravity there was a greater concentration of growth substance on the underside. The bending is due to a differential growth-rate of the two sides resulting from the uneven distribution of growth substance. It should be remembered that auxin stimulates growth at appropriate concentrations in the tissues concerned but at higher concentrations it is growth-inhibitory. The actual direction of the bending will therefore depend upon the tissue concerned and the absolute concentrations of auxin on the two sides of it (cf. Zimmerman & Hitchcock[14] and Thimann[13]). In *Avena*, rye-grass and rape, for example, the roots are positively geotropic and the shoots negatively geotropic.

The transverse migration of auxin follows after the setting up of a differential polarity under the influence of the stimulus, and Schrank[18, 19] pointed out that the origin of this differential electrical polarity was a primary problem in the understanding of tropic responses. He found that stimulation by gravity or by mechanical means resulted in the prompt establishment of a differential electrical polarity between the two sides. With *Avena* coleoptiles, mechanical stimulation establishes a negative polarity on the stimulated side. With *Avena* coleoptiles placed horizontally, the upper side became electrically negative relative to the lower side. After the establishment of the differential electrical polarity, there follows a redistribution of auxin, and finally a differential growth rate on the two sides. He also found that removal of the apical 3 mm. (which contains a source of auxin) did not prevent the establishment of the polarity though, of course, no bending occurred. The phototropic response was found to be in accord with the findings related to response to gravity and mechanical stimulation.

It can be seen, therefore, as pointed out by Schrank,[20] that chemical interference with the geotropic response of roots could occur through one or more of several mechanisms, which include: (i) hindering the establishment of the differential electrical polarity, (ii) an effect on the generation of auxin from its precursors or on its destruction or biochemical function, and (iii) an effect on the lateral transport of auxin.

It is difficult to understand how chemical substances of the types concerned could effect the setting up of the differential electrical polarity, since knowledge of the biophysical mechanism concerned in this is still scanty. The only test would be direct measurement, and this has not been done. There are more facts upon which to base an opinion about (ii). Destruction of auxin is probably a relevant factor in the photochemical response, but there is no reason to expect it with the geotropic response. Experiments in this Laboratory have established that the antigeotropic response is shown when the seed germination is done in complete darkness. If the antigeotropic response were an 'anti-auxin' effect, this should be demonstrable by direct experiment in the geotropic test as well as by the employment of other tests involving response to auxins. An 'anti-auxin' should stop growth altogether at an appropriate concentration, or at least diminish it. It is perhaps significant that 2 : 3 : 5-tri-iodobenzoic acid, which has been claimed to antagonize the action of auxin,[21, 22] is only antigeotropic in our tests at concentrations where marked reduction of root length occurred.[1] Many of the antigeotropic substances show strong activity at concentrations that have little effect on root growth.[1, 2] Attempts to abolish the antigeotropic activity of α-naphthylphthalamic acid and of 2 : 6-dibromo-4-tolylnitramine (at 0·1–10·0 p.p.m.) by adding to the agar medium indolylacetic acid at concentrations between 0·005 and 1 p.p.m. were unsuccessful. Further, our colleague Dr. W. G. Templeman has found no interference by several of our active compounds with the response of indolylacetic acid or 4-chloro-2-methylphenoxyacetic acid in the *Avena* auxin test. We have also found that roots and shoots of rape and rye-grass grown on agar containing 10 p.p.m. of α-naphthylphthalamic acid give the usual bending response when a

block of agar containing indolylacetic acid was placed in contact with one side. It seems clear, therefore, that antigeotropic activity is not necessarily connected with an interference with auxin function.

The third possibility, namely interference with lateral transport of auxin, offers a plausible explanation of the phenomena observed. We have now found at least 60 compounds that show antigeotropic activity at 10 p.p.m. or less. They are all either acids, or derivatives, such as esters, that might well become activated after hydrolysis *in vivo* to the free acids. Although the dissociation constants of all these acids are not known, there is sufficient general information on this point to conclude that all the antigeotropic acids will be almost completely ionized in plant tissues which have a pH of about 6. Indolylacetic acid ($K_\alpha = 2{\cdot}9 \times 10^{-5}$) is over 95% ionized at pH 6·0. Although the strengths of the *N*-arylphthalamic acids have not been measured, phthalamic acid itself has $K_\alpha = 1{\cdot}6 \times 10^{-4}$. 2-Benzoylbenzoic acid has $K_\alpha = 3{\cdot}7 \times 10^{-4}$. Measurements by our colleague Dr. A. K. Gupta indicate a similar strength for 4′-phenyl-2-benzoylbenzoic acid and $K_\alpha$ values for 2 : 4 : 6-tribromophenylnitramine and 2-chloro-9-fluorenol-9-carboxylic acid of $1{\cdot}35 \times 10^{-3}$ and $8{\cdot}5 \times 10^{-4}$ respectively. The antigeotropic compounds therefore resemble indolylacetic acid in their high degree of ionization within the plant tissues and in the fact that they are based on aromatic nuclei. It is possible, therefore, that they compete with indolylacetic acid in the lateral transport mechanism. The longitudinal transport will almost certainly occur by a different mechanism from that regulating lateral transport at the growing tip, and consequently will probably be governed by different structural considerations (cf. Clark[23]). Thus some structural specificity may be expected in antigeotropic compounds, since two quite different functions require to be performed, namely longitudinal transport from seed to growing point and lateral transport at the growing tip.

This discussion contains some speculative suggestions and relates almost entirely to the possible mode of action of acidic substances in the antitropic effects. The discovery of such substances places a new weapon in the hands of plant physiologists, and it is obvious that much more detailed investigation is called for. It so happens that the antitropic effects have been sought mainly among aromatic carboxylic acids, and it is by no means impossible that other chemical types will be found to have similar biological activity. An instance of this has already been found by Dr. Templeman in the compound phenylmercury acetate, and the possibility must be envisaged of more than one biochemical mechanism for the interference with tropism.

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# SEASONAL VARIATION IN THE QUALITY OF GRASS SILAGE

By A. M. SMITH

A study of the analytical results obtained with 1244 samples of silage, examined for advisory purposes during four seasons, has shown interesting relationships between dry matter, crude protein and pH values, and the influence of summer rainfall on these properties. A dry season tends to give a large proportion of samples that have a high dry-matter content and a low protein content and pH, whereas the converse holds good for a wet season. The results indicate that the best conservation is secured with herbage containing more than 20% of dry matter.

## Introduction

The investigation into conditions best suited for the successful conservation of grass and other crops by ensiling has steadily increased in the last twenty years, and, although there is still considerable uncertainty on the precise biochemical changes that take place during ensilage, sufficient evidence has been accumulated to establish the need for certain precautions if failure is to be avoided. In keeping with the important development of ensilage as an alternative to other feeding-stuffs, there has also been a large increase in the numbers of samples of silage examined by the agricultural advisory departments of various countries. The result is that much experience has been gained that provides a good basis for assessing the main features of the process.

Because of differences in climate and farming economy in various countries, results are not always strictly comparable, and there is little doubt that each country or area will eventually derive the most suitable technique for its farmers. This paper is concerned with a study of the samples of grass silage examined for advisory purposes in the East of Scotland area during the four years 1949–1953. The numbers of samples seemed to justify such a summary because, although details of the state of the crop, weather, process of ensiling and nature of the silo were not always known, the samples could be regarded as representing a fair cross-section of the good and bad silages produced by farmers both experienced and inexperienced in this method of conservation.

## Analysis of samples

Nearly all the samples were taken by the general advisory officers of the College. Various methods were adopted to obtain a reasonably representative sample from the silo; sometimes the sample consisted of slices taken from the face of the exposed silage, sometimes it was made up of vertical cores through the silage, and sometimes it comprised a sample of the silage being fed. For routine purposes, the following determinations were made: (1) the pH (by glass electrode) of an aqueous extract of a sub-sample, (2) the weight of dry matter by drying in an electric oven at 100° and (3) the nitrogen content of the dry matter by the Kjeldahl method. Normally no correction was made for loss of volatile compounds during drying, because it is known[1] that the losses are not of much consequence with well-preserved silage and, in any event, the field sampling errors are relatively large; further, the food value of badly preserved silage is probably as much dependent upon palatability as upon composition. Hence the dry matter and protein are both underestimated but the discrepancies may be regarded as of minor importance in the present review.

