THE BIOGENESIS OF CAROTENOIDS *

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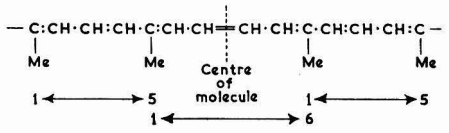
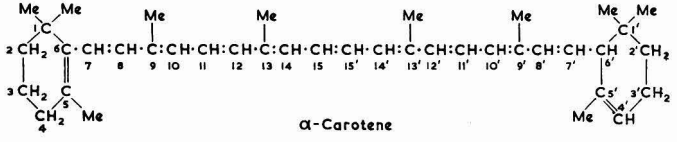
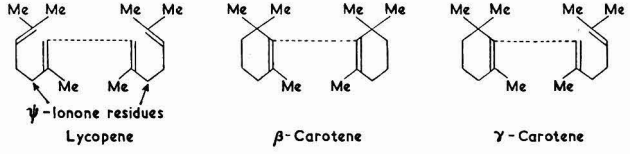
CAROTENOIDS are very widely distributed plant-pigments, insoluble in water but soluble in all the usual fat-solvents; they vary in colour from lemon-yellow to deep red. From their structure, brilliantly elucidated by Karrer and by Kuhn in the 1930s (see e.g. Karrer & Jucker[1]), they can be considered, on paper at least, to be built up from isoprene (CH_2:CH·CMe:CH_2) units, usually eight. Their main structural characteristic is that there is a 'reversal' of the direction of the C_5 (isoprene) units at the centre of the molecule, as if four C_5 units had combined head to tail to form a C_{20} unit, two of which then combined tail to tail to give a C_{40} carotenoid. This property is included in the definition of carotenoids issued by I.U.P.A.C.; it is stated that in carotenoids the isoprene units are 'linked so that the two methyl groups nearest the centre of the molecule are in positions 1 : 6, whilst all other lateral methyl groups are in positions 1 : 5':



Carotenoids are divided into two main groups: (*a*) carotenes, which are hydrocarbons, and (*b*) xanthophylls, which are oxygen-containing derivatives of carotenes: the oxygen atoms can exist in hydroxyl, epoxyl, carbonyl, carboxyl or methoxyl groups. The structure of α-carotene is given here to indicate the detailed structure of carotenoids and the numbering of the molecule now internationally accepted; it will be seen that the molecule contains an α- and a β-ionone residue and that it is numbered in two halves, the plain numerals being applied to the half containing the β-ionone residue; this rule applies to all carotenoids.



All naturally occurring C_{40} carotenoids contain the same linear C_{22} polyene chain as does α-carotene; they differ from each other in the terminal substituents of this polyene chain. The structures of lycopene and β- and γ-carotenes are given below and the structures of almost all of the pigments to be discussed here can be referred to these pigments.



For a long period the main interest of biochemists in carotenoids was in the conversion of certain members (chiefly β-carotene) into vitamin A. During the past decade considerable progress has been made in the carotene → vitamin A investigations, so much so that it can

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fairly be said that the problem is essentially solved. Thus, interest has been shifted to some extent to the other major outstanding problems in carotenoid biochemistry, namely, biogenesis and function (other than as vitamin-A precursors). Studies on the role of carotenoids in reproduction and in phototropic responses have not made very great progress, and indeed it now seems possible that carotenoids play only a minor part in the latter phenomenon.[2]

In the study of the biosynthesis of carotenoids, however, progress has been made; this is mainly due to the fact that the recent great expansion in general biochemistry has provided the present-day investigator of carotenogenesis with a very much greater 'striking power' than his predecessor of 5–10 years ago could command. Information about the biosynthesis of carotenoids was, up to 1950, mainly of the general type involving investigations into the cultural and environmental conditions controlling synthesis. It is not intended to discuss here these important basic studies in detail (see Goodwin[3] for a review), but to consider work carried out since 1950, the ultimate aim of which is to describe the biosynthesis of carotenoids at the molecular level.

Higher plants

(i) *Fruit*

It may seem a little illogical to discuss fruit before leaves, but, from the point of view of the biosynthesis of carotenoids, fruit can be considered as distinct units; further, much more is known about the synthesis of carotenoids in fruit, and this knowledge is of great help in considering the, as yet, meagre information about formation in leaves.

Lycopene is a carotene characteristic of fruit, for it is never found in leaves. It has been known for a long time that lycopene occurs in ripe tomatoes in large amounts, together with smaller amounts of α- and β-carotenes. Recently small amounts of carotenes less unsaturated than lycopene or β-carotene have been detected in tomatoes. These are neurosporene, ζ-carotene, phytofluene, phytoene and tetrahydrophytoene. (The last three are colourless and are perhaps better described as polyenes rather than carotenes.) Although the evidence at present available is not absolutely conclusive, it appears that these compounds are all structurally related to lycopene, each differing from the preceding one in the list given above in containing four more hydrogen atoms: neurosporene is tetrahydrolycopene, and ζ-carotene tetrahydroneurosporene, etc.

Porter & Lincoln,[4] on the basis of extended genetical studies on tomatoes, have concluded that lycopene is formed from an almost fully saturated precursor (eicosahydrolycopene) *via* the series given above ('the Porter–Lincoln series') of polyenes by the step-wise removal of four hydrogen atoms. It was further concluded that α- and β-carotenes are formed by the isomerization of lycopene and xanthophylls by its oxidation. Porter & Lincoln's scheme is outlined in Fig. 1; a critical discussion of the genetical basis of the scheme has been provided by Mackinney.[5] Assuming for the moment the validity of the Porter–Lincoln scheme, the question arises, how is the basic saturated precursor formed? There are two main possibilities: (*a*) two C_5 repeating units condense tail to tail to form the centre of the molecule and then the remaining six units condense head to tail, three on each side of the central C_{10}

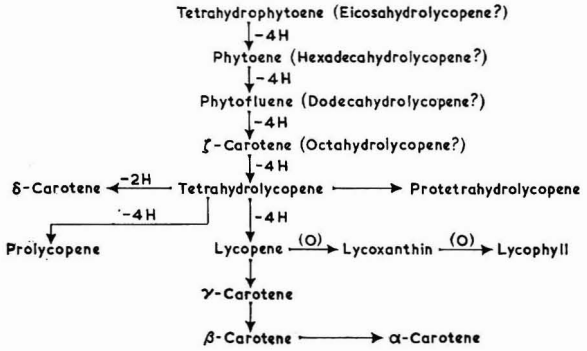


FIG. 1.—*Porter & Lincoln's scheme for carotenogenesis in tomatoes*

unit, or (*b*) that C_{20} units are first formed by the head-to-tail condensation of four C_5 units and that two of these C_{20} units then combine to form a saturated C_{40} compound. The C_{20} compound just envisaged would inevitably be closely related to phytol and thus we are brought back to the very first ideas on the synthesis of carotenoids. As chlorophyll disappears in ripening fruit and carotenoids increase, Willstätter & Mieg[6] thought that they might be formed from the phytol liberated by hydrolysis of chlorophylls a and b. This suggestion has now

$$H_3C\cdot CHMe\cdot[CH_2]_3\cdot CHMe\cdot[CH_2]_3\cdot CHMe\cdot[CH_2]_3\cdot CMe{:}CH\cdot CH_2\cdot OH$$

Phytol

been shown in a number of ways to be invalid : (*a*) there is not sufficient phytol liberated from chlorophyll to account for all the lycopene formed in ripening tomatoes ;[7] (*b*) lycopene continues to be formed for a considerable time after chlorophyll has disappeared ; and (*c*) although tomatoes produced in complete darkness have no chlorophyll when unripe, they do contain lycopene when mature.[8] Obviously these experiments do not rule out the possibility that lycopene is synthesized from a non-chlorophyll source of phytol.

The step-wise transformation of the basic compound into lycopene has been considered by Porter & Lincoln to take place possibly in two main ways : (*a*) different enzymes are necessary for each step, their production being controlled on the 'one gene, one enzyme' principle ; or (*b*) only one enzyme is concerned in all the steps, but a specific hydrogen-acceptor is necessary at each step ; in this event it would be the production of these hydrogen-acceptors that is genetically controlled. The fact that the conversion of a fully saturated molecule into lycopene involves a change in molecular shape from a coil to a rod[5] indicates that (*a*) is the likelier possibility. Another possibility for the synthesis of the Porter–Lincoln series is that they are produced, not step-wise from each other, but in a parallel fashion from a common precursor ;[9] lycopene could then be formed by the condensation of eight unsaturated C_5 residues (possibly β-methylcrotonaldehyde) and the other members by the appropriate condensations of saturated and unsaturated C_5 units[5] (Fig. 2). These condensations would be under genetic control.

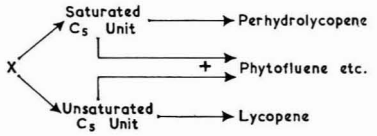


FIG. 2.—*The 'parallel synthesis' of the Porter–Lincoln series*

Further, when lycopene synthesis in tomatoes is inhibited by high temperature, all the Porter–Lincoln series are equally inhibited ;[10] if a step-wise process were functioning, an accumulation of one member of the series might have been expected just before the temperature-sensitive stage. The fact that some, but not all, members of the Porter–Lincoln series are missing from some berries[11] tends to support the idea of parallel syntheses.

About the postulated isomerization of lycopene to α- and β-carotene there is evidence to suggest strongly that this does not take place to any great extent in ordinary commercial tomatoes, but that the synthesis of β-carotene occurs *via* a completely independent pathway. It has long been known that tomatoes ripened at 30° produce very little lycopene, but normal amounts of β-carotene ;[10, 12, 13] similarly at low temperatures carotene synthesis is much less affected than lycopene synthesis ;[10] if they were connected one would have expected them to have been affected in the same way.

Two strains of tomatoes have recently been isolated one of which could be considered to support the idea of a separate pathway for carotene synthesis, and the other to support the idea of its synthesis from lycopene. In the first strain, containing the 'lutescent' gene, the fruit ripen for two weeks as yellow tomatoes, that is, with normal amounts of carotene but with very little lycopene ; then, within 24–36 hours, relatively enormous amounts of lycopene are synthesized.[5] In the second strain occurs the gene *B*, which expresses itself in a marked increase in β-carotene synthesis at the expense of lycopene.[14] The existence of these strains cannot be taken as providing unequivocal proof of the validity of either synthetic scheme, for in both it is probable that the site of genetic control is very far back in the

synthetic chain (possibly at C_5 unit stage), a conclusion already tentatively advanced by Mackinney.[5]

It will be seen, when carotenogenesis in fungi is considered, that the production of β-carotene *via* the Porter–Lincoln series almost certainly does not take place. It remains to discover whether this can be confirmed or not in tomatoes; for, although the available evidence points in this direction, the crucial experiment has not yet been carried out. Whatever the fate of the Porter–Lincoln concept, however, it has been of the greatest value in stimulating thought and investigations.

(ii) *Leaves*

Very little work has been carried out on the mode of synthesis of carotenoids in leaves, compared with that undertaken to describe the pattern of synthesis (see Goodwin[3]). Only recently has the isolated leaf been examined and found to be a suitable preparation for the study of carotenogenesis. Bandurski[15] was first in the field, and he found that the synthesis of carotene in bean leaves depends only indirectly on the presence of light, in so far as the leaves would normally utilize photosynthetic products as substrates for the enzymes concerned with carotene synthesis. Isolated leaves will synthesize carotene in the dark if they are provided with glucose or sucrose. Although neither pyruvate nor glycerol is effective in this way, it remains to be proved whether the leaf utilizes sucrose *in vivo* or possibly a smaller fragment produced 'en route' from carbon dioxide to sucrose. Fluoride, but not sulphanilamide, inhibits the synthesis of carotene from glucose.

The following have been shown to inhibit carotenogenesis in pea seedlings: streptomycin, guanidine hydrochloride, chloromycetin, *iso*nicotinic acid hydrazide, maleic hydrazide and tetronic acid,[16–18] but penicillin has little, if any, effect. The most interesting features emerging from these studies are: (*a*) tetronic acid is extremely potent, being about 60 times as active as streptomycin, and (*b*) whereas all the others have also an inhibitory effect on chlorophyll synthesis, *iso*nicotinic acid hydrazide is without any effect.

Glover & Redfearn[19] have recently begun a study of carotenogenesis in isolated tobacco leaves, with emphasis on the production of ^{14}C-labelled β-carotene with a high specific activity. They have found that in leaves kept in the dark for 24 hours in the absence of carbon dioxide there is a decrease in the β-carotene content but a rise in the level of the epoxides of β-carotene; on admitting carbon dioxide and light, the epoxide level almost immediately drops to normal, but there is a lag of about 24 hours before the β-carotene level begins to rise, until after 96 hours it attains its original value. These results are interpreted as indicating that the production of epoxides is the first step in the oxidative destruction of β-carotene in the leaf, but that the synthesis of β-carotene is not *via* epoxides. Experiments with $^{14}CO_2$ indicate that there is a fairly rapid turnover of β-carotene in the leaf.

(iii) *Petals*

In a number of flower petals carotenoid epoxides are the characteristic pigments present. It has been suggested by Karrer & Rutschmann[20] that they are formed by the oxidation of parent carotenes or hydroxycarotenes; the recent work of Glover & Redfearn[19] on leaves indicates that this may be true.

Schroeder[21] found that monkey flowers (*Mimulus longiflorus*) allowed to develop under natural conditions on the plant do not contain any *cis*-carotenoids; if, however, they are allowed to develop by keeping stems with buds in water for several days exposed to diffuse daylight, then considerable amounts of *pro*lycopene and *pro*-γ-carotene are formed. The significance of the production of *cis*-carotenoids from the point of view of carotenogenesis is not yet apparent [for a consideration of the chemistry of *cis*-carotenoids, see Zechmeister[22]].

(iv) *Roots*

Little is known about carotene synthesis in roots, except that, like fruit, carrots and sweet potatoes appear to contain the necessary enzymes and substrate(s) for direct synthesis and are not dependent on the leaf. Both vegetables after harvesting can synthesize considerable amounts of β-carotene in storage.[23, 24] Carrot roots grown in tissue culture, however, do not synthesize either β-carotene or colourless polyenes.[11]

Cryptograms: fungi

The carotenogenic ability of the Phycomycete *Phycomyces blakesleeanus* has recently been the subject of much investigation, and it is by means of this organism that most of our information about carotenogenesis in fungi has been obtained. *Phycomyces blakesleeanus* was first

shown to contain β-carotene by Schopfer & Jung,[25] and this has been repeatedly confirmed. It was not until recently, however, that the Porter–Lincoln series were also shown to be present in very small amounts.[8]

On a glucose/asparagine medium, *Phycomyces* takes 3–4 days to achieve full growth and during this time comparatively little carotene is synthesized; when it is fully grown it dissimilates any residual glucose and it is at this stage that most of the carotene is formed. If only sufficient glucose is present for growth, then the carotene content of the mycelium remains low.[26, 27] A similar pattern of synthesis has been observed qualitatively in *Rhodotorula rubra*.[28]

If well formed mats of *Phycomyces* are transferred to a medium containing glucose alone, then further considerable amounts of carotene are synthesized;[27] the process cannot, however, continue indefinitely, for the mycelium soon becomes saturated with carotene and synthesis ceases.[29] When glucose is dissimilated by *Phycomyces*, the pH of the medium, initially at about 6·0, falls to 3·2; if this drop is prevented by buffering the medium then little or no β-carotene is synthesized, although fat synthesis is stimulated.

Three main questions arise when β-carotene synthesis in *Phycomyces* is further considered: (*a*) what is the C_5 repeating unit; (*b*) how is it derived; and (*c*) what part does the Porter–Lincoln series play?