It has been customary to take the pH value of 4·5 as a convenient point to discriminate between good and poor conservation. A value of 4·2 might be better since butyric acid is absent or in very low concentration at greater degrees of acidity than this, but the amounts present at pH 4·5 are usually small compared with the amounts of lactic and acetic acids. For the estimation of food value, a measure of the dry matter is of over-riding importance —a fact that is often disregarded by farmers accustomed to feeding hay—and a figure of 20%, which is a fairly common level for grass silage, has been selected to differentiate between wet and dry samples. The value of a silage for maintenance or production is dependent upon its content of crude protein, which provides an estimate of both starch equivalent and digestible crude protein. In each of the four years considered the average value of crude protein has been close to 12·5% and this is indicative of the stage of growth regarded by farmers as the most suitable for bulk of herbage and ease of ensiling. There were also, of course, samples ensiled from very leafy or from mature grasses, and the limits for crude protein were between 6 and 30%. However, it is convenient to place the samples into the three categories, less than 11, 11 to 14, and over 14% of crude protein.

One of the merits frequently claimed for silage is that it can be made in weather that

is impossible for hay-making. Although this is true, it does not mean that a crop may be safely ensiled during continuous or heavy rain, for there is little doubt that excessive moisture increases the difficulties of securing a desirable fermentation, and the leaching of silage in a badly covered silo by rain may bring about a secondary fermentation, with the production of butyric acid from the lactic acid already present. The weather at the time of ensiling is often quite a good guide to the final condition of the silage. For example, it had been confidently and correctly predicted that the conservation in 1950 would not be so satisfactory as in 1949. To test this assumption, rainfall records from 11 stations in the area for the period May to September have been averaged to give 'summer' rainfall, and these figures do show an overall relationship with silage quality. This helps to explain some of the correlations in silage properties that have recently been reported.

## Results

In Table I, the samples are grouped according to pH, dry matter, and crude protein for each of the four years, and a summary of the figures in each main group, expressed as percentages of the annual totals, is given in Table II. Most grass silage is made in May and June but some is made later in the summer and a considerable quantity is made from aftermath in September. However, because of incomplete information about the samples when they were submitted for analysis during the winter months, it was not possible to subdivide them according to time of ensiling. The summer rainfall figures in Table II can, therefore, be used only as a first approximation to conditions at or about the time of ensiling. There was actually a greater precipitation in May and June during 1951 and 1952 than during the same period for 1950, the high rainfall for the summer of 1950 being due mainly to an abnormally wet September. But so many factors are involved, in addition to precipitation, such as the speed of ensiling and the finishing of the silo to throw off rain, and the organization of the work varies so much from farm to farm, that it is thought that the total summer rainfall is the best simple guide to climatic differences in the four seasons.

**Table I**

*Silage samples grouped according to pH, dry matter, and crude protein*

| | | pH over 4·5 Dry matter, % | | pH below 4·5 Dry matter, % | | |
|---|---|---|---|---|---|---|
| | Year | below 20 | over 20 | below 20 | over 20 | Total |
| Crude protein, below 11% | 1949 | 7 | 6 | 13 | 41 | 67 |
| | 1950 | 17 | 8 | 14 | 31 | 70 |
| | 1951 | 30 | 14 | 21 | 27 | 92 |
| | 1952 | 21 | 14 | 16 | 66 | 117 |
| | Total | 75 | 42 | 64 | 165 | 346 |
| Crude protein, 11–14% | 1949 | 12 | 7 | 10 | 34 | 63 |
| | 1950 | 28 | 17 | 19 | 39 | 103 |
| | 1951 | 41 | 28 | 42 | 51 | 162 |
| | 1952 | 34 | 22 | 33 | 92 | 181 |
| | Total | 115 | 74 | 104 | 216 | 509 |
| Crude protein, over 14% | 1949 | 0 | 5 | 5 | 10 | 20 |
| | 1950 | 45 | 18 | 31 | 34 | 128 |
| | 1951 | 33 | 29 | 19 | 38 | 119 |
| | 1952 | 19 | 27 | 25 | 51 | 122 |
| | Total | 97 | 79 | 80 | 133 | 389 |
| Grand total | | 287 | 195 | 248 | 514 | 1244 |

The outstanding results in Table II are as follows: 41% of all samples contained between 11 and 14% of crude protein but the dry year, 1949, had a relatively high proportion of samples poor in protein, and the wet year, 1950, a relatively high proportion rich in protein; 58% of all samples contained more than 20% of dry matter, but this was mainly due to the relatively high proportions of dry samples in 1949 and 1952; 63% of the samples could be regarded as well preserved but this was largely accounted for by the relatively good seasons in 1949 and 1952.

## Discussion of results

Scatter diagrams of pH against dry matter at different protein levels showed only one common feature, namely, that when the dry matter was high (over 25%) there were few

**Table II**

*Percentage of silage samples in various categories*

| Year .. .. .. | 1949 | 1950 | 1951 | 1952 | Average |
|---|---|---|---|---|---|
| Summer rainfall, in. | 9·2 | 15·6 | 12·9 | 11·2 | |
| No. of samples | 150 | 301 | 373 | 420 | |
| Crude protein, | | | | | |
| below 11% | 45 | 23 | 25 | 28 | 30 |
| 11–14% | 42 | 34 | 43 | 43 | 41 |
| over 14% | 13 | 43 | 32 | 29 | 29 |
| Dry matter, | | | | | |
| below 20% | 31 | 51 | 50 | 35 | 42 |
| over 20% | 69 | 49 | 50 | 65 | 58 |
| pH, | | | | | |
| over 4·5 | 25 | 44 | 47 | 33 | 37 |
| below 4·5 | 75 | 56 | 53 | 67 | 63 |

samples with a pH greater than 4·5. This is fairly clear from the results in Table I, which show that, of the samples with a pH above 4·5, the numbers with less than 20% of dry matter are almost invariably greater than the corresponding numbers with more than 20% of dry matter; conversely, of the samples having a pH below 4·5, the numbers with more than 20% of dry matter are always greater than the numbers with less than 20% of dry matter. This suggests that it is desirable to ensile material with a fairly high dry-matter content and supports the recommendation that has been made to allow the grass to wilt before collecting for ensiling. Weather conditions, of course, determine whether this step can be taken, but if the weather is fairly settled it would undoubtedly be advantageous to allow the grass to lie for a few hours, during which time the dry-matter percentage may rise from about 15 or 20 to about 25. At the same time it must be remembered that there may be losses of dry matter during wilting, and that when the material is rather dry there is sometimes trouble in preventing the temperature of the mass in the silo from rising rapidly and too high, and so reducing the food value of the product.

It has already been pointed out that the protein content tended to be higher than average in the wet season of 1950 and lower in the dry season of 1949. This might have been expected because grass is much more leafy in a good grass season and matures much more quickly in a dry season. That is borne out by the respective figures for the proportions of samples having below and above 20% of dry matter. It is not surprising, therefore, that there should be a relationship between rainfall, dry matter, and quality of silage—the rainfall in the growing season determining to a large extent the leafiness of the grass at the usual time for ensiling, the dry matter being responsible for the ease of ensiling and the quality of the product, and the quality in turn being closely connected with the degree of acidity produced by fermentation.

These relationships have been discussed by several investigators. For example Barnett,[2] in a study of 164 samples collected in one season, found that the percentage of nitrogen was related directly to pH and inversely to dry matter. He plotted those properties against each other and adapted regression curves from which he derived equations to predict the percentage of nitrogen from dry matter and pH, or pH from dry matter and nitrogen. In actual practice, however, it would be hazardous to use such equations because the level of significance was not very high and there are naturally many exceptions to the general rule. Crasemann & Heinzl[3] have reported that an attempt to obtain a dry-matter content of 32 to 35% by wilting made the ensilage process much more reliable. In an examination of silages during a period of 10 years, Martin, Reyntens & Buyesse[4] derived certain equations relating pH with the amount of butyric or lactic acid produced and with the volatile nitrogen. The total number of samples was only 120 and they did not find a correlation between dry matter and pH. Brown,[5] on the other hand, obtained a good correlation between these values.

It would seem, therefore, that there is little doubt that a good conservation, resulting from a satisfactory fermentation and a lowering in pH value to below 4·5, is most readily secured when the crop ensiled has a dry-matter content of over 20%. It would be to the advantage of farmers if they could avoid ensiling under very wet conditions and, if possible, allow the material to dry somewhat in the field before collecting and ensiling. This is particularly important when a very leafy crop is being cut. In this instance, of course, the production of acid is usually assisted by the addition of an easily fermentable material like molasses,

or the biochemical changes can be reduced by adding sufficient acid to lower the pH immediately to 4 or less. It is also important to remember the desirability of finishing a silo in such a way that rain is unable to permeate through the mass, and in this connexion it is interesting to note that the roofing of silos is becoming popular in certain areas.

Experimental evidence for the relationship between degree of preservation and food value is still lacking, and, although one would naturally expect that an apparently palatable food would be of more benefit to the animal than a badly preserved one, it is well known that some animals will greedily consume what would be regarded as very bad silage. A number of experiments on the digestibility of good and bad silage have been carried out but it is still too soon to draw any general conclusions.

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# EFFECT OF INSECT INFESTATION ON STORED GRAIN. I.—Studies on Soft Wheat

By S. V. PINGALE, M. NARAYANA RAO and M. SWAMINATHAN

Soft wheat, which is considered to be easily susceptible to insect damage during storage, was subjected to infestation by three species of insects, namely *Calandra oryzae* L. (weevils), *Trogoderma granaria* E. (khapra beetles) and *Ephestia cautella* W. (almond moth), and the changes brought about by them are reported. The results suggest that, although weevils cause heavy reduction in the weight of the grain, the moth, which feeds only on the germ portion, considerably reduces the grain's viability. The results of an investigation on the degree of unhygienic conditions brought about in the grains by the addition of impurities and body fragments of the insects have been presented. An increase in the acidity of fat, and a decrease in the thiamine content due to insect damage, were both appreciable, but the effect on other constituents of the grain, such as total nitrogen and reducing sugars, was not significant.

## Introduction

Insects are known to take a heavy toll of stored food grains. Cotton[1] estimated annual losses of cereals alone, due to insects, at 5% of the total production, which amounts to some million tons. These losses, however, did not take into account the deterioration in quality due to insect activity. Herford[2] pointed out that certain insects confined their damage to the germ portion of the grain which, although not amounting to much in quantity, is of great importance from the point of view of quality or nutritive value. He further considered the contamination of foodstuffs with insect fragments, which is an inevitable consequence of insect infestation and an indication of unhygienic conditions, and affects the quality.

Grain stored free from insects is also likely to undergo some changes whatever the conditions of storage. Zeleny[3] stated that grain stored under favourable conditions may undergo relatively minor changes for many years, whereas that stored under unfavourable conditions may be spoilt in a short time. Since insects, which are unfavourable factors, are almost always associated with the grain during storage, some changes, in addition to the normal ones, are likely to be produced by them. The nature of these changes, however, does not appear to have been investigated. It is generally believed that insect-damaged food is less nutritious, but there is no critical evidence to support this view. The present investigation was therefore undertaken to study the physical and biochemical changes in grain infested by some common species of insects.

## Experimental

Soft wheat, because of its ready susceptibility to insect damage, was chosen for the studies. The grain had an initial moisture content of 10·32% and a viability of about 89·7%. It was stored in closely woven bamboo bins in 70-lb. lots, and 100 adults each of *Calandra oryzae* L. (weevils), *Trogoderma granaria* E. (khapra beetles) and *Ephestia cautella* W. (almond moth), were introduced separately in different bins. These bins were then kept in isolated rooms. Humidity and temperature in these rooms were not controlled but varied between 48 and 62% and 78° and 84° F respectively, as shown by a hygro-thermograph.

All values were ascertained at monthly intervals when each bin was thoroughly mixed by hand and a 2½-lb. representative sample drawn for subsequent analysis. For the determination of insect populations, only larval, pupal and adult stages of respective insects were counted. Viability was determined by keeping a representative sample in moist filter-paper sheets. The insect-fragments count was made according to the method of Harris *et al.*[4]

For the chemical examination, grain samples were cleaned and ground in a laboratory flour-mill and passed through a 50-mesh sieve, and the flour was used for analysis.

Nitrogen, moisture, ash, fat, fat acidity and reducing sugars were determined according to the methods of the American Association of Cereal Chemists.[5]

Nitrogen soluble in 3·0% sodium chloride solution was determined by extracting a 10-g. sample of the flour with 200 ml. of sodium chloride solution at room temperature (29–30° C). The mixture was shaken at a uniform rate in a mechanical shaker for one hour, then centrifuged, and the nitrogen content of an aliquot of the extract was determined. Thiamine was determined by the method of Swaminathan.[6]

Khapra-infested samples showed signs of cross-infestation at the end of five months and further analysis with these was therefore discontinued.

## Results and discussion

*Loss in weight due to insect damage.*—Loss in weight was found to be great in insect-damaged samples, and particularly with weevil-infested grain. The insects commonly infesting stored grains bore into the grain leaving the outer coat intact, or eat away part of the grain, or eat away only the germ portion. Each of the insects used in the tests caused one of these types of damage. Weevil damage represented the first type, where the weight of the grain was affected but the volume remained almost unchanged. The khapra and moth damage represented the second and third types respectively, where both the volume and weight of the grain were affected. This made it difficult for the uniform application of the weight : volume ratio for expressing the loss in storage as mentioned by Pingale.[7] Total quantity was therefore weighed at the end of each month and losses were calculated on the dry matter.

The germ portion of the grain was another constituent affected by insects. Hinton[10] reported that the germ portion contains as much as 50–70% of the total thiamine in the grain, and therefore its loss meant a proportionate loss of thiamine from the grain. Damage to the germ portion was ascertained by the viability test, and from the results in Table II it will be seen that all the test insects damaged this part of the grain. *Ephestia* is known to feed only on the germ portion[8, 9] and therefore caused greater loss in viability.

*Hygienic conditions in the infested grains.*—Infested grains are likely to contain different stages of the living insects, the body fragments and excreta. When the grain is thoroughly cleaned some of these impurities are removed but not the insect fragments. The fragments, being hard particles, might contribute to digestive disturbances, but there is no definite evidence for this. The presence of the fragments is also considered to be an indication of unhygienic storage conditions, and their proportion as an index to the inferior quality of the grain. The population of living insects, the quantity of excreta in the grain, and the insect-fragment counts are shown in Tables I and II. It will be seen from the results that the weevils multiply relatively rapidly and that they add more impurities and body fragments to the grain over the same period. The moth, besides adding excreta and fragments, caused webbings (see Table II), which gave a filthy appearance to the grain.

*Loss of nutrients.*—Because of the loss in the quantity of the grain through insect damage, loss of nutrients from the grain was expected. The values for nutritionally important constituents were therefore worked out, and are given in Table III.

*Nitrogen.*—The results show that the total nitrogen remains practically unchanged in the insect-free sample. In the samples infested by *Calandra* and *Trogoderma* the endosperm of the grains was converted into fine powder which was lost during cleaning. The slight increase in the total nitrogen of these samples is due to this loss of endosperm, which is rich in carbohydrates and poor in proteins, and to the addition of insect fragments and eggs. The insect

## Table I

*Insect population and the extent of loss in the stored grain*

| Species of insects responsible for damage | % Loss in weight after | | Insect population, per 500 g., at the end of | | % Kernel damage at the end of | |
|---|---|---|---|---|---|---|
| | 3 months | 6 months | 3 months | 6 months | 3 months | 6 months |
| *C. oryzae* | 11·25 | 35·12 | 684·5 | 1006·4 | 21·71 | 89·30 |
| *T. granaria* | 4·31 | — | 28·62 | 123·3* | 6·32 | — |
| *E. cautella* | 1·73 | 5·61 | 9·3 | 25·2 | 22·49 | 62·32 |
| Insect-free grain | — | — | — | — | — | — |

* At the end of 5 months

## Table II

*The effect of insect damage on the hygienic condition and viability of the grain*

| Species of insects responsible for damage | Insect fragments per 500 g. of grain at the end of | | Impurities per 500 g. of grain at the end of | | % Viability at the end of | |
|---|---|---|---|---|---|---|
| | 3 months | 6 months | 3 months | 6 months | 3 months | 6 months |
| *C. oryzae* | 1972·6 | 13313·0 | 11·3 g. | 78·0 g. | 62·16 | 19·40 |
| *T. granaria* | 718·4 | 2121·4 | 4·42 g. | — | 76·8 | 34·12* |
| *E. cautella* | 281·6 | 712·3 | 0·27 g. | 3·5 g.† | 56·2 | 17·19 |
| Insect-free grain | — | — | — | — | 88·7 | 89·3 |