The nature of the C_5 unit

The first information about the nature of the C_5 fragment came from experiments in which leucine and valine were used to replace the normal nitrogen source (asparagine) in the standard cultural medium for *Phycomyces*. In a medium containing sufficient glucose (1%) to give fair growth but little β-carotene production, this substitution caused a fourfold stimulation of carotenogenesis with leucine and considerable, but slightly less, stimulation with valine;[30] the observations with leucine have recently been confirmed.[31] It was therefore considered that either *iso*valeraldehyde or β-methylcrotonaldehyde would be the repeating unit, according as a saturated C_{40} unit was first formed (Fig. 3) or not. Similarly, valine could be considered

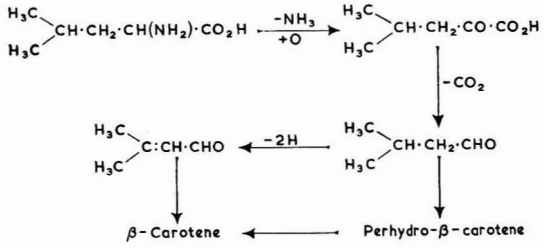


FIG. 3.—*A postulated scheme for the synthesis of β-carotene from leucine (Goodwin)*

to give β-methylcrotonaldehyde by the scheme illustrated in Fig. 4. It is easier to conceive of valine being converted into β-methylcrotonic acid, but, as will be seen later, this acid is probably inactive in stimulating carotenogenesis. The failure to observe stimulation of carotene synthesis by leucine or valine on a medium containing 3% of glucose is due to the fact that

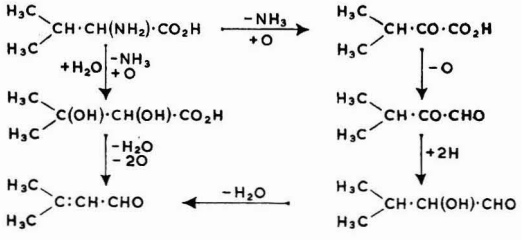


FIG. 4.—*Possible routes for the synthesis of β-methylcrotonaldehyde from valine*

the residual glucose provides sufficient precursor to saturate the organism with the pigment. In order to test the validity of the ideas about the stimulation of carotenogenesis by leucine and valine, various postulated precursors were synthesized and tested in growing *Phycomyces* cultured on a medium containing 1% of glucose. Almost all the materials tested were very toxic but stimulation was observed with low concentrations of α-keto*iso*caproic acid and β-methylcrotonaldehyde, though not with β-methylcrotonic acid ; α-hydroxy*iso*caproic acid and *iso*valeraldehyde produced slight stimulation.[32] Greatest stimulation was achieved with β-methylcrotonaldehyde, but it did not reach that observed with leucine ; before, therefore, β-methylcrotonaldehyde can be finally accepted as the C_5 repeating unit, these experiments must be repeated with labelled compounds. Preliminary isotope experiments indicate that *iso*valeraldehyde is probably not a precursor when acetate is the carbon source.[33] If this result is found to apply when leucine is the carbon source, then the scheme devised to explain the formation of β-methylcrotonaldehyde from leucine must be revised to exclude *iso*valeraldehyde ; the simplest possibility is to assume that α-keto*iso*caproic acid undergoes βγ-dehydrogenation before decarboxylation :

$$Me_2HC\cdot CH_2\cdot CO\cdot CO_2H \longrightarrow Me_2C{:}CH\cdot CO\cdot CO_2H \longrightarrow Me_2C{:}CH\cdot CHO$$

The synthesis of the C_5 unit from glucose

Assuming that β-methylcrotonaldehyde is the active C_5 fragment, the next question is, how does it arise from glucose ? The most likely route is shown in Fig. 5, although it must be stated that a mixture of acetone and acetaldehyde did not stimulate carotene synthesis under conditions in which β-methylcrotonaldehyde did.[32] It is reasonable to assume that acetate would be produced in *Phycomyces* by the normal glycolytic route. This scheme would

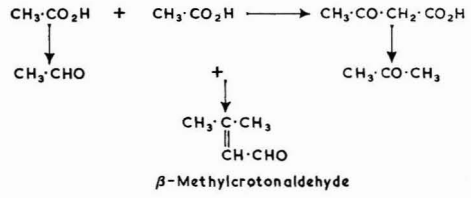


FIG. 5.—*A possible route for the synthesis of β-methylcrotonaldehyde from acetate*

then embrace the observations of Schopfer & his colleagues,[33–36] who consider acetate to be the fundamental precursor of β-carotene ; they base their conclusions on their observation that *Phycomyces*, growing on a medium containing acetate as the sole carbon source, will also synthesize β-carotene. Under such conditions, using acetate labelled with ^{14}C in either the carboxyl or methyl group, Grob *et al.*[36] found that both carbon atoms were incorporated into the β-carotene molecule. About 69% of the carotene atoms could be accounted for in these experiments ; it remains to be seen whether this comparatively low utilization was due to some losses of carotene during its isolation or whether atmospheric carbon dioxide can be fixed by *Phycomyces*. Preliminary tracer experiments with $^{14}CO_2$ seem to indicate that little or no incorporation of carbon dioxide into β-carotene takes place in *Phycomyces*.[33] Schopfer, Grob & Poretti[37] have found that acetate behaves in the same way towards carotenogenesis in *Mucor hiemalis* as in *Phycomyces*.

A somewhat puzzling observation of Schopfer & Grob[33] was that *Phycomyces* grew very sparsely on lactate as the sole carbon source, and synthesized no β-carotene. Normally one would have expected some lactate to have been converted into acetate *via* pyruvate and thus give rise to β-carotene. Goodwin & Griffiths[38] have confirmed that growth is very sparse on lactate alone, but have also found that, if a large enough crop of mycelium is examined, β-carotene can be extracted from it in the same concentration as that found in ' acetate mycelia ' Growth on lactate in culture vessels of normal size is so small that the traces of carotene present can easily be missed.

If acetate is the forerunner of β-carotene, it still remains to be explained why (*a*) the concentration of β-carotene in ' glucose mycelia ' is greater than in ' acetate mycelia '[38] and

(*b*), as shown by isotope studies, why acetate in the presence of glucose is not preferentially utilized for carotene synthesis.[39]

A possible explanation is that the acetate arising from glycolysis is ' active acetate ' (acetyl-phosphate–coenzyme A complex). This would fit in with the ideas of Willmer[40] and Arnaki & Stary,[41] that acetate is converted into the C_5 repeating unit *via* the tricarboxylic acid cycle, for it is now accepted that acetate is fed into this cycle as ' active acetate '. The reactions envisaged for the production of β-methylcrotonaldehyde *via* the tricarboxylic acid cycle are outlined in Fig. 6.

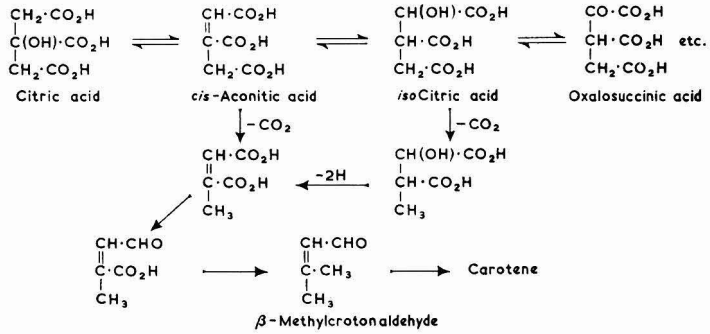


FIG. 6.—*A possible route for the synthesis of β-methylcrotonaldehyde* via *the tricarboxylic acid cycle*

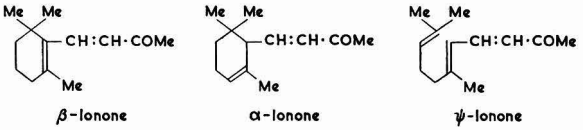
Although much work will have to be carried out before a decision can be made about the correctness of this interesting scheme, one existing piece of information can be interpreted as evidence against it. Streptomycin inhibits carotenogenesis, but not growth, in *Phycomyces* when glucose is the carbon source[37, 42] and, according to Goodwin & Griffiths,[42] both growth and carotenogenesis are inhibited when acetate is the carbon source ; Schopfer *et al.*[37] state that under the latter conditions growth but not carotenogenesis is inhibited. The observations of Umbreit, Smith & Oginsky[43] indicate that streptomycin does not act in *Escherichia coli* by inhibiting ' acetate '–oxaloacetate condensation to citric acid. Thus, if these observations are applicable to *Phycomyces*, streptomycin ought not to inhibit carotenogenesis if the synthetic route involves citrate.

An alternative mechanism

A further mechanism for carotene synthesis, which has been discussed by Mackinney,[5, 44] is that the two terminal residues of the molecule are synthesized separately from the middle portion. The evidence for this is that the addition of β-ionone to growing cultures of *Phycomyces* produces an eightfold increase in the amount of β-carotene synthesized during the next 7–12 hours, compared with untreated cultures : the difference between the two cultures lessens with increasing time, but is still twice as great in the β-ionone-treated mats 72 hours later. Similarly, but on a much smaller scale, citral and ψ-ionone stimulate lycopene production, but α-ionone has no effect on the synthesis of α-carotene.[31, 44, 45] The possibility that the C_{13} β-ionone unit is incorporated into the β-carotene molecule *in toto* appears to be ruled out by the interesting observation that β-ionone labelled with ^{14}C in the carbonyl group and tested on *Phycomyces* did not result in the production of labelled β-carotene.[44] This might be due to a degradation of the β-ionone to a C_{10} or C_{11} unit, which is then incorporated. The β-ionone stimulation might, of course, be due to an indirect effect.

Inhibitors of carotenogenesis

Apart from streptomycin, already discussed, and diphenylamine, to be discussed in the next section, other antibiotics and drugs have recently been tested on *Phycomyces*. Chlor-amphenicol (0·2 and 0·16%) has no effect on growth, but about 65% of carotene synthesis



is inhibited at the higher concentration, although no effect is noted at the lower; *iso*nicotinic acid hydrazide (0·01 and 0·05%) inhibited carotenogenesis by 50% at both levels, and growth was inhibited at the higher but not at the lower concentration; aureomycin completely inhibited growth at a concentration of 0·02%, but at a level of 0·001% it had no effect on either growth or pigmentation; neither penicillin nor sulphathiazole at a concentration of 0·02% had any effect on growth or carotene synthesis.[38] It is interesting that tetronic acid, which is such an effective inhibitor of carotene synthesis in seedlings, has no such effect in *Phycomyces*.[18]

Obviously much further work is necessary before the steps in carotenogenesis are finally elucidated; perhaps it would be wise, however, at the moment not to lean too heavily on any single scheme, even for the same organism. Recent investigations on streptomycin, just mentioned above, show that when glucose is the carbon source, a concentration of 0·07% of streptomycin inhibits carotenogenesis in *Phycomyces* by about 60%; increasing the concentration of streptomycin up to 1·6% does not cause any further diminution in carotene production.[42] It seems clear from these experiments that in *Phycomyces* there must be two synthetic routes, of which one is insensitive to streptomycin.

The role of the Porter–Lincoln series in the synthesis of β-carotene by Phycomyces

In 1936 Kharasch, Conway & Bloom[46] observed that diphenylamine (DPA) inhibited chromogenesis in a number of bacteria and fungi. Turian,[47] investigating this with *Mycobacterium phlei* (see under Bacteria for further details), found that carotenoid synthesis was inhibited and suggested that DPA was acting by inhibiting the dehydrogenation of colourless polyenes, such as phytofluene. In *Phycomyces* it was found that β-carotene synthesis was strongly inhibited by DPA, while synthesis of the Porter–Lincoln series was stimulated at the same time, the magnitude of the effect being proportional to the concentration of DPA.[8] Although these results provided good *prima facie* evidence that phytofluene etc. were the precursors of β-carotene, it was pointed out at the time that other explanations were possible; the most likely was that, instead of resulting from a step-wise synthesis one from the other, the series might be formed in parallel syntheses from a common precursor, thus:

Common precursor → { Phytoene, Phytofluene, ζ-Carotene, Neurosporene } Unaffected by DPA
{ α-Carotene, β-Carotene, γ-Carotene, Lycopene } Blocked by DPA

In the light of recent work, it appears that such a 'parallel mechanism' is much more likely, for all the evidence now available points away from phytofluene etc. being precursors of β-carotene in *Phycomyces*.[48] The reasons on which this conclusion is based are: (*a*) the pattern of synthesis of the Porter–Lincoln series and of β-carotene are quite different, the former reaching their maximal concentration during the early stages of growth whereas, as stated previously, β-carotene synthesis occurs mostly after completion of growth; (*b*) the addition of riboflavin or adenylic acid (adenosine-5'-phosphate, AMP) to a medium containing DPA overcomes the anticarotenogenic effect of DPA, β-carotene synthesis returning to normal; it does not, however, cause the Porter–Lincoln series to return to their normal low levels, the concentrations of these polyenes remaining almost as high as in 'DPA-only' cultures; (*c*) if well-formed mats of *Phycomyces* grown on a DPA medium and containing large amounts of the Porter–Lincoln series are washed free from DPA and transferred to a fresh medium containing dissimilable glucose but no DPA, there is no conversion of phytofluene etc. into β-carotene. As to the reversal of DPA inhibition by riboflavin, it should be pointed out that at a relatively high concentration (> 1/20,000) riboflavin itself inhibits carotenogenesis.

The exact mode of action of DPA in inhibiting carotenogenesis has not yet been worked out, but it must inhibit a reaction specific to β-carotene because, although it slows up growth, it has no effect on the final amount of growth achieved or on the amount of lipid synthesized. It is possible that the transfer of a high-energy phosphate group from a key intermediate to an acceptor is blocked and that this is overcome by the addition of AMP, which is a high-energy phosphate-acceptor. If this is true, then the role of riboflavin is not obvious, but it might allow an alternative route to come into action, which would by-pass the phosphate-transferring stage.

The use of mutants

Much less use has been made of mutants than one would have expected. Bonner, Sandoval, Tang & Zechmeister[49] examined seven ultra-violet mutants of the red yeast *Rhodotorula rubra* and found that in the orange mutants the synthesis of torulene (a pink pigment with the probable structure 3 : 3′-dimethoxy-3′ : 4′-dehydro-γ-carotene, and not 3 : 3′-dimethoxy-γ-carotene as given in error by Goodwin,[3] p. 105) was blocked and that synthesis of β- and γ-carotenes and phytofluene was also blocked in the colourless mutants. In an investigation not yet reported in full, Haxo[50], working with mutants of *Neurospora crassa*, has come to the conclusion that in this mould, as in *Phycomyces*, a parallel synthesis of carotenoids is more likely than a step-wise synthesis. Goodwin & Griffiths[51] examined a number of naturally occurring mutants of *Phycomyces* and found that they did not differ from the parent strain in the type of carotenoids synthesized and differed only slightly in the rates of synthesis.

Production of xanthophylls

It appears that in *Rhodotorula* species xanthophylls are synthesized by the oxidation of carotenes.[5, 52] Cultivation of these yeasts at low temperature results in the production of yellow cultures containing predominantly α- and β-carotene, but at higher temperatures the cultures are red because the xanthophylls torulene and torularhodin are in excess. Oxygen is necessary for xanthophyll synthesis ; in *R. glutinis*, for example, the orange cultures produced at 5° turn to reddish-orange when incubated at 25° only in the presence of oxygen ; no change takes place in an atmosphere of nitrogen. No changes in the types of carotenes produced by *Phycomyces* can be observed over the temperature range 18–30°, although less β-carotene is produced at 18° and 30° than at 25°.[53] Lower temperatures might produce qualitative changes*, for Fromageot & Tschang[54] also found no qualitative changes in *R. sanniei* over the range 14–28°, as compared with the marked changes noted by Nakamura in the closely related *R. glutinis* when the temperature was lowered to 5°. Haxo[50] considers that the xanthophylls of *Neurospora* are formed by oxidation of carotenes.