* At the end of 5 months

† Webbings are not included in the impurities here, which amount to 28 and 90 g. at the end of 3rd and 6th month respectively

## Table III

*Effect of insect infestation on the various constituents of the grain*

| Data summarized | Control (insect-free) at the end of | | | *C. oryzae* at the end of | | *T. granaria* at the end of | | *E. cautella* at the end of | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 month | 3 months | 6 months | 3 months | 6 months | 3 months | 5 months* | 3 months | 6 months |
| Total nitrogen, % | 1·60 | 1·61 | 1·61 | 1·79 | 1·87 | 1·68 | 1·80 | 1·70 | 1·67 |
| Nitrogen soluble in 3% NaCl solution, % | 0·71 | 0·67 | 0·62 | 0·63 | 0·54 | 0·56 | 0·53 | 0·63 | 0·51 |
| Reducing sugars (mg. of maltose per 100 g.) | 45·0 | 56·0 | 60·0 | 58·0 | 65·0 | 59·0 | 64·0 | 55·0 | 62·0 |
| Acidity of fat (mg. of KOH required to neutralize free fatty acids from 100 g. of grain) | 19·2 | 32·0 | 37·0 | 34·0 | 43·9 | 42·2 | 45·9 | 41·8 | 49·3 |
| Thiamine (μg./g.) | 4·5 | 4·4 | 4·2 | 2·9 | 1·8 | 2·1 | 2·0 | 2·0 | 1·7 |

* Owing to cross-infestation after the 5th month, the analysis of the sample infested with *T. granaria* could not be continued, so the results at the end of the 5-month period are given

fragments and eggs could not be completely removed from the grains used for analysis. Shutt[11] observed a progressive, though small, increase in the protein content of wheat during prolonged storage. This was shown to be due to loss of carbohydrates by respiration. The solubility of protein in 3% sodium chloride solution decreased as a result of storage and insect damage. Jones & Gersdorf[12] thought that the decrease in solubility was probably due to denaturation of proteins whereby they became progressively less soluble. The decrease was found to be relatively greater in insect-damaged grain.

*Reducing sugars.*—The reducing sugar content in all the samples showed an increase which, in the insect-damaged grain, was relatively greater. Leevitt & Le Clerc[13] showed that the total sugar content of wheat increased during storage.

*Fat acidity.*—Zeleny[3] considered the free fatty acid content of the grain to be a sensitive index of its deterioration. High fat acidity has been shown by other workers to be associated with high content of damaged kernel,[14] low viability,[15] and poor bread-baking quality.[16] In these tests all the insect-damaged samples showed relatively higher fatty acid contents. Off-flavour, indicative of oxidative rancidity, was not evident at any stage of the tests.

*Thiamine.*—Cereal grains are an important source of B-group vitamins and loss of these is serious, particularly in a country like India where cereals constitute the main diet of the

people. In these tests, values of thiamine alone were assessed but even these could serve, to some extent, as an index of the loss of other B-vitamins. Fraenkel & Blewett[6] have shown the need for five other constituents of B-group vitamins in addition to thiamine in the insect diet. From Table II it will be evident that the thiamine content of the grain is severely affected by insects, particularly so when only the germ portion is eaten, as by moths.

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# STUDIES ON COMPOSTS PREPARED FROM WASTE MATERIALS. I.—Preparation, Nitrogen Losses and Changes in 'Soluble Nitrogen'

By DELPHINE A. HOYLE and G. E. G. MATTINGLY*

1. Changes in the pH and total soluble nitrogen of composts from waste materials have been examined during decomposition for periods up to two years. Losses of nitrogen, dry matter and organic matter after 14–16 weeks were also determined.

2. The rate of decomposition, based on the level of soluble nitrogen, in composts from straw and sewage sludge, increased as the initial nitrogen content increased from 1·10 to 1·97%; above 1·97% there was no increase in rate of decomposition and losses of nitrogen became excessive.

3. Nearly all the soluble nitrogen in mature composts was present as nitrate; the pH of the mature composts varied approximately inversely as the nitrate content.

4. Supplementary aeration slightly decreased the level of soluble nitrogen in mature composts and considerably increased losses of nitrogen in 14–16 weeks. Aeration had little effect on losses of dry matter.

5. The soluble nitrogen in mature composts from straw and ammonium sulphate was much greater than in composts from straw and sludge prepared with the same initial nitrogen content.

6. The optimum conditions for preparing composts from straw and sludge in small cells are discussed, and the importance of the level of nitrogen, minimal aeration, duration of storage period and the influence of soluble nitrogen in the starting materials are emphasized.

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## Introduction

In the last decade much attention has been given to composts prepared from various waste materials. Stoughton[1] described the use of habitation wastes in agriculture and horticulture, and Bould[2, 3] and Vick[4] the preparation of composts from sewage sludge and town's refuse; similar investigations have been carried out in South Africa,[5, 6] Germany[7, 8] and Holland[9–12] where town's refuse is now composted on a large scale. Crowther & Bunting[13, 14] have described the use of sludge and composts from straw and sludge in large-scale field trials; much of this work in Great Britain has been summarized in a Technical Communication by the Ministry of Agriculture.[15]

Very few studies, however, have been made of chemical changes in composts from waste materials. Bould[2, 3] examined the effect of composting and storage conditions on losses of nitrogen from composts prepared from sludge and town's refuse. He showed the importance of adequate aeration in the early stages of decomposition but found aeration also favoured loss of nitrogen, probably as ammonia. He further found that decomposition was retarded at moisture contents greater than about 65% initially. He concluded that the nitrogen in mature composts was largely unavailable, at least in the year of application. With composts from straw and sludge less nitrogen is lost during composting and useful composts have been prepared from these materials.[3, 15] Some recommendations on the preparation of composts from straw and sewage sludge in large heaps have been published by several authors.[3, 15, 32]

Over thirty years ago Hutchinson & Richards[16] showed that straw requires about 0·7 part of soluble nitrogen per 100 parts of straw, adequate aeration and a neutral or slightly alkaline reaction for satisfactory composting. Much subsequent work on chemical changes in composts of this type, and in farmyard manure, has been reviewed by Waksman.[17]

The level of inorganic nutrients, other than nitrogen, in composts of habitation wastes does not usually appear to be a limiting factor in decomposition. Bould[2] showed that addition of phosphate to refuse–sludge composts slightly increased production of carbon dioxide, and gave a small positive effect on the rate of decomposition of large heaps that was maintained for about 20 weeks. He commented, however, that the increase did not warrant the expense of adding the phosphate.

The investigations described here deal mainly with nitrogen changes in composts prepared from straw and sewage sludge, and straw and ammonium sulphate. They were undertaken to determine how initial composition and method of composting affected the pH and soluble nitrogen of composts, and the losses of dry matter, organic matter and nitrogen. Subsequent papers will deal with the nature of the organic nitrogen in composts at various stages of decomposition, the availability of nitrogen in composts and pot and field experiments. Preliminary accounts of some of this work have already appeared.[18, 19]

## Experimental

### *Materials*

A single sample of wheat straw, chaffed to facilitate sampling, was used throughout the main series of experiments to provide a standard material for composting. The source of nitrogen was either primary sedimentation sludge, in a partially dried state from the drying beds at Maidenhead sewage works, or ammonium sulphate. Analytical figures of the materials used are given in Table I.

**Table I**

*Analytical figures of materials used for composting*

| Material | | Dry matter, % | Ash | Loss on ignition | Total nitrogen |
|---|---|---|---|---|---|
| | | | (as % of dry matter) | | |
| Wheat straw | WS2 | 89·5* | 12·4 | 87·6 | 0·569 |
| Sludge | SS4 | 27·0 | 39·9 | 60·1 | 3·34 |
| ,, | SS5 | 25·7 | 37·7 | 62·3 | 3·46 |
| ,, | SS10 | 32·9 | 47·9 | 52·1 | 2·68 |
| ,, | SS11 | 37·0 | 52·9 | 47·1 | 2·82 |

*Determined separately for each experiment

### *Composting unit*

Composting was carried out on a small scale under controlled and reproducible conditions in a unit consisting of 8 brick cells each 2 ft. × 2 ft. × 2 ft., built on a concrete floor, and lined with asbestos sheets to provide insulation.

Supplementary aeration was provided, when required, through 2-in.-diameter land drains placed on the floor of the cells; throughout this paper the term 'aeration' is used to describe this method of supplementing normal aeration. The composts were covered with bitumen-bonded fibre-glass 'mats' enclosed in hessian covers to prevent loss of heat from the surface.

*Method of composting*

Composts of straw and sludge were prepared by mixing the required quantities of materials (dry-weight basis) in the fresh state; water was added to give a moisture content of 65%. Straw to be composted with ammonium sulphate was damped and compressed for a day or two before the nitrogen (as ammonium sulphate), dissolved in water, was applied. Great care was taken that there was no loss of water or nutrient by drainage. The dry weight of materials used per cell is given in Table II.

**Table II**

*Initial nitrogen content of composts and weight of materials used for composting*

| Compost | Total nitrogen as % of dry matter | Sludge used | Straw dry matter (lb. per cell) | Sludge dry matter (lb. per cell) |
|---|---|---|---|---|
| Straw–sludge | 2·44 | SS4 + SS5 | 10·7 | 21·5 |
| ,, | 1·97 | SS4 + SS5 | 16·1 | 16·1 |
| ,, | 1·70 | SS11 | 26·9 | 27·0 |
| ,, | 1·35 | SS10 | 26·5 | 15·6 |
| ,, | 1·10 | SS10 | 26·5 | 8·9 |
| Straw–sludge–ammonium sulphate | 1·63 | SS11 | 26·9* | 7·6 |
| Straw–ammonium sulphate | 1·55 | — | 26·9† | — |

* Plus 1·06 lb. of $(NH_4)_2SO_4$ and 0·75 lb. of $CaCO_3$
† Plus 1·48 lb. of $(NH_4)_2SO_4$ and 1·5 lb. of $CaCO_3$

Composts were weighed, turned and mixed after 7–21 days, and sufficient water was added to bring the moisture content up to 65%. At the end of the composting period (usually 14–16 weeks) the composts were again weighed and transferred to covered storage bins.

*Temperature records*

Temperatures were taken daily at 9.30 a.m. at a depth of 9 in. with long-stemmed thermometers inserted in glass tubes sunk centrally in the heaps during the initial preparation of the composts.

Some typical records for four different composts are given in Fig. 1. These show that reasonably high temperatures were reached and maintained in the small heaps used.

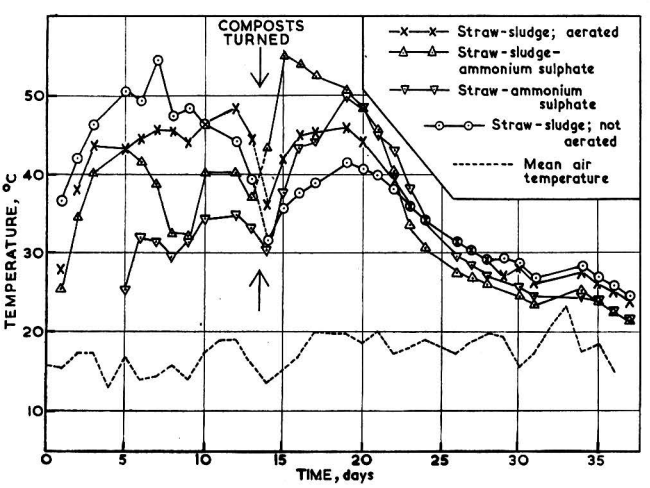


FIG. 1.—*Mean daily temperatures during the early stages of composting for four different composts*

*Sampling*

Sampling was carried out with a hollow auger, 2 ft. long and 1½ in. in diameter. A number of 'cores' were taken vertically from top to bottom in various parts of the heap; a square wire grid placed over the cell was used to avoid taking samples from the same place on two successive occasions. This method provided representative samples of the heterogeneous materials used without disturbing the rest of the heap and was completely satisfactory except in the early stages of composting materials containing a large proportion of straw. Here difficulty in cutting through the straw was experienced and sampling was done by hand. The 'cores' were transferred to covered containers, well mixed, and chopped by hand before sub-sampling for analysis.

*Analytical methods*

*Moisture* was determined by drying to constant weight for 48–72 hours at 60°. Samples for analysis were ground to pass a 40-mesh sieve.

*Ash* was determined by igniting a 40-mesh sample in silica basins at 510–520°. At this temperature a sensibly constant weight was attained in 2 hours. The 'loss on ignition' figures were not corrected for carbonates in the ash and were taken as approximately equivalent to organic matter and used to determine loss of organic matter on composting.

*pH determinations* were made in duplicate by means of glass and calomel electrodes pressed into the fresh compost. No water was added to the compost.

*Total nitrogen* was determined on the ground samples by the Kjeldahl method, as recommended by Chibnall, Rees & Williams.[20] A macro-scale digestion (4 hours) was employed, and aliquots of the digest (0·1–0·2 mg. of nitrogen), after being cooled and diluted, were distilled in a Markham micro-distillation apparatus.[21] Salicylic acid (1 g./30 ml. of sulphuric acid) was used for materials containing nitrates; the nitro-derivative was reduced with sodium thiosulphate.

All nitrogen figures are corrected for ammonia lost on drying. Ammonia-nitrogen was determined (*a*) on the fresh material and (*b*) on the oven-dried material by the method described below. The difference between these results was taken as the ammonia-nitrogen lost on drying and was added in all instances to the total nitrogen determined on the oven-dried material.

'*Soluble nitrogen*' was extracted from the fresh material with 0·1N-hydrochloric acid in the ratio of approximately 1 g. of fresh compost to 10 ml. of acid. The extraction bottle was shaken intermittently during the day, set aside overnight and the contents were filtered without suction through No. 541 Whatman filter papers. The first 20–30 ml. of filtrate was rejected. Portions of the filtrate were analysed for total soluble nitrogen by the Kjeldahl method. When nitrates were present in the extract they were reduced with finely divided iron, as described by Pucher, Leavenworth & Vickery.[22] 'Soluble nitrogen' in this paper refers to nitrate- plus ammonia- and organic-nitrogen extracted under the conditions defined above.

*Ammonia-nitrogen* was determined on another portion (1–3 ml.) of the filtrate from the 0·1N-hydrochloric acid extraction by distillation in the Markham apparatus with 3 ml. of alkaline borax (5 g. of sodium borate in 100 ml. of 0·5N-caustic soda, pH 9·6 approx.) for 5 minutes.

*Nitrate-nitrogen* was determined by colorimetric estimation with phenoldisulphonic acid.[23] Some extracts, which were slightly coloured, were decolorized with hydrogen peroxide.[24]

*Sampling errors*

A statistical examination was made of the results of estimations from duplicate cells of straw–sludge composts. The error sum of squares was calculated from the variation between cells that received the same treatments.

The standard errors of the mean of duplicates for the percentage of total nitrogen, soluble nitrogen and ash in the dry matter are given below for samplings made after 3 weeks and 3 months.

| | After 3 weeks | After 3 months |
|---|---|---|
| Total nitrogen, % of dry matter | ± 0·044 | ± 0·048 |
| Soluble nitrogen, ,, ,, ,, | ± 0·018 | ± 0·004 |
| Ash, ,, ,, ,, | ± 0·79 | ± 1·14 |
| Degrees of freedom for error | 9 | 8 |

There is no significant difference between the errors at the two sampling times for total nitrogen and ash; the standard errors have accordingly been pooled below to calculate fiducial

limits for these estimations. The standard error for soluble nitrogen is significantly greater ($P = 0.05$) after 3 weeks than after 3 months. This is discussed below.

The 95% fiducial limits of the mean of two cells are as follows: total nitrogen, ± 0·099; ash, ± 1·96; and soluble nitrogen, ± 0·041 after 3 weeks and ± 0·009 after 3 months.

## Results

The composts, prepared in duplicate for each treatment, were analysed 8–10 times during the period of decomposition and storage, which, in some instances, was up to two years. The analytical figures of ten composts, after 14–16 weeks' decomposition in the composting cells, and at the final sampling after further storage in bins, are given in Table III.