Non-photosynthetic bacteria

Following on the important observation of Turian[47] that DPA reduced carotenoid synthesis in *Mycobact. phlei*, it has been shown that this reduction is accompanied by the appearance of the more saturated polyenes, such as phytoene, phytofluene and ζ-carotene, which do not occur in normal cultures.[55, 56] Turian[57] has further shown that phenol and, to a lesser extent, resorcinol also inhibit carotenogenesis ; α-naphthylamine, thiourea, potassium cyanide and salicylaldoxime, on the other hand, are inactive, but 2 : 4-dinitrophenol appears to stimulate carotenoid synthesis. It is not known whether phenol and resorcinol also cause the production of phytoene, phytofluene and ζ-carotene.

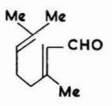
In contrast with the situation in *Phycomyces*, AMP and riboflavin will not counteract to any significant extent the inhibitory effect of DPA on *Mycobact. phlei* and do not cause the disappearance of the more saturated polyenes.[56]

Arkani & Stary[41] have been investigating carotenogenesis in *Sarcina lutea*. They find that, on a basal medium containing sodium lactate and ammonium sulphate, pigment production is greatly stimulated by the addition of pyruvate, aspartic acid or glycine ; valine, leucine and acetate are not stimulatory. These observations should be compared with those made on *Phycomyces*. The failure of acetate to stimulate carotenogenesis is explained by assuming that the organism can convert pyruvate but not acetate into the acetylphosphate–coenzyme-A complex, which can then be metabolized *via* the tricarboxylic acid cycle. The inactivity of valine and leucine may be due to their not being metabolized by this organism. Similarly,

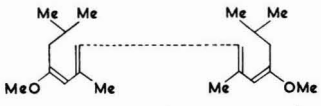
* Recent experiments at 3–5° with *Phycomyces* show no qualitative change

the only reason for the inactivity of alanine, which is usually deaminated to pyruvate, is that it is not metabolized.

Recently it has been observed (see Mackinney[5]) that *Corynebacterium poinsettiae*, cultured in a medium low in thiamine (0·1 μg./100 ml.), is pink and contains mostly spirilloxanthin and lycoxanthin (3-hydroxylycopene). On a high-thiamine (100 μg./100 ml.) medium, orange colonies are formed, which contain much less spirilloxanthin, but in which cryptoxanthin (3-hydroxy-β-carotene) was detected ; the lycoxanthin content did not alter. Although thiazole and, to a lesser extent, pyrimidine can replace thiamine for growth, only thiazole was active in controlling carotenoid synthesis. Whether the suggestion that thiamine controls the ring closure of an alicyclic precursor (e.g. lycoxanthin → cryptoxanthin) is valid remains for future work to decide. In this connexion it is interesting to note that, although β-carotene synthesis is probably reduced in *Phycomyces* grown on media low in thiamine, there appear to be no qualitative changes in pigment synthesis.[53]



Citral



Spirilloxanthin (probable formula)

Photosynthetic bacteria

Rhodospirillum rubrum, which synthesizes only one carotenoid, spirilloxanthin, together with traces of its *cis*-isomers,[58] and no detectable colourless polyenes, has recently been investigated from the point of view of carotenogenesis.[59, 60] When cultured in the light on a malate/glutamate/salts/biotin medium, *R. rubrum* synthesizes much more pigment under anaerobic than under aerobic conditions ; similarly, aerobic-light cultures produce more pigment than aerobic-dark cultures (the organism will not grow anaerobically in the dark). As in *Phycomyces* and *Mycobact. phlei* DPA inhibits carotenogenesis (and also the synthesis of bacteriochlorophyll) ; up to a DPA concentration of 1/140,000 growth is not affected and, although pigmentation is reduced considerably, it is not completely inhibited ; above this concentration the organism becomes colourless, and growth is reduced to the level observed in cultures grown in the dark (i.e. in the absence of photosynthesis). In such colourless cultures, traces of phytofluene and other pigments, including ζ-carotene, have been observed. In contrast with what occurs in *Phycomyces* and *Mycobacterium*, in *R. rubrum* neither AMP nor riboflavin overcomes the inhibition produced by DPA.

R. rubrum is much more sensitive to streptomycin than is *Phycomyces*, growth being completely inhibited at a concentration of 10^{-3}. Over the range 10^{-4}–10^{-5}, however, pigmentation is inhibited to a greater extent than is growth.

If cells of *R. rubrum* cultured on the normal medium are transferred to media containing available carbon sources (acetate, malate, succinate), no pigment is synthesized ; if such cells are transferred to a medium containing a carbon and nitrogen source, then, as the cells multiply, pigment synthesis is resumed. The reason for the failure of resting cells to produce pigment is probably this : as all the spirilloxanthin present in *R. rubrum* is attached to a protein (cf. *Phycomyces*, in which β-carotene is mainly in the fat droplets) that occurs in the chromoplasts,[61] it is reasonable to assume that under normal cultural conditions all the available protein carrier is utilized ; for example, when resting cells assimilate acetate, none is diverted to spirilloxanthin synthesis, because there is no protein carrier available.

If 3–4-day-old *R. rubrum* cells grown on DPA and, thus almost colourless, are washed free from DPA and resuspended anaerobically in phosphate buffer in the light, spirilloxanthin (and also bacteriochlorophyll) immediately begins to be synthesized, and within 24 hours its concentration approaches that found in normal cultures. Similarly, if DPA cultures are incubated aerobically in the dark, the same amount of pigment is formed, although, as stated above, normal aerobic-dark cultures synthesize much less pigment than anaerobic-light cultures. Further, only little pigment is produced anaerobically in the dark by resting DPA cells, and intermediate amounts aerobically in the light. Growing DPA cultures must accumulate a colourless precursor of spirilloxanthin, which can be transformed into the pigment only when energy is available. This energy can come from respiration (aerobic-dark) or from photochemical reactions (anaerobic-light). The failure to produce pigment anaerobically in the dark

would thus be explained. The lowered production in aerobic-light cultures may be due to the destruction of the pigment in the presence of both light and oxygen. This explanation is made more likely by the recent observations that washed resting DPA cells suspended in buffer take up considerable amounts of oxygen, whereas the oxygen uptake of normal resting cells is little or none.[62]

Attempts to identify the precursor are being carried out at present, and so far it does not appear to be a member of the Porter–Lincoln series or any analogous polyene. Traces of these are found in DPA cultures, but they are still present after the regeneration of spirilloxanthin. It is important to compare the failure to obtain regeneration of β-carotene from washed DPA cultures of *Phycomyces* with the foregoing observations.

If the explanation of the failure of normal resting cells of *R. rubrum* to synthesize further amounts of spirilloxanthin is true, then it must be assumed that DPA cells produce normal amounts of protein (or ' colourless chromoplasts '), which is available for chromoprotein synthesis when the DPA is removed. This would also be consistent with the observations that addition of various carbon substrates (acetate, malate, citrate etc.) to the phosphate buffer, in which the washed DPA cells are suspended, does not increase the amount of pigment synthesized; with buffer alone the saturation concentration has already been reached.

As production of spirilloxanthin or bacteriochlorophyll is equally inhibited by DPA, and this inhibition is overcome when it is removed, the possibility exists that DPA exerts its major inhibitory effect either (*a*) far back in the metabolic chain in the utilization of a common precursor (? acetate), or (*b*) by inhibiting a type of enzymic reaction common to both syntheses but involving different substrates in each.

van Niel[63] found that the synthesis of the yellow and red pigments in *Rhodopseudomonas spheroides* varied with cultural conditions. Under anaerobic conditions in light or darkness, the yellow component predominates; under aerobic conditions the same total amount of pigment is formed, but in this case the red component predominates.

Algae

Little work has been carried out recently on carotenogenesis in this important group of micro-organisms. They have considerable potentialities and should provide a fruitful field of study as the following observations (almost all that exist) suggest.

Carotenogenesis in *Haematococcus pluvialis* growing on a medium containing asparagine or peptone is stimulated by the addition of acetate.[64] *Chlorella pyrenoidosa* cultured in low-intensity illumination contains more α- than β-carotene, the situation being reversed under high illumination.[65] When the flagellate *Dunaliella salina* is cultured in saturated saline (25%), only β-carotene is synthesized, whereas in solutions only $\frac{1}{2}$–$\frac{2}{3}$ saturated with saline much less β-carotene is produced, but considerable amounts of chlorophyll are synthesized.[66]

Conclusion

It will be agreed that in the last five years a start has been made on the problems of describing carotenogenesis at the molecular level and of relating it to the general metabolic processes of the organisms studied. Although considerable information has been accumulated, it will be obvious that most of the problems still remain unsolved. The immense technical developments made recently in biochemistry—in column and paper chromatography, in isotope techniques, in methods of synthesis and degradation of labelled molecules, to name but a few —combined with the new knowledge of the general metabolic processes occurring in living organisms, should, if applied to the present problem with assiduity, lead to the solution of many if not all of its numerous aspects. It should also lead, as do most fundamental problems, to the solution en route of many other apparently unrelated problems. For example, one is hopeful that a study, in progress at the moment, of the inhibition of carotenogenesis in *Phycomyces* by streptomycin will give information about the general mode of action of this antibiotic.

Acknowledgment

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THE FREE AMINO-ACIDS AND PEPTIDES OF PLANT TISSUES

By N. O. BATHURST

Free amino-acids were extracted from pasture plants with 80% alcohol, and the residue was extracted with water to remove bound amino-acids (peptide fraction). The alcohol extracts and the hydrolysed water-extracts were assayed microbiologically for 17 amino-acids. Total amino-nitrogen was also estimated in these extracts by Van Slyke's nitrous acid method. Most of the free amino-nitrogen was due to relatively few amino-acids, chiefly the dicarboxylic acids (and their amides), alanine and serine. A larger number of amino-acids contributed significantly to the peptide fraction. Treatment of the grasses with different nitrogenous fertilizers resulted in some differences in the amino-acid patterns. Larger differences were noted between young and more mature tissues. Of the total amino-nitrogen measured by Van Slyke's method about 70% in the free amino-acid fraction and about 95% in the peptide fraction were accounted for by assay of individual amino-acids.

In the study of nitrogen metabolism in plants considerable attention has been paid to the soluble nitrogenous constituents, particularly the amides glutamine and asparagine, which have been shown to occupy important roles in the metabolic system. Other nitrogenous constituents, apart from ammonia and nitrate, have been investigated less thoroughly, and different systems of ' group analyses ' have been used by different workers. Vickery and co-workers, who investigated tobacco,[1] rhubarb[2] and narcissus,[3] measured total soluble nitrogen, free amino-nitrogen, peptide nitrogen and ' other soluble nitrogen ', in addition to ammonia, nitrate and the amides. Wood, Cruickshank & Kuchel,[4] working with Sudan grass and Kikuyu grass, used a similar fractionation. Viets, Moxon & Whitehead,[5] investigating maize, also measured non-amino basic nitrogen. Although much useful information can be obtained from these group analyses, it is highly desirable that the groups should be fractionated further, and this is particularly true of the amino-nitrogen group, which constitutes up to 80% of the total soluble nitrogen.[6]

It is only within the last ten years that reasonably precise methods applicable on a routine basis have been available for amino-acid analyses. These methods have been recently reviewed by Dunn,[7] who gives a comprehensive bibliography, and hence they do not require discussion. Few investigations have, however, been made of the amino-acids of plant tissue by means of modern techniques, and no report has appeared in which the soluble free and combined amino-acids of leaf tissues have been determined. Folkes,[8] by the microbiological method, measured amino-acids in the protein of germinating barley. Armstrong[9] used the same method for estimating tryptophan, tyrosine, cystine, methionine, lysine and histidine in the proteins of 15 herbage species. Here the total plant tissue was hydrolysed so that the results could include both free and combined amino-acids, and losses would almost certainly occur owing to protein being hydrolysed in the presence of carbohydrate.[10]

A number of workers have used paper chromatography qualitatively and semi-quantitatively in the investigation of different plant-tissue extracts. Of the quantitative methods proposed, that of Thompson, Zacharius & Steward[11] (also Thompson & Steward[12]) would appear to be the most satisfactory. An investigation of the amino-acids in protein extracted from several grassland species, cereals and bracken was carried out by Smith & Agiza[13] by means of paper chromatography.

Peptides in plant extracts have usually been determined by measuring the amino-nitrogen before and after hydrolysis in N-acid for six hours, the difference being assumed to be peptide nitrogen.[3] Although measurements by this method often indicate that an appreciable portion of the soluble nitrogen is in peptide form, little work has been done to characterize this fraction more definitely. Synge,[14] with a four-compartment cell, separated various nitrogenous fractions ionophoretically, and identified bound amino-acids by a paper-chromatographic examination of the appropriate fraction after hydrolysis.

A more definite characterization, both qualitative and quantitative, of the free and bound amino-acids in the ' soluble nitrogen ' fraction of plant tissues, was the purpose of the present investigation.

Experimental

Tissue investigated.—Grasses were chosen as experimental material because of the relative ease of sampling the fresh material, and also because of the interest of this Division in pasture plant metabolism.

Plots 13, 14 and 15, for which analyses are given in the Tables, were sown with perennial rye-grass to which regular top-dressings of a complete fertilizer were added. Nitrogen was supplied in different forms but in equivalent amounts, namely ammonium sulphate for plot 13, sodium nitrate for plot 14 and urea for plot 15. In all cases dressings equivalent to 750 lb. of nitrogen per acre per year were applied, 1/11 of this quantity being used each month except February. The yields from each of these three plots were very similar, averaging close to 12,000 lb. of dry matter per acre per year. Rye-grass U was grown in a box in the glasshouse, the nitrogen being applied as urea. The cocksfoot sample was drawn from a pure stand grown for seed-production purposes. Rye-grass used for the experiment, the results of which are shown in Table I, was cut when about 4 in. high, whereas the cocksfoot was cut at the 6-in. stage. The young rye-grass of Table II was cut at a height of about 1 in., 5 days after mowing. Rye-grass U (Table II) was about 6 in. high when cut.

Table I

Free amino-nitrogen (NH_2-N) in rye-grass and cocksfoot

	Rye-grass 13 (ammonium sulphate)		Rye-grass 14 (sodium nitrate)		Rye-grass 15 (urea)		Cocksfoot	
	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N
Alanine	32·0	20·9	28·0	22·4	22·0	22·9	7·9	13·6
Arginine	2·1	1·4	1·1	0·9	1·0	1·0	1·4	2·3
Aspartic acid (total)	28·3	18·5	9·6	7·7	8·3	8·7	7·8	13·4
Glutamic acid (total)	23·0	15·0	25·0	20·0	21·0	21·9	11·1	18·9
Glycine	3·4	2·2	4·7	3·8	4·3	4·5	4·0	6·9
Histidine	0·9	0·6	0·6	0·5	0·7	0·7	0·4	0·7
*iso*Leucine	2·5	1·6	2·4	1·9	2·1	2·2	1·6	2·7
Leucine	2·6	1·7	2·7	2·2	2·4	2·5	1·1	1·9
Lysine	1·2	0·8	0·9	0·7	1·2	1·2	0·3	0·5
Methionine	0·8	0·5	0·9	0·7	0·8	0·8	0·5	0·9
Phenylalanine	0·9	0·6	0·8	0·6	0·8	0·8	1·7	2·9
Proline	23·0	15·0	7·1	5·7	3·1	3·2	2·2	3·8
Serine	20·0	13·1	28·0	22·4	18·0	18·7	10·4	17·8
Threonine	5·9	3·9	7·1	5·7	4·7	4·9	4·5	7·7
Tryptophan	0·7	0·5	0·5	0·4	0·5	0·5	0·6	1·0
Tyrosine	0·6	0·4	0·5	0·4	0·4	0·4	0·9	1·5
Valine	5·2	3·4	5·2	4·2	4·8	5·0	1·9	3·3
Total	153·1		125·1		96·1		58·3	
Total (Van Slyke)	215		187		137		94	
Recovery, %	71		67		70		62	

Extraction, fractionation and preparation for analysis.—Immediately after cutting, samples were freeze-dried for 48 hours and stored in tightly stoppered, paraffin-sealed jars till required.