### Table III

*Analytical figures of composts* (a) *after* 14–16 *weeks and* (b) *at final sampling*

| Type of compost | Initial nitrogen, % | Aeration, weeks | Age at sampling, weeks | pH | Loss on ignition | Total nitrogen | Total soluble nitrogen | Total nitrogen (as % loss on ignition) |
|---|---|---|---|---|---|---|---|---|
| | | | | | (as % dry matter) | | | |
| Straw–sludge | 2·44 | 16 | 16 | 7·5 | 53·8 | 2·69 | 0·161 | 5·00 |
| | | | 67 | 5·7 | 51·3 | 3·22 | 0·418 | 6·31 |
| ,, | 1·97 | 16 | 16 | 7·5 | 55·4 | 2·64 | 0·184 | 4·76 |
| | | | 109 | 5·9 | 51·0 | 3·00 | 0·629 | 5·88 |
| ,, | 1·70 | 2 | 14 | 7·1 | 51·8 | 2·19 | 0·057 | 4·24 |
| | | | 49 | 6·3 | 48·5 | 2·24 | 0·255 | 4·61 |
| ,, | 1·70 | nil | 14 | 7·0 | 50·8 | 2·22 | 0·055 | 4·37 |
| | | | 49 | 6·2 | 47·1 | 2·27 | 0·273 | 4·82 |
| ,, | 1·35 | 15 | 15 | 6·7 | 60·5 | 2·04 | 0·101 | 3·37 |
| | | | 51 | 6·3 | 56·9 | 2·30 | 0·176 | 4·04 |
| ,, | 1·35 | 3 | 15 | 6·9 | 60·2 | 2·08 | 0·091 | 3·46 |
| | | | 51 | 6·1 | 55·5 | 2·31 | 0·233 | 4·16 |
| ,, | 1·10 | 15 | 15 | 6·5 | 68·2 | 1·59 | 0·097 | 2·33 |
| | | | 51 | 6·8 | 59·7 | 2·05 | 0·107 | 3·44 |
| ,, | 1·10 | 3 | 15 | 6·9 | 66·0 | 1·69 | 0·087 | 2·57 |
| | | | 51 | 7·0 | 59·2 | 1·98 | 0·061 | 3·35 |
| Straw–sludge–ammonium sulphate | 1·63 | 2 | 14 | 6·4 | 61·1 | 2·09 | 0·184 | 3·42 |
| | | | 49 | 5·9 | 58·3 | 2·21 | 0·396 | 3·79 |
| Straw–ammonium sulphate | 1·55 | 2 | 14 | 7·0 | 71·5 | 2·16 | 0·552 | 3·02 |
| | | | 49 | 6·3 | 66·7 | 2·37 | 0·827 | 3·56 |

The effects of varying the composition of the starting materials and the method of composting are considered in detail below.

1. *Initial level of nitrogen*

(*a*) *Effect on soluble nitrogen.*—The changes in soluble nitrogen during decomposition followed a similar pattern in composts of wheat straw and sewage sludge prepared with initial nitrogen contents ranging from 1·10 to 2·44% of the dry matter.

There was little difference in the total soluble nitrogen in composts prepared with 1·97 and 2·44% of nitrogen (Table IV). Differences, however, were greater between composts prepared with 1·10 and 1·35% of initial nitrogen. It is clear from these results and from Fig. 2 that the composts with the lowest level of nitrogen released soluble nitrogen very slowly.

### Table IV

*Effect of initial level of nitrogen on total soluble nitrogen in straw–sludge composts: all composts received supplementary aeration for* 15–16 *weeks*

| Initial nitrogen (% of dry matter) | Soluble nitrogen as per cent. of total nitrogen | | | | | |
|---|---|---|---|---|---|---|
| Period of decomposition, weeks .. .. | 16 | 24 | 35 | 50 | 67 | 109 |
| 2·44 | 6·0 | 7·0 | 8·7 | — | 13·0 | — |
| 1·97 | 7·0 | 8·0 | 9·2 | — | 11·7 | 21·0 |
| 1·35 | — | 6·2 | — | 10·1 | — | — |
| 1·10 | — | 3·6 | — | 3·1 | — | — |

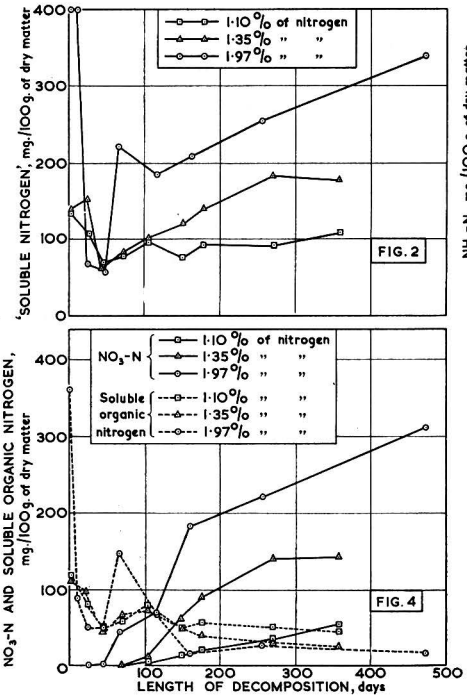


FIG. 2.—*Changes in ' soluble nitrogen ' in straw–sludge composts ; effect of initial level of nitrogen*

FIG. 4.—*Changes in nitrate-nitrogen and soluble organic nitrogen in straw–sludge composts ; effect of initial level of nitrogen*

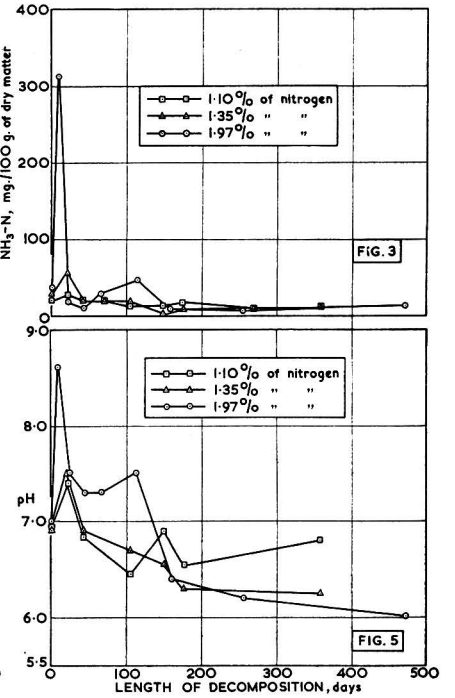


FIG. 3.—*Changes in ammonia-nitrogen in straw–sludge composts ; effect of initial level of nitrogen*

FIG. 5.—*Changes in pH in straw–sludge composts ; effect of initial level of nitrogen*

The changes in soluble nitrogen can be divided into four separate stages (i) ammonification, (ii) rapid decrease in soluble nitrogen, (iii) period of minimum soluble nitrogen, (iv) period of steady increase in soluble nitrogen. Figs. 3 and 4 show the changes in the amount of ammonia-nitrogen, soluble organic nitrogen and nitrate-nitrogen in the composts. Soluble organic nitrogen was calculated as the difference between total soluble nitrogen and the sum of nitrate- and ammonia-nitrogen. The total soluble nitrogen was present largely as ammonia in the early stages of composting. Nitrification began between 6 and 9 weeks in the 1·97 and 2·44% nitrogen composts and increased steadily ; almost all the soluble nitrogen was present as nitrate at the end of the experiment. Fig. 4 shows that the soluble organic nitrogen decreased steadily during nitrification. The chemical nature of this nitrogen was not determined, but qualitative tests showed the absence of free amino-nitrogen (ninhydrin and biuret tests) and the presence of basic nitrogenous substances (precipitate with phosphotungstic acid). No precipitates were obtained, however, with trichloroacetic acid, picric acid or flavianic acid.

In the composts prepared with lower percentages of initial nitrogen, no nitrate was detected until 9–10 weeks from the start, and when the initial nitrogen content was 1·10%, nitrate increased only very slowly between 22 and 50 weeks (Table V). A similar, though less well defined, decrease in soluble organic nitrogen was observed in these composts (Fig. 4).

(*b*) *Effect on pH.*—Fig. 5 shows the variation of pH with time in three straw–sludge composts prepared with different initial levels of nitrogen. These variations followed a similar pattern in the three composts.

In the early stages of decomposition, the pH increased, probably because of ammonification, and then decreased rapidly at first, falling finally in two composts to 5·7 and 6·2. The rapid fall in pH in the 1·97%-nitrogen compost appeared to coincide with the onset of nitrification (see Figs. 4 and 5). The pH of the compost with the lowest level of nitrogen did not fall appreciably during decomposition, nor did nitrate accumulate.

**Table V**

*Effect of initial level of nitrogen on accumulation of nitrate-nitrogen in straw–sludge composts : all composts received supplementary aeration for 2–3 weeks*

| Initial nitrogen (% of dry matter) | $NO_3$-N, as per cent. of total nitrogen | |
|---|---|---|
| | 22 weeks | 50 weeks |
| 1·70 | 1·8 | 10·7 |
| 1·35 | 3·35 | 8·75 |
| 1·10 | 0·3 | 1·3 |

(*c*) *Effect on loss of nitrogen.*—In general, losses of nitrogen were heaviest in composts prepared with the highest initial level of nitrogen (Table VI). The effect of the period of aeration is discussed below.

**Table VI**

*Effect of level of nitrogen on losses of nitrogen, dry matter and organic matter in 15–16 weeks in straw–sludge composts : all composts received supplementary aeration for 15–16 weeks*

| Initial nitrogen (% of dry matter) | Loss of nitrogen (% of initial nitrogen) | Loss of dry matter (% of initial dry matter) | Loss of organic matter (% of initial organic matter) |
|---|---|---|---|
| 2·44 | 26·5 | 32·6 | 48·5 |
| 1·97 | 19·7 | 39·3 | 54·5 |
| 1·35 | 7·6 | 38·5 | 49·5 |
| 1·10 | — | 37·2 | 45·8 |

(*d*) *Effect on losses of dry matter and organic matter.*—The losses of dry matter and organic matter in 15–16 weeks, shown in Table VI, appear to be substantially independent of nitrogen level between 1·10 and 1·97% of nitrogen. The smaller loss for the compost (2·44% of nitrogen) is attributed to the high ash content of the sludge used (Table I). Although loss of organic matter was greatest in the compost with 1·97% of initial nitrogen and least in that with 1·10% of nitrogen, the differences were not nearly so pronounced as the different levels of soluble nitrogen reached in these composts during decomposition (Fig. 2).

2. *Aeration*

(*a*) *Effect on soluble nitrogen.*—The soluble nitrogen in the composts decreased slightly with increased periods of aeration ; the results for two pairs of composts are given in Table VII.

**Table VII**

*Effect of period of aeration on pH and soluble nitrogen (% of total nitrogen) in straw–sludge composts : results for two composts each at two different periods of aeration*

| Period of aeration, weeks | Initial nitrogen (% of dry matter) | Soluble nitrogen | | pH | |
|---|---|---|---|---|---|
| | | 24 weeks | 50 weeks | 24 weeks | 50 weeks |
| Nil | 1·70 | 8·0 | 12·0 | 6·40 | 6·15 |
| 2 | 1·70 | 5·7 | 11·4 | 6·55 | 6·30 |
| 3 | 1·35 | — | 10·1 | — | 6·10 |
| 15 | 1·35 | — | 7·65 | — | 6·25 |

(*b*) *Effect on pH.*—The differences in pH due to different periods of aeration were very closely related to the amount of soluble nitrogen (largely nitrate-nitrogen) present, as shown in Table VII, the pH falling as nitrate-nitrogen increased.

(*c*) *Effect on loss of nitrogen.*—Increasing the period of aeration (Table VIII) increased losses of nitrogen from the composts.

**Table VIII**

*Effect of the period of aeration on loss of nitrogen (as % of initial nitrogen) from straw–sludge composts in 14–15 weeks : results for two composts each at two different periods of aeration*

| Initial nitrogen (% of dry matter) | Period of aeration, weeks | | | |
|---|---|---|---|---|
| | nil | 2 | 3 | 15 |
| 1·35 | — | — | 3·8 | 7·6 |
| 1·70 | 5·3 | 8·3 | — | — |

(*d*) *Effect on losses of dry matter and organic matter.*—The period of aeration had little effect on the loss of dry matter or organic matter during composting (Table IX). The lower loss of dry matter in the 1·7%-nitrogen compost was due to the high percentage of ash (52·9%) present in the sludge used in its preparation. The results further indicate that, for composts prepared with 1·10 to 1·70% of initial nitrogen, loss of organic matter in 14 to 15 weeks is nearly constant.

## Table IX

*Effect of the period of aeration on losses of dry matter and organic matter from straw–sludge composts in 14–15 weeks: results for three composts and two periods of aeration*

| Period of aeration, weeks | Initial nitrogen (% of dry matter) | Loss of dry matter (% of initial dry matter) | Loss of organic matter (% of initial organic matter) |
|---|---|---|---|
| Nil | 1·70 | 27·1 | 44·4 |
| 2 | 1·70 | 28·6 | 44·5 |
| 3 | 1·35 | 37·3 | 48·9 |
| 15 | 1·35 | 38·5 | 49·8 |
| 3 | 1·10 | 38·6 | 48·4 |
| 15 | 1·10 | 37·2 | 45·3 |

3. *Nature of nitrogen source*

In the experiments described above, wheat straw was composted with sewage sludge at different levels of application and with different periods of aeration. Further experiments were carried out to compare the rate of breakdown of straw with sludge and inorganic nitrogen alone or in combination. Three composts were prepared, in duplicate, from wheat straw and (A) sludge alone, (B) sludge plus ammonium sulphate and (C) ammonium sulphate alone. Calcium carbonate was added to B and C (Table II). The mean initial nitrogen content was 1·63 ± 0·07% and the composts were aerated for two weeks.

(*a*) *Effect on soluble nitrogen.*—The changes in the soluble nitrogen due to these treatments are shown graphically in Fig. 6. The rates at which soluble nitrogen increased did not differ greatly between treatments. The minimum level of soluble nitrogen reached during decomposition was, however, much higher in composts prepared with ammonium sulphate than in composts with sludge as the source of nitrogen.

(*b*) *Effect on pH.*—The changes in pH were more complex in these composts than in the straw–sludge composts discussed above. pH was again closely related with nitrate level, as shown in Table X, but in the early stages of composting the reaction of the straw plus ammonium sulphate compost remained high even when the ammonia concentration was decreasing, probably owing to the presence of the calcium carbonate.

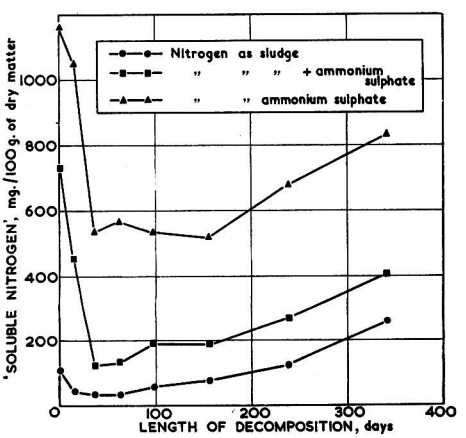


FIG. 6.—*Changes in 'soluble nitrogen' in composts from wheat straw with different sources of nitrogen (initial nitrogen = 1·63 ± 0·07%)*

## Table X

*Effect of source of nitrogen on pH and nitrate-nitrogen (% of total nitrogen) during composting: initial nitrogen = 1·63 ± 0·07%*

| Compost | pH, weeks | | | $NO_3$-N, weeks | | |
|---|---|---|---|---|---|---|
| | 9 | 34 | 49 | 9 | 34 | 49 |
| Straw–sludge | 7·25 | 6·55 | 6·3 | nil | 4·5 | 10·7 |
| Straw–sludge–ammonium sulphate | 6·45 | 5·9 | 5·9 | 2·5 | 11·5 | 16·7 |
| Straw–ammonium sulphate | 7·35 | 5·9 | 6·3 | 3·3 | 29·5 | 33·6 |

(*c*) *Effect on loss of nitrogen.*—Loss of total nitrogen from the three composts A, B and C in 14 weeks was 8·3, 21·6 and 7·5% respectively. It is difficult to account for the heavy loss of nitrogen in the compost of straw plus sludge and ammonium sulphate. From subsequent work on the fractionation of nitrogen in these composts, to be discussed in a later paper, it appeared that this compost behaved similarly to a straw–sludge compost with a high initial content of soluble nitrogen, and the loss of nitrogen from this compost is comparable with losses from the sludge composts (initial nitrogen 1·97 and 2·44%, Table VI).