Free amino-acids were extracted by a modification of the method used by Dent, Stepka & Steward[15] for potato. Twenty-five grams of freeze-dried material were extracted in the Waring Blendor with four portions of about 500 ml. each of 80% alcohol. The combined alcohol extracts were concentrated under reduced pressure until all the alcohol was removed, the residue being made to 50 ml. with water. Portions of the filtered aqueous extract (from 1 to 10 ml.) were placed into small glass tubes and freeze-dried.

The residue unextractable with 80% alcohol was then blended with 500 ml. water and centrifuged. Acetic acid was added until no further precipitate appeared, after which the extract was again centrifuged and the supernatant liquid concentrated to 50 ml. A 5-ml. sample was removed for chromatography and amino-nitrogen determination, and the remainder refluxed for 8 hours after the addition of an equal volume of concentrated hydrochloric acid. After hydrolysis the extract was concentrated to a syrup, diluted with water and again concentrated to remove most of the hydrochloric acid. It was then taken up in 80% alcohol and neutralized with sodium hydroxide. The inorganic salts that were precipitated were removed by centrifugation, washed several times with 80% alcohol (the washings being added to the extract) and discarded. The alcohol was then removed from the extract by evaporation, and water added to bring the extract to the volume it possessed before hydrolysis.

Amino-nitrogen.—The nitrous acid method of Van Slyke was used to determine the total free amino-nitrogen in the alcohol extracts, and peptide nitrogen in the water extracts. The

reaction time was 4½ minutes at 19°. Ammonia was always removed before the estimation by distillation *in vacuo* for 15 minutes in phosphate–borate buffer at pH 9·5, the extract then being neutralized with sulphuric acid. The amounts of urea present were so low as to cause negligible error.

Microbiological assay of amino-acids.—In order to economize in amino-acids, the assays were performed in 4-in. × ⅜-in. test-tubes, the total volume of each tube being made up to 1 ml. The response was measured by titration of the acid produced with 0·05N-sodium hydroxide, the maximum titration usually reaching 2–3 ml.

The organisms used were: *Leuconostoc mesenteroides*, for aspartic acid, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, threonine and tyrosine; *Lactobacillus arabinosus*, for glutamic acid, *iso*leucine, leucine, tryptophan and valine; *Streptococcus faecalis*, for arginine, and *Leuconostoc citrovorum* for alanine.

The use of *Leuconostoc citrovorum* for the assay of alanine followed its recommendation by Sauberlich & Baumann.[16] In place of their liver-extract supplement, a supplement was prepared thus: A concentrated aqueous extract of liver meal was applied in a narrow band 2½ in. from the narrow edge of a 22½-in. × 18¼-in. sheet of Whatman No. 1 filter paper. A marker spot of alanine was placed at one end of the band. The paper was then subjected to one-way chromatography with butanol–acetic acid–water (25 : 6 : 25 by volume) for 24 hours. The alanine band, the position of which was revealed by treating the marked end of the paper with ninhydrin, was cut out and the remainder extracted with water, concentrated and freeze-dried.

For assays of methionine, tyrosine, and tryptophan the hydrogen peroxide-treated peptone medium of Lyman, Moseley, Wood & Hale[17] was used.

An improved medium for glutamic acid assays was obtained when, instead of individual pure amino-acids, a preparation derived from acid-hydrolysed casein was used. This preparation consisted of two different mixtures which were both added to the basal medium. The first mixture was obtained by autoclaving the casein hydrolysate for 4 hours at 120° at pH 3·5, thereby converting most of the glutamic acid into pyrrolidone carboxylic acid. The pH was then brought to between 6 and 7, the hydrolysate concentrated and allowed to crystallize. The resultant crystalline mixture, after washing with water, provided the first part of the preparation used in the medium. The second part was prepared from another portion of the original casein hydrolysate by precipitating with phosphotungstic acid, discarding the supernatant liquid and decomposing the precipitate with barium hydroxide. The solution thus obtained provided the basic amino-acids as well as others, and was used in conjunction with the first mixture to supply most of the amino-acids required for the medium. It was necessary to add only alanine, aspartic acid, glycine, serine and tryptophan to complete the amino-acid content of the medium. An alternative medium for glutamic acid assays could be prepared by adding to the normal medium a supplement of glutamic acid-free yeast extract prepared in a similar way to the alanine supplement described above.

Except where otherwise specified, the media used were essentially those of Dunn, Shankman, Camien, Frankl & Rockland.[18]

Tryptophan was not measured in the hydrolysed peptide fraction, as this amino-acid is destroyed by acid hydrolysis.

Hydrolysis of asparagine and glutamine.—Portions of the alcohol extracts were made normal with respect to sulphuric acid, heated in a boiling-water bath for two hours, neutralized and diluted to a suitable volume. Provided the extract was diluted at least five times after hydrolysis (the minimum dilution used in the present investigation) there was no interference with the assay due to the salt content.

Alcohol extracts used for the assay of amino-acids other than aspartic and glutamic acids were left unhydrolysed.

Paper chromatography.—This was used qualitatively only. For two-way chromatograms, butanol–acetic acid–water (25 : 6 : 25 by volume) was used in one direction followed by water-saturated phenol in the other.

Results and discussion

In Table I the amount of each amino-acid is expressed in mg. of amino-nitrogen per 100 g. of dry matter in order to allow for summation and comparison of the total free amino-nitrogen obtained in this way with the corresponding value obtained by Van Slyke's nitrous acid method. In addition, the amount of amino-nitrogen due to each amino-acid is expressed as a percentage of the total free amino-nitrogen obtained by summation. Recoveries are also shown, based

Table II

Free and peptide amino-acids in rye-grass

	Rye-grass 13 (ammonium sulphate)				Rye-grass 14 (sodium nitrate)				Rye-grass 15 (urea)				Rye-grass U (urea)			
	Free		Peptide		Free		Peptide		Free		Peptide		Free		Peptide	
	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N	mg. NH_2-N/ 100 g.	% of total N
Alanine	20·1	8·5	4·1	9·0	26·4	10·9	6·0	9·1	26·4	13·2	5·2	9·5	20·1	15·6	4·4	12·2
Arginine	5·0	2·1	2·3	5·0	2·4	1·0	3·1	4·7	4·3	2·2	3·1	5·6	3·1	2·4	2·7	7·5
Aspartic acid (total)	69·5	29·4	6·4	14·1	81·0	33·4	5·7	8·6	55·6	27·8	4·5	8·2	10·7	15·3	3·3	9·2
Glutamic acid (total)	84·6	35·8	9·0	19·8	55·1	22·7	12·8	19·4	51·8	25·9	10·0	18·2	26·2	20·3	1·5	4·2
Glycine	7·3	3·1	5·2	11·4	10·7	4·4	6·9	10·5	12·7	6·4	7·1	13·0	2·8	2·2	3·6	10·0
Histidine ..	1·4	0·6	0·7	1·5	2·7	1·1	1·2	1·8	1·8	0·9	0·8	1·5	1·4	1·1	0·7	1·9
*iso*Leucine ..	2·0	0·8	1·4	3·1	3·6	1·5	2·6	3·9	3·2	1·6	2·1	3·8	3·9	3·0	2·0	5·5
Leucine	3·2	1·4	1·9	4·2	4·2	1·7	3·4	5·1	2·8	1·4	2·9	5·3	3·3	2·6	2·8	7·8
Lysine	1·6	0·7	2·7	5·9	1·3	0·5	5·3	8·0	1·4	0·7	4·0	7·3	2·0	1·6	2·9	8·0
Methionine ..	0·4	0·2	0·5	1·1	0·5	0·2	0·8	1·2	0·4	0·2	0·7	1·3	0·5	0·4	0·6	1·7
Phenylalanine ..	1·0	0·4	1·0	2·2	1·6	0·7	2·0	3·0	1·7	0·9	1·0	1·8	1·7	1·3	1·0	3·0
Proline	10·9	4·6	2·3	5·0	7·1	2·9	3·4	5·1	6·6	3·3	3·3	6·0	10·7	8·3	2·6	7·2
Serine	12·8	5·4	3·1	6·8	25·3	10·4	4·9	7·4	17·3	8·6	3·0	5·5	17·3	13·4	2·0	5·5
Threonine ..	10·3	4·4	2·4	5·3	12·2	5·0	3·9	5·9	7·7	3·9	3·4	6·2	9·4	7·3	2·8	7·8
Tryptophan ..	0·3	0·1	—	—	0·5	0·2	—	—	0·6	0·3	—	—	1·1	0·9	—	—
Tyrosine	0·7	0·3	0·6	1·3	0·7	0·3	1·2	1·8	0·6	0·3	1·2	2·2	0·9	0·7	0·9	2·5
Valine	5·3	2·2	1·9	4·2	7·1	2·9	2·8	4·2	4·8	2·4	2·5	4·6	4·7	3·6	2·2	6·1
Total	236·4		45·5		242·4		66·0		199·7		54·8		128·8		36·0	
Total (Van Slyke)	414		47·6		317		66·7		300		57·6		177		45·4	
Recovery ..	57%		96%		77%		99%		67%		95%		73%		80%	
Total soluble N	580		92		489		*		351		93		255		78	

Rye-grasses 13, 14 and 15 cut at 5-day growth stage

*Undetermined

Table III

Amino-nitrogen (Van Slyke) in 'peptide' extracts

	Unhydrolysed extract, mg. NH_2-N/100 g.	Hydrolysed extract, mg. NH_2-N/100 g.	Unhydrolysed as percentage of hydrolysed
Rye-grass 13	24·3	47·6	51
Rye-grass 14	25·9	66·7	39
Rye-grass 15	25·4	57·6	44
Rye-grass U	15·3	45·4	34

on the total amino-nitrogen by summation expressed as a percentage of the total amino-nitrogen by the Van Slyke method.

In Table II are presented analytical results for three samples of rye-grass cut at a very young stage and also a rye-grass sample grown in the glasshouse. In addition to free amino-acids, the amino-acids in the peptide fraction are also shown, calculations being on the same basis as for Table I. Figures for total soluble nitrogen are also shown in Table II.

Only one of the amino groups of lysine is included in the figures for this amino-acid. For proline, although its nitrogen does not belong to a primary amino-group and is not measured in the Van Slyke reaction, the nitrogen is included in Tables I and II, as proline is accepted as an amino-acid. The percentages of nitrogen recovered therefore show a slightly exaggerated degree of concordance between the figures obtained by summation of individual amino-acids and those of the Van Slyke estimation.

Extraction with 80% alcohol has been found[6] to give results for total free amino-nitrogen closely similar to those for water extracts. A number of alcohol extracts were examined for the presence of peptides by measuring the amino-nitrogen by Van Slyke's method before and after hydrolysis with 6N-acid for eight hours. In no case was any measurable increase found, so that it is concluded that 80% alcohol extracts are virtually free of peptides. This freedom from peptides and the low concentration of inorganic salts makes alcoholic extraction particularly suitable for microbiological assays.

The water extract of the alcohol-insoluble residue has been referred to in this paper as the ' peptide ' fraction. Whether this is truly peptide or also contains some soluble protein, it is not possible at present to say. Paper chromatograms of the unhydrolysed water extracts showed usually only two or three very faint spots, the spots being greatly increased in number and intensity by hydrolysis. The amino-nitrogen content of the unhydrolysed extracts, measured by Van Slyke's method, was about 40% of that of the extracts after hydrolysis (see Table III). This would indicate that at least a considerable part of the nitrogen of the ' peptide ' fraction is really in peptide form. Further evidence for the peptide nature of extracts prepared in this way was obtained with another set of extracts, where trichloroacetic acid was used instead of acetic acid to precipitate protein. Trichloroacetic acid did not cause a reduction in the soluble nitrogen greater than that caused by acetic acid.

The Van Slyke nitrous acid method for estimating amino-nitrogen is convenient for routine estimations, but it is known to have deficiencies in that some substances not containing α-amino-nitrogen react with nitrous acid to evolve nitrogen. Glycine gives recoveries of about 110% and lysine also gives high results. γ-Aminobutyric acid has been found to give most of its nitrogen as α-amino-nitrogen under the conditions of the estimation. Other methods, e.g. formol titration and ninhydrin–carbon dioxide, have been tried, but none has given more satisfactory results than the nitrous acid method.

Under the conditions of the aspartic acid assay asparagine shows about 10% of the activity of aspartic acid. Asparagine frequently occurs at higher concentrations in plant tissues than does aspartic acid and would lead to major errors. Hence the amides glutamine and asparagine were not estimated separately but were hydrolysed and measured together with the free acids. In any case, any asparagine or glutamine present in the peptide fraction would be converted into the corresponding dicarboxylic acid by the hydrolysis to which this fraction was subjected.

Examination of Table I, which shows results for free amino-acids in rye-grass and cocksfoot, makes it apparent that the bulk of the amino-acid fraction is made up of relatively few amino-acids. The dicarboxylic acids (and their amides), alanine and serine, together account for about 70% of the amino-nitrogen. The basic amino-acids, arginine, histidine and lysine, are present in only small amounts. Proline in the rye-grass varies considerably according to the form in which nitrogen is supplied, and is highest for ammonium sulphate and lowest for urea. Of the total free amino-nitrogen measured by Van Slyke's method, from 62–71% can be accounted for by summation of the amino-nitrogen of the individual amino-acids measured.

Results recorded in Table II include amino-acids in the peptide fraction as well as those in the free state. Free amino-acids in sample U follow much the same pattern as for sample 15, some exceptions being that sample U is higher in aspartic acid and threonine and lower in serine.

When a comparison is made between the free amino-acids of the five-day-growth samples and the corresponding four-inch samples (of rye-grasses 13, 14 and 15), some striking differences are revealed. In the young grass in each case aspartic and glutamic acids and their amides make up over half the total free amino-nitrogen, whereas alanine and serine, though still major constituents, make up a much lower proportion.

The composition of the peptide fraction as shown in Table II is markedly different from

the corresponding free amino-acid fraction. The dicarboxylic acids, although still present in appreciable amounts, make up a much lower percentage of the total than they do in the alcohol extracts. In rye-grass U, for example, the two dicarboxylic acids (with their amides) together account for only 13·4% of the total peptide amino-nitrogen, whereas for the free amino-nitrogen the corresponding figure is 35·6%. The basic amino-acids are present in a much greater proportion in the peptide fractions than in the alcohol extracts, lysine showing the greatest increase and histidine the smallest. Glycine, too, accounts for a much higher proportion of the peptide amino-nitrogen than of the free amino-nitrogen.

It is difficult to compare directly the patterns of amino-acid content in the alcohol and water extracts respectively with those of typical plant proteins, because of the unavailability of complete amino-acid analyses of the plant proteins. A summary of various workers' analyses of plant proteins is given by Lugg.[10] In general, it appears that the peptide fractions of the samples analysed in the present investigation are, in amino-acid composition, roughly intermediate between the free amino-acid fractions and leaf protein.

Recoveries for the free amino-acids in Table II are comparable with those in Table I, i.e. about 70%. Recoveries for the peptide fractions, however, are much higher, being usually over 90%. Low recoveries in the alcohol extracts could be due to the presence of amino-acids not assayed (e.g. γ-aminobutyric acid, cystine), or to the inclusion of some non-amino nitrogen in the Van Slyke estimation. In the water extracts any cystine originally present would be largely or wholly destroyed by the acid hydrolysis, and no chromatographic evidence for the presence of γ-aminobutyric acid was obtained. The presence of substances containing non-amino nitrogen that would be measured in the Van Slyke estimation would also be less likely in the peptide fraction, so that higher recoveries could be expected.

Table II also includes figures for total soluble nitrogen (except for the peptide fraction of rye-grass 14, where insufficient sample material was available for this analysis). It can be seen that usually about 50% of the total soluble nitrogen can be accounted for as actual compounds, whereas with the older 'group analyses' the greater part of the amino-nitrogen fraction was of unknown composition.

Although this paper is concerned with methods rather than physiology, it is of interest to note the differences in the amino-acid pattern resulting from the application of nitrogen in different forms, although generalizations cannot be made at this stage owing to the small number of samples examined. The amino-acids which have shown the greatest variations in amount with different fertilizer treatments are the dicarboxylic acids (and their amides), glycine, proline and serine. Differences between young and mature tissues have already been referred to.

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CHEMICAL ASPECTS OF THERMAL DAMAGE TO THE NUTRITIVE VALUE OF VEGETABLE OILS. I.—Formation of Intrapolymeric Glycerides during Heat Polymerization of Linseed and Soya-bean Oils

By L. WISEBLATT, A. F. WELLS and R. H. COMMON

Observations on the nutritionally deleterious effects of heat-polymerized vegetable oils and various fractions of them prompted an examination of the chemical nature of the products of polymerization. Analytical data for linseed and soya-bean oils and for stand oils prepared from them are reported. The data suggest that linolenic acid reacts in a selective manner during thermal polymerization of linseed oil until the concentration of linolenic acid is greatly reduced. The relationship between content of polymeric acyl radicals and mean molecular weights of heated linseed and soya-bean oils is discussed in connexion with the existence of intrapolymers. The refractive indices of heated linseed and soya-bean oils bear a linear relationship to their content of dimeric acyl radicals. This provides a simple method of polymerizing to a desired content of dimeric acids.

Introduction

Linseed was the only domestic oil-seed crop available in Canada in substantial quantities in 1939. Consequently the possibility of utilizing linseed oil as a food fat was examined in Canada during the period 1939–45 and subsequently. Hydrogenated linseed oil was, in fact, used for food purposes in Canada to a limited extent during and immediately after the period 1939–45. The product was peculiarly susceptible to rapid deterioration of flavour or ' flavour reversion '.[1] This feature is associated with the high linolenic acid content of linseed oil, and in all likelihood with the presence of *iso*linoleic acids in the hydrogenated oil.[2]

Subsequent work[3, 4] showed that preliminary thermal polymerization reduced the degree of flavour-reversion of the shortening obtained by subsequent hydrogenation. Another high-temperature process for the production of edible oil from fish oil was used in Norway during the war.[5] The oil is said to have been alkali-refined and bleached, and then deodorized–polymerized at 280–300° for 8 to 12 hours *in vacuo*. Such treatment reduces susceptibility to flavour-reversion. It is doubtful whether susceptibility to oxidation is reduced. The heat-treated oil is less unsaturated, but it seems likely that natural stabilizers are either destroyed or removed.

Interest in the subject was stimulated by the observation that shortenings prepared from hydrogenated linseed oil subjected to preliminary polymerization[3] proved to be nutritionally deleterious to rats.[6] Since the polymerized constituents of the oil could have been responsible for these deleterious effects, heat-polymerized linseed oil was segregated into (*a*) an acetone-soluble and mainly monomeric glyceride fraction, and (*b*) an insoluble and mainly polymeric fraction. However, the acetone-soluble segregates were nutritionally deleterious, even when hydrogenated and deodorized.[4]

At this stage it seemed possible that the nutritionally deleterious effect might be associated with the presence of dimeric fatty acid radicals. Studies on the fatty acid composition of the heated oil fractions were begun, and the present paper deals with the results. The work was originally directed to the examination of the acetone-soluble fraction for the possible presence of dimeric acid radicals.

Experimental methods

Samples of commercial alkali-refined linseed oil and of alkali-refined soya-bean oil were heated at 275° in a current of carbon dioxide for various periods of time. The current of carbon dioxide was enough to keep the oil surging freely and the free flow of the gas also served to sweep out volatile products of pyrolysis.

Portions of the whole heated oils were brought into solution by adding 7 vol. of acetone and warming on the water bath. The mixture was left in the cold room overnight, and the acetone-soluble segregate separated by decantation. Acetone was removed from both fractions by heating on the water bath under reduced pressure. The following determinations were performed on both the whole oil and the acetone-soluble segregates: iodine value by the ' rapid Wijs' ' method,[7] percentage of free fatty acids, refractive index, and mean molecular weight by cryoscopy in *cyclo*hexane. It was found essential to purify the *cyclo*hexane by successive treatments with fuming sulphuric acid and barium hydroxide, followed by distillation

in a Stedman column. The purified *cyclo*hexane had m.p. 6·4° and $[n]_D^{25}$ 1·4237. Glasgow, Murphy, Wallingham & Rossini[8] have given values of m.p. 6·547 and $[n]_D^{25}$ 1·42354 for very highly purified *cyclo*hexane. Observed mean molecular weights were corrected for the presence of free fatty acids, as suggested by Bernstein,[9] who assumed almost complete association of the fatty acids and assigned to them a mean molecular weight of 558.

Portions of all the heated oils, of their acetone-soluble segregates and of the unheated oils were converted into the methyl esters by methanolysis[10] and distilled under reduced pressure. Simple distilling bulbs and a bath filled with glycerol or bath wax were used. The distilling bulb was filled with glass wool to reduce frothing and bumping. The distillation-temperature limits were ascertained by preliminary distillation of the esters of the whole unheated oil. Conducted in this way, the distillation effected a separation of esters of monomeric acids from esters of polymeric acids. The separations were checked by determinations of mean molecular weight. For quantitative measurements of percentage of distillable esters, it was found convenient to use a small bulb blown on a piece of Pyrex glass tubing (7 to 9 mm. in diameter) furnished with a short side-arm and bulb to act as receiver. The bulbs were of a size to hold conveniently a charge of 0·5 to 0·7 g. After distillation the bulb and side-arm were broken apart and weighed. The residue and distillate were then removed by ether and the weights of distillate and residue obtained by difference. This simple technique was found to give consistent quantitative results, and there was little or no loss of material.

Refractive indices, iodine values and mean molecular weights were determined on all these ester fractions. In addition monomeric ester fractions were analysed spectrometrically for fatty acid composition by the standard procedure of the American Oil Chemists Society.[11] The spectrometric results are expressed as ' linoleic ' acid and ' linolenic ' acid. This use of the terms ' linoleic ' and ' linolenic ' acid does not imply that the diene and triene acids present before the alkali isomerization were none other than ' linoleic ' and ' linolenic ' acids, excepting, of course, in the unheated oils. Isomerization to other diene and triene acids is likely to have taken place during heating, and *cis–trans* isomerization may have shifted the absorption peaks. Such isomers will not necessarily conjugate at the same rate as ' linoleic ' and ' linolenic ' acids during alkali isomerization. The expression of the spectrometric results in terms of ' linoleic ' and ' linolenic ' acids is thus a means of expressing the order of magnitude of the amounts of acids capable of diene and triene conjugation which were present in the monomeric acid fractions. Spectrometric analyses were not performed on the polymeric ester fractions, since the influence of polymeric acid residues on the absorption maxima was unknown.

The analytical data for the linseed stand oils are set out in Table I and for the soya-bean oils in Table II. The yields of polymeric esters were known, as well as the ' linolenic ' and ' linoleic ' acids as measured by spectroscopy on the monomeric ester fraction. It was thus possible to calculate fatty acid compositions in terms of the total esters. Cowan *et al.*[12] have pointed out the inconsistency of iodine values in the presence of dimeric molecules. Even in the present experiments, where the monomeric ester fractions only were considered, the calculated values for oleic acid were abnormally high and have been discarded. In order to secure an approximate value for monoethenoid acid, the saturated acid values for all samples were assumed not to differ greatly from that for the unheated oil, and the monoethenoid (oleic acid) values were obtained by difference.

Table I

Analytical data for linseed stand oils

Time heated, h.	0	5	9	13	17
Iodine value	182	140	130	121	112
Refractive index (25°)	1·4788	1·4840	1·4852	1·4869	1·4886
Free fatty acid, %	0·17	0·83	0·88	0·85	0·91
Mean mol. wt.	873	1140	1300	1490	1840
Viscosity (25°), poises	0·45	2·72	4·70	10·30	27·00

Table II

Analytical data for soya-bean stand oils

Time heated, h.	0	3	9	15	21	30
Iodine value	145	131	124	119	110	100
Refractive index (25°)	1·4733	1·4737	1·4744	1·4754	1·4767	1·4786
Free fatty acid, %	0·04	0·66	0·43	0·56	0·75	0·69
Mean mol. wt.	867	863	912	1000	1103	1302

The data for the composition of the monomeric acid fractions of the linseed stand oils are presented in Figs. 1 and 2, and for the soya-bean oils in Figs. 3 and 4. In Figs. 1 and 3 the data are presented as percentages of the amounts of the constituents present, and in Figs. 2 and 4 as percentages of the amounts originally present in the unheated oil. Iodine values of the stand oils were selected as abscissae rather than heating times because of the difficulty of assigning a precise value to the heating times. [Refractive indices might also have been used, a point which will be discussed further below.] The iodine values are regarded as measures of relative rather than absolute unsaturation.

The proportions of polymeric acids are indicated on Figs. 1 and 3 as dimeric acids. Marcusson[13] and Petit[14] have claimed that heat-bodied vegetable oils do not contain appreciable amounts of acids higher than dimeric acids, although more recent work[15] suggests that appreciable amounts of trimeric acid may be present after prolonged heat-bodying.

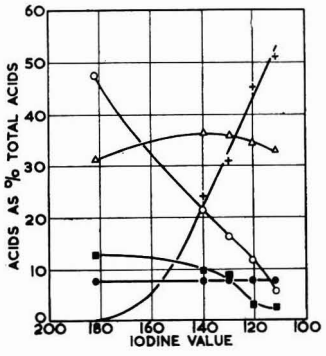

FIG. 1.—*Changes in fatty acid composition during heat polymerization of linseed oil*

● = saturated acids
○ = 'linolenic' acid
■ = 'linoleic' acid
△ = 'oleic' acid
+ = dimeric acids

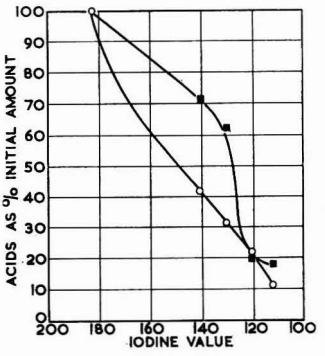

FIG. 2.—*Relative decline in 'linolenic' and 'linoleic' acids during heat polymerization of linseed oil*

○ = 'linolenic' acid
■ = 'linoleic' acid

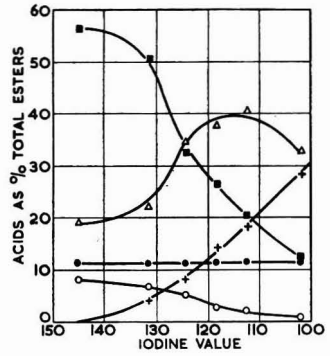

FIG. 3.—*Changes in fatty acid composition of soya-bean oil during thermal polymerization*

● = saturated acids
○ = 'linolenic' acid
■ = 'linoleic' acid
△ = 'oleic' acid
+ = dimeric acids

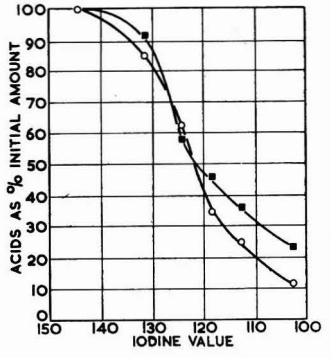

FIG. 4.—*Relative decline in 'linolenic' and 'linoleic' acids during heat polymerization of soya-bean oil*

○ = 'linolenic' acid
■ = 'linoleic' acid

Discussion of results

The data presented in Figs. 1 and 2 show that ' linolenic ' acid decreased rapidly throughout the heating period. The relative rate of decrease was considerably greater than that of ' linoleic ' acid until the iodine value had declined to about 130. Beyond that iodine value, the rate of disappearance of ' linoleic ' acid appears to have increased. It is assumed that, in the heat-treatment, no monomeric diene acid is produced from the ' linolenic ' acid which on alkali-isomerization would be recorded as ' linoleic ' acid, and hence mask any disappearance of the ' linoleic ' acid originally present.

Figs. 3 and 4, relating to the soya-bean oils, do not reveal any tendency for ' linolenic ' acid to decline more rapidly than ' linoleic ' until an iodine value of around 130 is reached. The data suggest that ' linolenic ' acid may have disappeared more rapidly than ' linoleic ' acid afterwards, but the data are subject to errors in determining the small amounts of triene conjugation left at this stage. In addition, some of the triene acid present after heat treatment may, on alkali isomerization, give ratios of extinctions at 268 mμ–234 mμ different from that for ' linolenic ' acid itself; this increases the difficulties of interpreting spectrometric data. The relative increase in monoethenoid (' oleic ') acid with progressive decline in iodine value was much greater for soya-bean oil than for linseed oil, as might be expected from the relatively high initial content of ' linoleic ' acid in the soya-bean oil.

' Linoleic ' acid apparently decreases more rapidly once the apparent iodine value has declined to about 130, this effect being evident in both oils. The combined effects are reflected in the curve for dimeric acid content, as determined by distillation of the methyl esters. The results, more especially those for linseed oil, provide some evidence in support of the view that polymerization proceeds by stages and that it is, in the early stages at least, ' selective '

Evidence from the present experiments for the existence of ' intrapolymeric ' glycerides

Barker, Crawford & Hilditch[16] have discussed some of the previous evidence for the presence of so-called ' intrapolymers ' in heat-bodied oils, and a tentative consideration of the present results suggests that they may constitute evidence for the existence of intrapolymers.

The possible modes of heat polymerization are disconcertingly numerous, the more so as the validity of the Scheiber–Kappelmeier theory of Diels–Alder condensations is still in question. However, the argument can be restricted to the relations between mean molecular weight of the glycerides and the percentage of dimeric acids, assuming for purposes of argument that the only polymeric acids formed in appreciable amounts during earlier stages of thermal polymerization are dimeric acids. The simplest way in which dimerization of fatty acid radicals could take place in the earlier stages of heat-bodying may be considered. Adams & Powers[17] have treated this matter generally in terms of functionality, but the point can be illustrated more simply. In a system of 10 triglyceride molecules, where the fatty acids are members of the C_{18} series and of mean molecular weight x, the union of two fatty acid radicals in adjacent glyceride molecules would increase the mean molecular weight to $10x/9$, i.e. to $1{\cdot}11x$. The percentage of dimers in the total fatty acids would be 6·7, since there are 30 fatty acid radicals in the original 10 triglyceride molecules. If the absence of intrapolymer-formation be postulated, a hypothetical relation between mean molecular weight and percentage of dimeric acids can be calculated. It will make no difference to this relation whether succeeding stages of dimerization involve union between two monomeric triglyceride molecules, between a polymeric glyceride and a monomeric glyceride, or between two polymeric glycerides, for in each case the number of molecular entities is decreased by one for each union of a pair of fatty acid radicals. The equation for this hypothetical relation is $y = 66{\cdot}7 - 66{\cdot}7M/x$, where y is the percentage of dimeric acids in the total acids, x the mean molecular weight of the heat-bodied glycerides, and M the mean molecular weight of the triglycerides before heating.

The relation implies (*a*) that acids higher than dimeric acids are not formed in appreciable amounts and (*b*) that formation of these dimeric acids does not involve formation of intrapolymers. The hypothetical curve for the sample of linseed oil is presented in Fig. 5, and that for the soya-bean oil in Fig. 6. If the foregoing argument be accepted, then any serious disagreement between the hypothetical and experimental may provide evidence of intrapolymer-formation.

Intrapolymerization could take place conceivably as follows:

(*a*) Two fatty acid radicals in the same monomeric glyceride molecule might unite. This

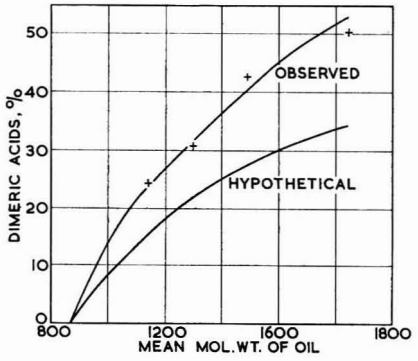

FIG. 5.—*Relationship between percentages of dimeric acids in heated linseed oils and mean molecular weights of the oils*

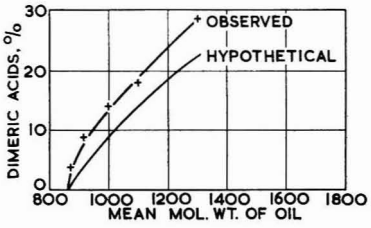

FIG. 6.—*Relationship between percentages of dimeric acids in heated soya-bean oils and mean molecular weights of the oils*

simplest type of intrapolymer, involving no change in mean molecular weight of the glycerides, may be designated ' Type-1 intrapolymer '.