(*d*) *Effect on loss of dry matter and organic matter.*—Differences between the composts for losses after 14 weeks were only small, and were least in the straw–sludge compost with a high ash content (Table XI).

**Table XI**

*Effect of source of nitrogen on losses of dry matter and organic matter in 14 weeks: initial nitrogen = 1·63 ± 0·07%*

| Compost | Loss of dry matter (% of initial dry matter) | Loss of organic matter (% of initial organic matter) |
|---|---|---|
| Straw–sludge | 28·6 | 44·5 |
| Straw–sludge–ammonium sulphate | 38·6 | 50·0 |
| Straw–ammonium sulphate | 33·0 | 40·1 |

4. *Size of cells used in composting*

In addition to composting on a small scale, several composts of straw and sludge were prepared in large cells 5 ft. × 5 ft. × 4 ft. 6 in. at Maidenhead Sewage Works, by the method described by Bould.[2, 3] Liquid sludge was used in the preparation of these composts, and since it was found preferable to sludge the straw in small quantities over several days, rather than apply the whole amount in one application, it was difficult to determine precisely the initial nitrogen content.

The composition of three such composts is given in Table XII. The results show that the composition of the composts after 3 to 6 months is similar to that obtained for composts, of a similar initial nitrogen content, prepared in the small cells (Table III).

**Table XII**

*Analytical figures of straw–sludge composts prepared at Maidenhead in large cells*

| Compost | Initial nitrogen, % | Aeration, weeks | Age at sampling, weeks | pH | Loss on ignition | Total nitrogen | Total soluble nitrogen | Total nitrogen (as % loss on ignition) |
|---|---|---|---|---|---|---|---|---|
| | | | | | (as % dry matter) | | | |
| C13 | 1·20 | 7 | 14 | 7·5 | 61·5 | 2·01 | 0·069 | 3·27 |
| C16 | — | 3·5 | 16 | — | 65·0 | 1·86 | 0·064 | 2·86 |
| C44 | — | 4·5 | 26 | — | 59·5 | 2·84 | 0·229 | 4·77 |

## Discussion

1. *Level of initial nitrogen*

The changes in soluble nitrogen in straw–sludge composts due both to the initial level of nitrogen applied and to the length of the period of decomposition were very marked and followed a clearly defined pattern. Soluble nitrogen increased steadily with time and with level of initial nitrogen up to a maximum in composts with an initial nitrogen content of about 2%. Most work on changes in composts has been limited to periods of decomposition of 3–6 months, though it is clear from these experiments that the level of soluble nitrogen can increase three-fold in straw–sludge composts on storage between 4 months and 2 years (Table IV).

The experiments described suggest that there is no advantage in increasing the initial level of nitrogen above 2% in straw–sludge composts, for above this level the rate of decomposition of the compost, as measured by the level of soluble nitrogen, did not increase, but the losses of total nitrogen in the early stages of composting increased substantially. Minimum loss of nitrogen occurred when the initial percentage of nitrogen was about 1·35; this figure corresponds closely with that determined by Scheffer & Karapurkar[25] for composts of straw and lucerne.

As the initial nitrogen content increased in straw–sludge composts from 1·10 to 1·97%, losses of organic matter in 14–16 weeks increased from 45·3 to 54·5%. Hutchinson & Richards[16]

and Waksman & Gerretsen[26] also showed that losses of organic matter increased on composting straw with increasing amounts of inorganic nitrogen. The changes, on composting, in soluble nitrogen, or the nitrogen losses, appear from this work, however, to indicate more accurately the optimum level of nitrogen required during composting.

It has been shown[27] that nitrate is confined to the outer and drier layers in large heaps of manure. Most of the soluble nitrogen in the composts prepared in these experiments, however, nitrified almost completely during storage, probably because of the relatively low moisture content (65%) and the small bulk of the material.

No determinations of losses were made beyond 16 weeks because of errors introduced into weighings by continuous sampling. In almost all instances, however, the total nitrogen as a percentage of the dry matter, and the ash content, increased steadily, and it is not considered likely that losses were high during storage.

2. *Aeration*

The supplementary aeration provided by land drains did not increase the rate of decomposition, i.e. the level of soluble nitrogen. In fact, there was evidence that the soluble nitrogen was slightly lower in aerated composts, owing probably to the heavier losses of nitrogen that accompanied aeration. It has been shown[16, 28] that dry-matter losses are smaller in straw rotted in the absence of air, and Bould[3] found losses of dry matter were smaller in composts prepared in large cells without supplementary aeration from below. Since there was no evidence that aeration increased losses of either dry matter or organic matter, it seems certain that sufficient aeration was obtained by convection in the small cells used in this work without supplementary aeration.

3. *Nitrogen source used in composting*

There were considerable differences between the levels of soluble nitrogen in mature composts of straw with sludge, sludge and ammonium sulphate, and ammonium sulphate respectively with approximately the same initial percentage of nitrogen ($1{\cdot}63 \pm 0{\cdot}07\%$). This figure (1·63%) is considerably higher than that recommended by Hutchinson & Richards[16] for composts from straw and inorganic nitrogen (1·25%), but lower than the optimum determined in this work for maximum soluble nitrogen in composts of straw and sludge (2%). The minimum level of soluble nitrogen in the composts prepared with ammonium sulphate did not fall below 25% of the total nitrogen, and in the compost from sludge the corresponding figure was 2·5%.

Although the retention of ammonia by straw agrees with the findings of Hutchinson & Richards[16] it is not clear by what mechanism it is retained. Clayson[29] showed that colloidal silica was present in straw and might be responsible for the retention of ammonia. This view is difficult to reconcile with the results from straw–sludge composts where ammonia-nitrogen reached 15% of the total nitrogen yet still fell rapidly to a low level (Fig. 3). Bould,[3] also, did not report any retention of ammonia in much larger heaps of straw–sludge and refuse–sludge composts.

Acharya *et al.*,[30] working in India with different composts, suggested that, for rapid liberation of nitrogen, it was an advantage in composting to use materials rich in soluble nitrogen, and the results here support that view. The comparatively small losses of nitrogen (7·5%) and organic matter (40%) in 14 weeks, obtained with the compost from straw and ammonium sulphate, applied at a level higher than that usually recommended, agree closely with the findings of Hesse & Schmalfuss[31] with straw composts prepared with twice the usual addition of soluble nitrogen (1·4%).

4. *Comparison of composts from small and large cells*

Considerable advantages were found by the use of small cells for composting experiments. It was possible to mix the materials more completely than in the larger heaps, and weighing and sampling could be carried out more easily and accurately. The standard errors of the mean of duplicate cells were considerably smaller than those reported for larger heaps by Burrows,[33] who drew attention to the need for estimating errors in sampling bulky organic materials. The high standard error for soluble nitrogen after 3 weeks is probably due to the rapid rate at which this variable is changing in the first few weeks of composting (Fig. 2); this error decreased significantly with samplings made after three months when soluble nitrogen was changing slowly.

Although little evidence has been given to justify comparison of composts prepared in small and large cells, it appears from the results in Table XII that no major differences exist.

This conclusion is supported by the results obtained by Bould[3] for changes occurring in large heaps. Maximum temperatures reached during decomposition were a little lower than is usual in larger heaps.

5. *Optimum conditions for composting in small cells*

The experiments described here suggest that to prepare composts on a small scale from primary sedimentation sludge and straw, with the minimum loss of nitrogen and the maximum level of soluble nitrogen in the mature compost, the following conditions must be fulfilled:

(1) The initial nitrogen should lie between 1·35 and 1·97%.
(2) Supplementary aeration should be limited to a minimum.
(3) Storage should be prolonged, and be preferably for at least a year.

The work described also emphasizes the advantages of using soluble nitrogen compounds for composting to increase the level of soluble nitrogen in the mature compost.

If it is assumed that partly drained sludges contain about 3% of nitrogen on dry matter and 70% of moisture, the conditions recommended for preparing composts from straw containing 0·5% of nitrogen, based on dry matter, and 10% of moisture, are the use of five parts of fresh sludge to one part of straw. The initial nitrogen content will then be about 2% on dry matter and the moisture content about 58–60%, which can be raised to 65% or higher by damping the straw.

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## CONTENTS