(*b*) The union might take place between two fatty acid radicals that have not yet participated in polymerization and that are located in a dimeric glyceride. Such a polymerization step would give a percentage of dimeric acid twice as great as that expected from the mean molecular weight if no intrapolymer-formation is assumed.

(*c*) The union might take place between two fatty acid radicals in a higher polymeric glyceride. Here the percentage of dimeric fatty acids will be less than twice that expected when no intrapolymer-formation is assumed, but will still be greater than this hypothetical value.

For convenience in discussion, cases (*b*) and (*c*) will be designated as formation of Type-2 intrapolymers.

The relevant observed values have been inserted in Figs. 5 and 6. The striking feature of the comparisons of observed and hypothetical values is not the divergences themselves but the magnitude of the divergences, especially for the shorter times of heating where the formation of trimeric acids is least likely to reach appreciable levels. It is believed, therefore, that the results provide some evidence in support of the view that intrapolymerization occurs during the earlier stages of thermal polymerization.

However, if trimeric fatty acids were formed in the absence of intrapolymerization, formation of a trimeric acid would produce a trimeric glyceride while uniting three fatty acid radicals. This theoretical consideration of polymerization, in which absence of intrapolymerization is assumed, also includes the possibility that four fatty acid radicals could be united in two pairs to form a trimeric glyceride. Either mode of polymerization would tend to give percentages of non-distillable esters lower, not higher, than those expected *ex hypothesi*.

A further objection to the foregoing interpretation of the results as evidence for intrapolymerization arises from the possibility of formation of dimeric glycerides by union of three fatty acids of one glyceride molecule with three fatty acids of another, each to each. This seems unnecessarily complex, and it would require a substantial proportion of such material to account for the divergences recorded in Figs. 5 and 6. It seems reasonable to regard the existence of such considerable divergences as evidence for the existence of Type-1 intrapolymers, at least in the early stages of polymerization. This explanation would accord with the views of Adams & Power,[19] of Kass[18] and of Barker, Crawford & Hilditch,[16] though not with the views expressed by Bernstein.[9]

Refractive index as a measure of dimeric acid content of thermally polymerized oils

Thermal polymerization of linseed or soya-bean oil to a desired content of dimeric acid radicals could be achieved by carrying out a preliminary series of heatings to establish a relationship between time of heating and dimeric acid content. It is possible to reduce the

number of determinations necessary and to avoid difficulties in fixing heating times by utilizing the relationship between refractive index and content of dimeric acids. In Fig. 7 the relationship between refractive index of the heated oils and percentage of dimeric esters in the total esters is given. The relationship is linear for the heated samples of both oils; the unheated oils apparently tend to lie slightly off this line. If it is desired to polymerize to a specified dimeric acid content, then it should be sufficient to fix two experimental points on this curve. The use of the unheated oil to secure a third point will not lead to great error. This conclusion was verified in the course of the preparation of polymerized linseed and soya-bean oils to specified contents of 9 and 18% of dimeric acid radicals. The relevant data are presented in Tables III and IV. The procedure enabled the desired dimeric acid contents to be attained with reasonable precision. Preliminary heatings are necessary for each different sample and the conditions of heating should be maintained as constant as possible.

Table III

Refractive indices and dimeric acid contents of thermally polymerized linseed and soya-bean oils

Oil	Approx. time of heating, h.	$[n]_D^{25}$	Dimeric acids, % (by distillation of esters)
Linseed	0	1·4790	0
	6·5	1·4829	16·9
	12·0	1·4861	26·7
Soya-bean	0	1·4732	0
	12	1·4748	10·4
	24	1·4766	20·2

Table IV

Production of specified dimeric acid content of thermally polymerized oil by heating to predetermined refractive-index value

Oil	Dimeric acid specified, %	$[n]_D^{25}$ corresponding to specified dimeric acid content	Dimeric acid, % obtained
Linseed	9	1·4809	9·5
	18	1·4832	18·0
Soya-bean	9	1·4746	8·9
	18	1·4762	17·8

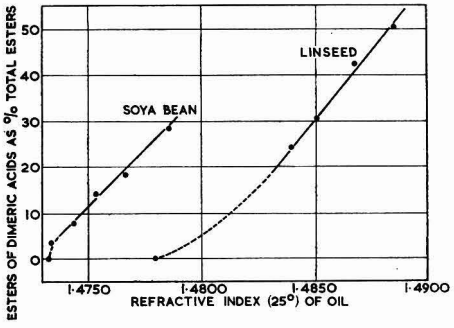

FIG. 7.—*Relationship between refractive indices of heated linseed oils and heated soya-bean oils and the percentages of dimeric acid radicals in the oils*

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CHEMICAL ASPECTS OF THERMAL DAMAGE TO THE NUTRITIVE VALUE OF VEGETABLE OILS. II.—The Possible Formation of Cyclized or Branched Monomeric Acyl Radicals

By A. F. WELLS and R. H. COMMON

Distillation of the ethyl esters of heat-polymerized linseed oil at low pressures gives a monomeric distillate, but approximately one-fifth of this distillate is incapable of forming urea adducts. Acetyl-value determinations indicate that this fraction does not represent hydroxy-acids. Estimations of terminal methyl groups support the tentative conclusion that branched acyl chains are absent. Data are presented for the ultra-violet absorption of the non-adduct-forming distillate fraction before and after alkali-isomerization. Some tentative views on the possible presence of cyclic acids are advanced.

Introduction

Nutritional trials[1] have shown that part of the reported deleterious effects of polymerized linseed oil on the nutrition of the rat was associated with the monomeric fatty acid fraction recovered by distillation of the ethyl esters of the whole heated oil. Reasons have been advanced[2] for the view that monomeric glycerides containing dimeric fatty acid radicals (so-called 'intrapolymers') are formed in the early stages of thermal polymerization. The possibility that cyclized fatty acid radicals might also be formed prompted an examination of the

ability of the distillable fraction of the esters of whole heat-polymerized linseed oil to form urea adducts.[3] Preliminary experiments with the acids of chaulmoogra oil (U.S.P.) showed that apparently only some 15% of the mixed fatty acids were capable of forming urea adducts. Moreno *et al.*[4] have also reported failure of the cyclic acids of chaulmoogra oil to form urea adducts. Thus, if the monomeric acids of polymerized linseed oil contain cyclized acids, these would be expected to be incapable of forming urea adducts.

Experimental

Commercial alkali-refined linseed oil was thermally polymerized at 275° for 12 hours, as described previously.[2] The whole oil was converted into the mixed esters by ethanolysis. A portion of the esters was distilled at a pressure of about 0·5 mm. of mercury, so as to provide a measure of the proportions of monomeric and dimeric material present. Another portion of the esters was fractionated by urea-adduct formation, to give an estimate of the proportions of adduct-forming esters and non-adduct-forming esters.

Bulk preparations of adduct-forming and non-adduct-forming fractions were performed as follows: A weight of 500 g. of esters was dissolved in 2 l. of absolute ethanol. Urea (2 kg.) was added slowly with stirring. The mixture was held at approximately 50° for half an hour and then left to cool slowly to room temperature overnight. Next morning the precipitate of adduct was removed on a large Buchner funnel and washed with several portions of absolute ethanol saturated with urea. (The use of ethanol instead of methanol has been found advantageous because esters of highly polymerized material are more soluble in ethanol, and hence more readily washed away from the adduct by ethanol.)

The adduct was thrown into a large excess of warm water, and the esters were separated in a large funnel. It was found that the addition of small amounts of sodium chloride aided the separation of the two phases and reduced losses due to emulsification. The ester was then washed with water and dried under reduced pressure. The filtrate was diluted with large volumes of water. The non-adduct-forming esters were recovered, washed with water and dried under reduced pressure. This method involves some loss by emulsification, but it is advantageous for the preparation of fractions intended for feeding trials. The fractions are not exposed to high temperatures and no traces of solvents of objectionable flavour are left.

For quantitative work, the esters were recovered from the aqueous mixture formed when the adduct was decomposed, or when the non-adduct-forming esters were thrown out of solution with water, by extraction with peroxide-free ethyl ether. The ether was removed under an atmosphere of carbon dioxide. The results of the two fractionations of the total esters of the heated oil are given in Table I.

Table I

Yields of fractions obtained from esters of thermally polymerized oil by distillation under reduced pressure and by urea-adduct formation

Ester fraction of total esters	Yield, %
Distillable (presumptive monomeric) fraction	65
Non-distillable (presumptive dimeric) fraction	35
Adduct-forming fraction	50
Non-adduct-forming fraction	50

Although 65% of the total esters was distillable, only 50% formed adducts. This observation suggested the presence of non-linear monomeric material in the distillable fraction. This possibility received some support from the report[5] that the monomeric C_{18} fraction of methyl linoleate which had been thermally polymerized for 96 hours contained some 60% of material incapable of yielding stearic acid on exhaustive hydrogenation.

The experiment was repeated with some modifications. Methyl esters were prepared from both heated and unheated linseed oil by methanolysis. The following fractions were prepared from the esters: Distillable and non-distillable fractions of the total esters, adduct-forming and non-adduct-forming fractions of the total esters, and adduct-forming and non-adduct-forming fractions of the distillable esters. The yields of these fractions are shown in Table II.

The results shown in Table II confirmed the presence of an appreciable amount of non-adduct-forming material in the distillable monomeric ester fraction.

The preceding experiment was repeated carefully for the preparation of a series of fractions for a feeding trial to be reported elsewhere.[1] The results are presented in Table III.

Table II

Yields of various fractions of methyl esters of thermally polymerized linseed oil

Source	Fraction	Yield, %	Yield mixed total esters, %
Mixed total esters	Distillable	72	72
	Non-distillable	28	28
Mixed total esters	Adduct-forming	58	58
	Non-adduct-forming	42	42
Distillable ester	Adduct-forming	83	60
	Non-adduct-forming	17	12

Table III

Yields and characteristics of certain fractions of ethyl esters of thermally polymerized linseed oil

Fraction	Yield, as % of total esters	Iodine value*	$[n]_D^{25}$	Mean mol. wt.†	Hydroxyl value, %
Adduct-forming fraction of total esters	46	118·2	1·45345	293	0·23
Non-adduct-forming fraction of total esters	54	162·7	1·47561	472	0·46
Distillable fraction of whole esters ..	60	130·1	1·45684	294	0·22
Adduct-forming fraction of distillable esters	49	124·8	1·45494	293	0·14
Non-adduct-forming fraction of distillable esters	11	170·7	1·46986	300	0·34
Non-distillable fraction of total esters (dimers)	40	159·9	1·48017	550	0·64

* Method of Benham & Klee,[6] with 1-h. reaction time[7]
† Cryoscopically, with *cyclo*hexane as solvent

In this experiment 11% of the total esters consisted of distillable and essentially monomeric material incapable of forming urea adducts. Among the possible reasons for this failure to form urea adducts the following were considered: (*a*) the presence of hydroxy-acids, (*b*) the presence of fatty acids having a branched chain and (*c*) the presence of cyclized acids.

Possibility (*a*) was practically excluded by the fact that the non-adduct-forming fraction of the distillate had a hydroxyl number of only 0·34. (This hydroxyl value may have been due to small amounts of monoglyceride, which would be concentrated in this fraction. The actual hydroxyl value would correspond to about 3·5% of monoglyceride in the fraction.)

Possibility (*b*) was examined by applying the Kuhn–Roth method of estimating the proportion of terminal methyl groups by chromic acid oxidation to acetic acid.[8] The non-adduct-forming distillate gave a terminal-methyl number of 1·70, and the adduct-forming distillate gave a terminal-methyl number of 1·74. These values are about the same as those expected from methyl esters of straight-chain fatty acids. Both fractions gave practically the same value. This observation does not exclude completely the possible presence of branching, for it has recently been shown[9] that the Kuhn–Roth oxidation yields one mole of acetic and (62–99%) yield for $—CMe_3$, $>CMe_2$ or $\geqslant CMe$. It does, however, restrict the possible types of branching. Thus it excludes the possibility that a single methyl or other alkyl group present on the main carbon chain was responsible for failure to form an adduct; for if this were so, the non-adduct-forming fraction should have given the higher terminal-methyl number.

The presence of cyclic acids would lead to two possibilities: (1) that the ring was terminal, when the non-adduct-forming fraction should have given an appreciably lower terminal-methyl number; (2) that the ring was not terminal, when the yields of acetic acid from the two fractions of the distillate should have been the same. The experimental results accord with the view that the presence of a non-terminal ring structure was the reason for failure to form an adduct.

The iodine value of the non-adduct-forming distillate (170·7) suggested a predominantly dienoic nature. The ultra-violet-absorption curve of the material was examined before and after alkali-isomerization for 25 minutes at 180°, and after six hours' isomerization at 180°. The absorption spectra are shown in Fig. 1. The use of the longer isomerization period was prompted by the observation of Paschke, Jackson & Wheeler[10] that *trans–trans*-linoleate requires six hours to attain maximum isomerization. Values for the content of diene and triene acids of the fraction were calculated from the absorption data by the method of the American Oil Chemists Society.[11] The values are reported in Table IV. It should be emphasized that the values for ' linoleic ' and ' linolenic ' will include all isomers of these acids capable of giving diene and triene conjugation on alkali-isomerization.

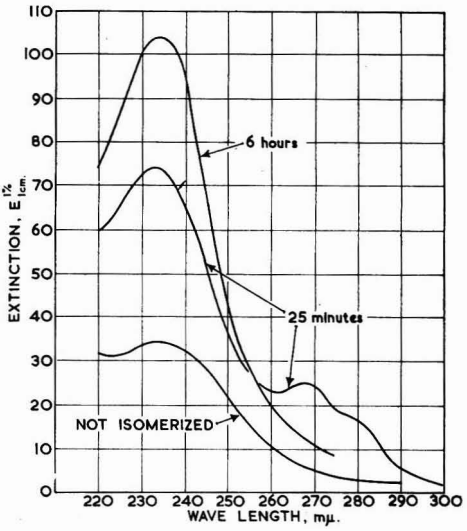

FIG. 1.—*Absorption spectra of non-adduct-forming fraction of the distillable fraction of the esters of thermally polymerized linseed oil (before alkali-isomerization, alkali-isomerized for 25 min. at 180°, and alkali-isomerized for 6 h. at 180°)*

Table IV

Fatty acid composition of the non-adduct-forming fraction of the distillable ethyl esters of thermally polymerized linseed oil

Conjugated diene, %	2·7*
Conjugated triene, %	0·0*
' Linoleic ' acid, %	2·5†
' Linolenic ' acid, %	3·1†
Total	8·3

* Calc. from data for fraction before alkali-isomerization
† ,, ,, ,, ,, ,, after ,, ,, for 25 min.

Less than 10% of the fraction could be accounted for as ' linoleic ', ' linolenic ' or conjugated diene acids. Even if ' linoleic ' acid was calculated from the six-hour isomerization, this proportion was only slightly increased. It thus appears that the fraction contained a large proportion of a dienoic acid whose double bonds could not be conjugated even by six-hour isomerization.

The foregoing evidence is clearly insufficient to establish the nature of this fraction, but

some speculations on its nature may be offered. A non-terminal cyclic system might arise from ' linolenic ' acyl chains by a free-radical or Whitmore rearrangement mechanism, which would account for the iodine value and the failure of the fraction to display a high diene-absorption at 234 mμ, even after prolonged alkali-isomerization. The mechanism of direct carbon-to-carbon linkage suggested here as a speculation has analogies with the theory of polymerization mechanism put forward by Barker, Crawford & Hilditch.[12]

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THROUGH-CIRCULATION DRYING OF SEAWEED
II.—*Laminaria cloustoni* frond

By R. G. GARDNER and T. J. MITCHELL

The drying characteristics of freshly harvested *Laminaria cloustoni* frond have been investigated in a through-circulation drier.

Drying factors investigated were bed depth (0·5–4 in.), particle size (minced and shredded), air velocity [3–7 lb./(sq. ft.) (min.)], air temperature (130–220° F), air wet-bulb depression (53–123° F), and the seasonal variation of drying times. In most of the tests reported, the fronds were minced before drying.

The general effect of air velocity, air temperature and humidity on the drying time is similar to that for *L. cloustoni* stipe, and a comparison test showed that minced frond dried slightly faster than the shredded material. Drying rates of frond beds at average water contents of 2·5–0·2 lb./lb. (dry basis) have been found to be directly proportional to the wet-bulb depressions of the air.

Two outstanding differences in the behaviour of frond and stipe are the effects of bed depth and seasonal variation. The frond-output/dry-loading curve rises to a well defined optimum point at 0·45 lb. of B.D.S. (bone-dry solid)/sq. ft. (1–1½ in.) for minced and 0·6 lb. of B.D.S./sq. ft. (4–4½ in.) for shredded frond. It is suggested that the low optimum loading is a result of the stickiness and lack of rigidity of the frond bed.

Drying times may vary three- or four-fold, according to the month in which the frond was harvested. This fluctuation has been tentatively related to the salt content and water ratio of the frond, which vary in a similar manner to the drying times. The maximum drying times for frond are encountered in May–June.

A drying test conducted on a commercial through-circulation grass drier demonstrated that frond can be dried on the large scale by through-circulation drying.

The maximum air temperature that *L. cloustoni* frond can withstand for reasonably long periods without scorching is 225° F.

A formula is given for the calculation of the static pressure drop of air passing through beds of dried minced *L. cloustoni* frond 1–9 in. deep (random packing).

Raw material

The fronds of the sublittoral seaweed species *Laminaria cloustoni* used in this investigation formed flat brown strips 18–24 in. long by 3–6 in. wide and about $\frac{1}{16}$ in. thick.

The fronds received 24 hours after harvesting, were covered with a mucilage containing sodium alginate, which caused the blades to adhere to each other. They decomposed within about 48 hours of harvesting if stored wet, and decomposition was often most marked at points where two pieces had adhered.

Differences in chemical constitution between frond and stipe arise from their different functions, e.g. most of the photosynthesis takes place in the frond. These differences are shown in Table I compiled from a paper by Black.[1]

Table I

L. cloustoni, *harvested Oban*, 1947

	Frond	Stipe
Water content (lb. water/lb. of B.D.S.*)	2·7–6·6	4·6–6·1
Total ash (dry basis), %	13–37	30–38
Alginic acid (dry basis), %	8–19	19–23
Laminarin (dry basis), %	1–32	Nil
Mannitol (dry basis), %	6–23	5–9
Average weight (450 samples), lb.	1·5	2·6

* Bone-dry solid

These figures are the minimum and maximum values obtained for 1947.

Extraction of the various constituents has been hindered in the past by the difficulty of harvesting and drying fresh seaweed. Cast seaweed is not so suitable, as some of the soluble constituents may be leached out by the sea or by rain water. If the frond is not dried promptly, many of the organic compounds are degraded by bacterial action.

Dewar[2] has outlined the various chemical constituents present in the seaweed, together with methods of extraction and possible industrial uses, and Woodward[3] has discussed the value of seaweed as an animal feeding-stuff.

Previous work on frond drying

Preliminary work on the natural air drying of fresh *L. saccharina* and *L. digitata* was carried out in this laboratory by McLean & White[4] and Black & Duthie.[5] *L. saccharina* frond, which had been soaked in water to a moisture content of approximately 50%, has been successfully dried in a pilot-plant rotary louvre drier.[6] Tests on a similar drier with freshly harvested minced *L. cloustoni* frond were only partly successful, owing to the excessive mucilage content of the raw material. Gardner, Mitchell & Scott,[7] using a radioactive-tracer technique, showed that the seaweed did not receive uniform treatment in this drier because of frond particles sticking to the drum.

A test on a large-scale grass drier, using fresh *L. cloustoni* as the feed has been described by Gardner.[8] This grass drier (Pehrson Dual-Process) had a pneumatic drying tower followed by two rotary-drying sections.

Experimental procedure

The drier used in the present investigation consists of a centrifugal fan which blows air over electric heaters and then vertically through the seaweed bed. The seaweed is contained in a metal basket which can be rapidly removed for weighing. A fuller description of this plant and the drying procedure has been given in a previous paper.[9]

The prepared frond was weighed into the basket and the bed levelled off without any pressure being applied. No method of arranging the pieces was possible, so the bed was packed at random. During drying, the dark-brown frond changed to a dark-green colour. When steam injection was used to humidify the inlet air, the colour changed initially to a bright-green shade which ultimately became dark green.

The seaweed bed contracted during drying, and, after about one-third of the drying time had elapsed, the bed had moved away from the basket side, allowing part of the air to short-circuit the bed. As the mucilage dried, the various particles were cemented together until, at the end of the test, the entire bed could be lifted out as a mat.

Results

In most of the 25 tests reported, the seaweed frond was minced before drying. The factors studied were: bed depth, particle size, air velocity, air temperature and wet-bulb depression, and the seasonal variation of the drying times. Drying conditions noted on the graphs are expressed as the standard deviations from the average of the set of runs. Physical differences between the frond and stipe have a profound effect on their drying characteristics.

Frond particles are sticky and flexible in contrast with the uniformly-sized, rigid, non-sticky stipe slices. This causes the frond bed to show a marked edge-effect, produced by the shrinkage of the materials from the edges of the basket; hence there is short-circuiting of the air through the resulting spaces, leading to uneven drying. In the bed of stipe, on the other hand, the slices are sufficiently mobile to fill up any gaps formed during drying.

The stipes have a reasonably uniform water content throughout the year, but the frond water content can vary up to three times its minimum value. In addition to the increased drying time caused by the greater evaporation load, the frond also offers more resistance to drying at the time of year when the water content is highest. With the stipe, no marked seasonal change of drying time was observed.

Bed depth

The effect of increase of bed depth was studied for minced and shredded frond at an inlet air temperature of 156° F. The curves of drying-time/dry-loading are given in Fig. 1. Both curves are concave upwards, showing that the drying time increases rapidly for small increments of depth. When the output/bed-loading curve is plotted (Fig. 2) for minced frond it may be seen that there is a marked optimum loading at 0·45 lb. of B.D.S./sq. ft. or 1–1½ in. deep. (Intermediate points on the output curve were interpolated from the curve in Fig. 1.)

The points for the shredded seaweed are more scattered but it is probable that the output curve is of a similar form to that for minced frond, with a slightly higher optimum point at 0·6 lb./sq. ft. (4–4½ in. deep). It would at first appear from these curves that shredding was superior to mincing in giving speedier drying, but these tests are not comparable as the place and time of harvesting were different. The comparison test between the two forms of pre-cutting showed that mincing was faster.

It is suggested that the disproportionate increase of drying time with increasing bed depth and the low optimum loading are the result of two factors: (1) the stickiness of the frond, (2) the lack of rigidity of the material.

The mucilage tends to fill the interstices between the individual particles, preventing the ready access of air, and the lack of rigidity of the fresh frond allows the lower strata to be compressed into a compact mass. This is partly confirmed by three tests with minced *L. cloustoni* frond which had been pre-dried in a rotary drier at 300° F. The particles, at a water content of 0·5 lb./lb., had lost all stickiness, but were still flexible. Beds of this material 1¼, 2½ and 5 in. thick were successfully dried, showing that heavy loadings may be practised when the effect of the mucilage is minimized. The drying-time/loading curve (Fig. 3) still shows a slight upward curvature, probably caused by compression of the lower layers with heavier loadings.

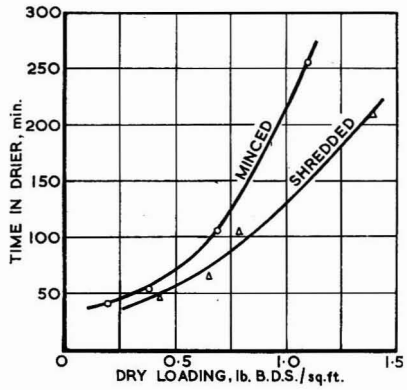

FIG. 1.—L. cloustoni *frond*

○ $G = 7{\cdot}1 \pm 0{\cdot}25$ lb./(sq. ft.)(min.)
△ $G = 8{\cdot}05 \pm 0{\cdot}15$,, ,, ,,
D.B.T., 156° F; W.B.D., 75° F

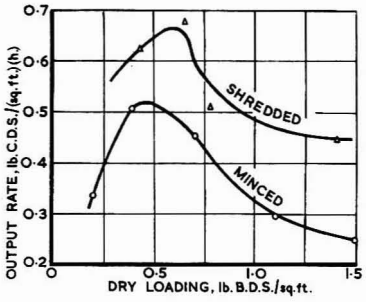

FIG. 2.—L. cloustoni *frond*

△ Inchcolm (February)
○ Oban (May)

Particle size

Earlier experiments showed that 6-in. lengths of frond could not be dried satisfactorily as a bed, as the outside of the layer was often crisp while the inner parts were wet and sticky. Where two strips had stuck together, the air could not penetrate and the drying time became excessive. It was apparent that some form of subdivision of the frond was required, not only to increase the specific surface, but also to allow the drying air to reach all the particles forming the bed. If the pieces are too finely cut or are crushed, more mucilage tends to exude from the fragments and some of the advantages of size reduction are lost.

Mincing is effective for disintegration of the frond, but the design of the mincer has a noticeable effect. Where the material is extruded through small holes, considerable amounts of mucilage are produced and the seaweed becomes like a sticky paste. The domestic mincer used in the present work has an external cutting ring which gives more of a shearing action. In all the tests on minced frond, the largest cutter was used giving pieces about $\frac{3}{8}$ in. × $\frac{1}{2}$ in. × $\frac{1}{16}$ in.

Tests were also carried out on frond which had been shredded in a rotary bean-cutter. This machine produced frond strips $\frac{3}{16}$ in. wide by about 6 in. long. This method of pre-cutting seemed to cause less exudation of mucilage than mincing. A direct comparison

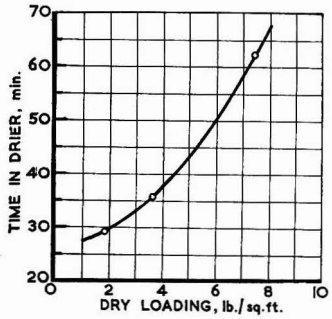

FIG. 3.—L. cloustoni *frond (minced and pre-dried): effect of bed depth on drying time* (T, 0·5 to T, 0·1)

D.B.T., 155° F; W.B.D., 71° F; G, $7{\cdot}9 \pm 0{\cdot}3$ lb./(sq. ft.)(min.)

between shredded and minced frond was made from tests on the same batch of seaweed (see Table II).

Table II

L. cloustoni *frond harvested at Inchcolm on* 14 *March*, 1952

Dry bulb = 156° F Wet loading = 4·375 lb./sq. ft.

	Shredded	Minced
Bed depth, in.	5	2
Dry loading, lb. of B.D.S./sq. ft.	0·65	0·68
Wet-bulb depression, ° F	76	74
Mass air flow, lb./(sq. ft.)(min.)	8·1	7·9
Drying time ($T = 5$ to $T = 0{\cdot}15$),* min.	76·5	62·5
Static pressure-drop, in. (water)	0·17	0·36

* T = total water content, lb. water/lb. of B.D.S. (i.e. water ratio)

This shows that minced frond dries more rapidly than the strips for plants harvested in March (when the alginic acid content is highest). Comparable drying times for *L. cloustoni* stipe are 68 minutes for minced stipe and 110 minutes for stipe slices, $\frac{1}{8}$ in. thick. It should be noted that the bulk density and static pressure drop of the shredded frond bed are much lower than the values for the minced frond.

Trouble was experienced with the fronds slipping on the feed drum of the rotary shredder, and in the remaining tests (except bed-depth runs) minced frond was used to reduce the preparation time. It is thought that this difficulty could be overcome on industrial shredding machinery, possibly by the use of spiked feed-drums. It may here be observed that, unlike the stipe, no water can be separated from the minced frond by centrifuging.

Seasonal variation

The seasonal change in water content, which is of especial interest as regards drying, is plotted as the water ratio (lb./lb.) in Fig. 4. It can readily be seen that the water content is highest in the spring (April–May) and lowest in the autumn (September–October). The mineral matter content, expressed as total ash, exhibits similar maxima and minima (Fig. 4) as also does alginic acid (March). On the other hand, laminarin and mannitol are at a maximum in the autumn, the laminarin curve being the inverse of the total ash graph.

Seven drying tests under similar conditions were made at different times between March and September. When the drying times for the water-content range 5–0·15 are plotted (Fig. 4,

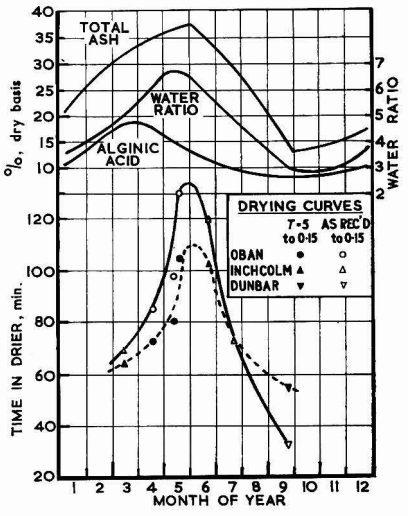

FIG. 4.—L. cloustoni *frond: seasonal variation of drying times (drying curves)*

D.B.T., 156° F; W.B.D., 73° F; L_d; 0·72 ± 0·1 lb./(sq. ft.); G, 7·4 ± 0·4 lb./(sq. ft.)(min.)

broken line) it can be seen that the drying time reaches a maximum in the spring and appears to be at a minimum in the autumn. (The time for September was obtained by extrapolation, as the initial water content was only 2·05.) This curve follows the total-ash line, and the increased drying time is probably related to the reduced vapour pressures caused by the higher salt concentrations.

The total ash of the fronds consists of soluble and insoluble salts. Black[10] found that the principal seasonal variation occurred in the water-soluble constituents which were presumably in solution in the cell sap, and they will naturally influence the vapour pressure. The insoluble mineral matter does not vary widely throughout the year and is comparatively low (the average value for the seven drying tests being 5% of the weight of the bone-dry frond). This means that the soluble ash content will follow the total-ash curve, but the actual values will be about 5% less. The total-ash content has been retained, as little information is available on the soluble ash content of seaweed.

The maximum drying time may be related also to the alginic acid content. The alginic acid would be expected to reduce the drying rate of a seaweed bed in two ways, externally by the production of mucilage on the surface of the frond pieces, and internally by its affinity for water. The latter effect would probably be more noticeable in the later stages of drying. It appears, however, that the salt concentration is the overriding factor controlling drying time, since the peak value for alginic acid occurred two months before the maximum drying time was reached, whereas the total ash maximum coincided with this maximum drying time.

The comparison of frond-drying times for a given water content range (i.e. 5–0·15 lb./lb. water content) gives some indication of the resistance to drying, provided of course that all other conditions are similar. In view of the wide variation of the initial water content of the frond, it is more realistic to compare the times required to dry from the initial condition to a standard final moisture content. This curve is shown in Fig. 4 (full line) and indicates that the minimum drying time may be about one-quarter of the peak value (i.e. 130 minutes in May–June to 33 minutes in September).

If the frond was being harvested and dried for the eventual extraction of any one particular compound, the seaweed would naturally be collected in the month when the constituent was at its maximum. None of the major constituents appear to have their peak value at the time of the lowest drying rate, but if the harvesting of frond for alginic acid is continued after March, the seaweed will become progressively more difficult to dry. Alginic acid is at present the principal commercial algal chemical, but it is almost invariably extracted from the stipe.

The curves for water content, total ash and alginic acid (Fig. 4, in which L_d = dry loading, lb. of B.D.S./sq. ft.) are taken from results reported by Black[1] for frond harvested at Oban in 1947. Determinations made on the material from the drying tests reported agree in general with Black's results.

This seasonal change in the drying rate of frond precludes any rigorous comparison, but the general effect of any one variable will probably be the same at any time of the year, although the actual value of the drying rates will have altered.

Owing to harvesting difficulties caused by bad weather, it was necessary to collect plants from different areas, so that the seasonal variation tests are not strictly comparable, but they give some indication of the fluctuations to be expected. It is apparent that any drier for seaweed must be sufficiently flexible in operation to cope with these variations.

Air velocity

This series of tests was done on 2-in. beds of minced frond, which, although greater than the optimum bed-loading, are nevertheless sufficiently close to be of value. At the time of testing, the maximum air flow of the drier was about 7 lb./(sq. ft.)(min.), so that only four runs were attempted.

The effect of mass air flow on the drying time (Fig. 5) has a similarity to the corresponding curve for stipe, and it is probable that this frond curve will also level off at higher air flows. It may be concluded that the minimum air flow should be 7 lb./(sq. ft.)(min.).

The plot of constant drying rate against air flow shown in Fig. 6 appears to be linear, but differs from that of stipe in that the straight line would intercept the x-axis if produced.

Temperature and humidity

Seven tests were carried out at an average dry-loading of 1·10 lb./sq. ft. (1½ in. approx.).

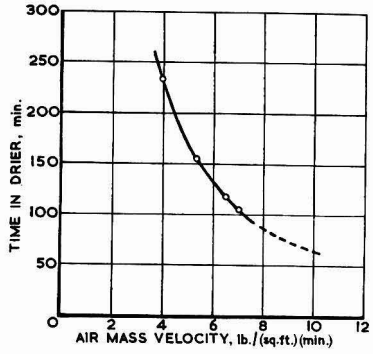

FIG. 5.—L. cloustoni *frond*

Oban (May) D.B.T., 156° F; W.B.D., 75° F;
L_d, 0·75 ± 0·04 lb./(sq. ft.)

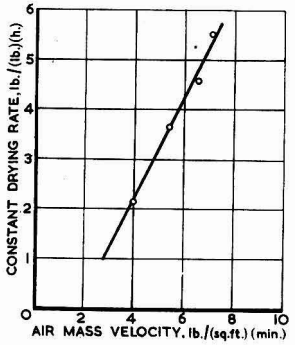

FIG. 6.—L. cloustoni *frond*

As the seaweed was harvested in August and September, the water ratio was only 2·4–3·0. The curves of water content versus time were drawn and the instantaneous drying rates at water ratios of 2·5, 0·6 and 0·2 measured with a tangentimeter.[11]

These drying rates (Fig. 7) show a linear relationship to the wet-bulb depression (W.B.D.) similar to that for stipe. It is most likely that the drying rates at higher water contents will obey the same rule. A test using humidified air (170° F dry-bulb) agrees reasonably well with the other points obtained from tests using heated air of normal room humidity.

As the drying time of the frond varies so widely with the season, it was considered unwise to construct a curve for unit W.B.D. on which to base a drier design. If, however, the drying time for a given W.B.D. is known then the drying time for any other W.B.D. may be estimated by proportion with reasonable accuracy.

Large-scale test

Tests on a commercial through-circulation grass drier (Templewood Mark 2) demonstrated the feasibility of this method for drying seaweed frond on full-scale equipment. This drier[12]

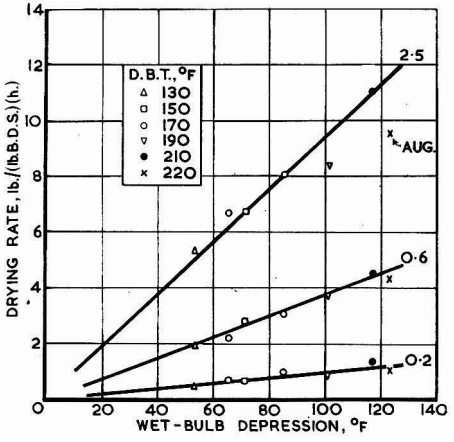

FIG. 7.—L. cloustoni *frond: effect of wet-bulb depression on drying rate (all D.B.T. figures are therefore Dunbar in September, except D.B.T. 220° F, which is for August; numbers on graph refer to water ratios)*

L_d, 1·1 ± 0·032 lb./(sq. ft.)
G., 7·6 ± 0·33 lb./(sq. ft.)(min.)

has two wire-mesh conveyor-belts 5·4 ft. wide × 25 ft. long, so arranged that the partly dried material drops from the upper to the lower belt. The drier is designed so that the hot air passes upward through the partly dried material on the lower conveyor, and then through the wetter feed on the top band. About one-third of the exhaust air is discharged direct to the atmosphere and the remainder is recirculated through the fan inlet. A discharge conveyor belt passes the dried product to a pre-breaker and hammer mill. The National Institute of Agricultural Engineering tested the prototype of this type of drier with grass, and the results have been reported.[13]

As the slowest belt speed on the grass drier did not allow sufficient time for the seaweed frond to dry, the drier was operated intermittently. At the end of the first stage of drying the frond pieces had formed a rigid bed which broke into short sections as it reached the end of the conveyor, thereby exposing the wetter fractions of the charge and promoting more uniform drying. Runs at different temperatures on this grass drier established that the limiting drying temperatures for *L. cloustoni* frond is slightly lower than that for stipe, namely 225° F.

This drier produced an excellent seaweed meal when an air temperature of 225° F was used. It was found in this test that a chaff-cutter was not the most satisfactory machine for cutting seaweed frond, although it could probably be readily modified to produce a smaller particle size.

It is suggested that shredding, if practicable on a large scale, would provide a much more suitable seaweed bed owing to its greater porosity and lower bulk density. Although the comparison tests showed mincing to be superior to shredding, the strips would probably break up more easily at the turnover. The lower static-pressure drop of the bed of shreds will mean less fan-power.

The field test and later work on *L. digitata* frond have shown the advantages of breaking up the partly dried frond bed. This is especially important for frond since, unlike the stipe, the individual particles are not discrete, but act as a mass causing a marked edge-effect. If this leakage is not rectified an appreciable fraction of the hot air will be ineffective.

It is suggested that a through-circulation drier for frond should have from two to four drying stages, with arrangements to break up the material bed at intervals, e.g. by rotating spiked rollers at the end of a stage.

It is apparent from the seasonal change in drying times that the drier must be sufficiently flexible in operation to cope with these fluctuations, so that the belt speeds should be variable within wide limits. If this were done, the same drier would be suitable for drying stipe and frond.

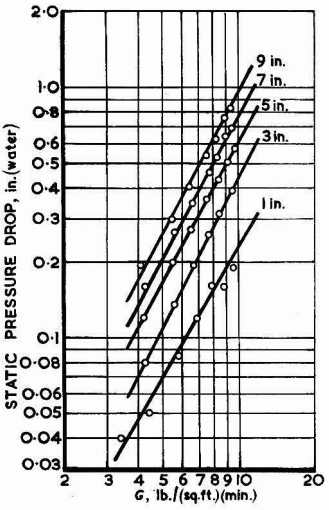

FIG. 8.—L. cloustoni *frond (minced and dried) : static-pressure drop* versus *air flow*

Static-pressure drops

The static-pressure drops of beds of dried minced *L. cloustoni* frond were measured by noting the draught-gauge reading when the bed was in position with the drier closed, and deducting the resistance of the drier and basket at the same air-flow rate. Plots of static-pressure drop against air flow are given in Fig. 8, with air at room temperature. These results

could be directly applied to the design of finishing bins which are used to condition nearly dry material to a standard moisture content. The main features of these bins are that deep beds and low air velocities are used.

The deepest bed (9 in.) was prepared first and weighed so that the remaining layers could be measured by weight, as this was more accurate than direct linear measurement. Static-pressure drops for wet frond are shown in Table III. It can be seen from Table III that the static-pressure drop per unit of bed depth decreases to a constant value as the bed loading is increased. This characteristic was observed with beds of *L. cloustoni* stipe slices.[9]

The velocity index also tends to a constant value with increasing depths, and the average value (1·85) is close to the indices for stipe (1·83 and 1·80 for dried and fresh slices respectively). A formula relating the pressure drop to air flow for dried frond beds (random packing) has been derived from the pressure drop of the 9-in. bed and the average exponent, i.e. $P = 0{\cdot}0183G^{1{\cdot}85}$, where P = static-pressure drop, in. (water)/ft. of bed, and G = air mass velocity, lb./(sq. ft.)(min.).

Table III

Static-pressure drops of beds of L. cloustoni *frond (minced and dried)*

$G = 9$ lb./(sq. ft.)(min.)

Bed depth, in.	1	3	5	7	9
Pressure drop, in. (water)/ft. bed	2·28	1·40	1·20	1·08	1·07
Velocity index	1·70	1·98	1·85	1·85	1·87

Nomenclature

The term ' commercial dry solid ' (C.D.S.) used in this work refers to seaweed having a water ratio of 0·15 (13·04% of water on the wet basis). At this water content the seaweed can be readily ground and stored safely.

Acknowledgments

One of us (R. G. G.) is indebted to the Institute of Seaweed Research for an extra-mural research scholarship. This work forms part of a programme of research and development on seaweed undertaken by the Institute of Seaweed Research, and the authors are indebted to the Director for permission to publish. Acknowledgment is also made of practical assistance from Gilbert H. Stewart.

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NOTE ON RECENT CHANGES IN THE A.O.A.C.* METHOD FOR THE ASSAY OF PYRETHRINS

By Wm. MITCHELL

Experimental evidence is advanced to show that the changes recently adopted[1] in the A.O.A.C.* (7th Edition) method[2] for the assay of pyrethrins † were an improvement; and that their subsequent official cancellation is unfortunate and should be reconsidered.

Important changes were made[1] in the methods authorized[2] by the Association of Official Agricultural Chemists for the assay of pyrethrum flowers and extracts. More recently these changes, in so far as they refer to the determination of pyrethrin I, have been rescinded by a special notice circulated to those on the Association's mailing list for 'Changes in Methods'.

Briefly, the change that has been rescinded involved the substitution of hydrochloric acid for sulphuric acid to liberate the chrysanthemum acids before the extraction of chrysanthemummonocarboxylic (chrysanthemic) acid with light petroleum. This change brought the method into line with that published by the Colonial Products Advisory Bureau's Consultative Committee on Insecticide Materials of Vegetable Origin through its Standing Sub-Committee on Methods of Analysis of Vegetable Insecticides;[3] and with the method adopted in the British Pharmaceutical Codex 1949.[4] It had the advantage that it simplified the process by obviating the need for removing the barium sulphate arising from the presence of an excess of barium chloride from an earlier stage of the procedure. However, the main reason for the change was based on the finding, on good authority, that use of hydrochloric acid gave significantly higher results for pyrethrin I; and the change was thus made in the belief that the accuracy of the determination was thereby increased. It is believed that the changes were rescinded as a result of representations, made by certain users of pyrethrum flowers, that the higher assay results were not reflected in the biological potencies, i.e. that the extra 'pyrethrin I' recorded was not in fact insecticidally active.

It therefore seems desirable to put on record some experimental work on this subject that was carried out some years ago in these Laboratories, but not published. This work was undertaken in an effort to account for the observed higher pyrethrin I results (on flowers or extracts) when hydrochloric acid was substituted for sulphuric acid. Table I gives typical results (on extracts) by the two procedures.

Table I

Comparison of results on pyrethrum extracts assayed by the A.O.A.C. method, with (a) *sulphuric acid and* (b) *hydrochloric acid*

Sample	Pyrethrin I, %		Pyrethrin II, %		Total pyrethrins, %	
	(a)	(b)	(a)	(b)	(a)	(b)
1	3·5	3·7	2·6	2·6	6·1	6·3
2	16·9	18·1	13·6	13·5	30·5	31·6
3	13·3	14·9	11·5	11·5	24·8	26·4

It will be seen that, whereas the figures for pyrethrin II are unaltered, those for pyrethrin I are increased to a substantial and variable extent when hydrochloric acid is used.

In a further series of analyses, the assay of pyrethrin I was carried out exactly as laid down in the A.O.A.C. method. In addition, the washed residue of barium sulphate on Filter-Cel (that is normally discarded) was in each case extracted by washing thoroughly with small portions of warm aqueous N-sodium hydroxide, followed by warm water. The combined washings were acidified with sulphuric acid and afterwards submitted to the same treatment as the main filtrate for the determination of pyrethrin I. The colour changes observed after the addition of Denigè's reagent were typical of those normally observed with chrysanthemic acid. The results obtained in this two-stage procedure are set out in Table II, along with the results obtained on the same extracts in one stage with hydrochloric acid instead of sulphuric

* Association of Official Agricultural Chemists (Washington)

† The terms 'pyrethrin I' and 'pyrethrin II' refer not to single substances but to groups of esters of varying molecular size and insecticidal potency[2a, 2b]

acid. The figures for pyrethrin II are not included, since in all cases none was obtained in the second stage, and those in the first stage agreed very closely with the results obtained with hydrochloric acid.

Table II

Comparison of pyrethrin I results obtained by the A.O.A.C. method with (a) *hydrochloric acid,* (b) *sulphuric acid, and* (c) *from the barium sulphate residues from* (b)

Sample	Pyrethrin I, %		
	(*a*)	(*b*)	(*c*)
4	18·6	17·4	1·0
5	15·7	14·1	1·5
6	18·2	15·9	2·4
7	17·1	14·9	2·2

These results show clearly that the acid recoverable from the barium sulphate residues, and assayed by the A.O.A.C. procedure, in each case accounted almost exactly for the difference between the results obtained with hydrochloric acid and sulphuric acid respectively. For samples 6 and 7 the assays were conducted, with five times the usual quantities of extract and reagents, to the stages where the main filtrate and subsidiary washings were obtained. These were each diluted to standard volumes and one-fifth aliquots used to complete the determinations of pyrethrin I as usual. The remaining four-fifths aliquot of each barium sulphate washing was acidified to Congo red with sulphuric acid and extracted with light petroleum (b.p. 40–60°; three portions, each of 50 ml.). The extracts were washed in turn with water (two portions, each of 20 ml.), the solvent was recovered and the residual acid titrated with 0·02N-sodium hydroxide against phenolphthalein. The results, calculated as pyrethrin I, were respectively 2·3 and 2·1%. Thus, they agreed very well with those obtained by the mercury-reduction procedure. Since the latter appears to be more or less specific for chrysanthemic acid, and since the figures by alkalimetry were slightly lower, it seems unlikely that extraneous acids were present in any significant amount.

The acids present in these titration liquids were then converted, by standard procedures, in the one case into the *p*-phenylphenacyl ester and in the other into the amide, the purified derivatives being obtained in yields upward of 50% in each case. The ester had m.p. 64°, alone or mixed with authentic material of m.p. 65°. The amide had m.p. 129°, alone or mixed with authentic material of m.p. 131°. Thus it was considered proved that the acid recovered from the barium sulphate residues was substantially pure chrysanthemic acid.

Discussion

Since the structurally similar chrysanthemumdicarboxylic acid does not appear to be held at all by the barium sulphate, it seems unlikely that the latter retains chrysanthemic acid by adsorption. Since the dicarboxylic acid is soluble in water, whereas chrysanthemic acid is only very sparingly soluble, it seems more likely that chrysanthemic acid is partially filtered out on the barium sulphate. Variations in the temperature of the liquids and in the mode of addition of the sulphuric acid may, by affecting the particle sizes both of the barium sulphate and of the liberated chrysanthemic acid globules, account for the variable degree of retention.

Chrysanthemic acid itself is of course devoid of insecticidal activity. It is one of the weaknesses of the mercury-reduction (or for that matter the Seil) assay that the total acid is determined and calculated as 'pyrethrin I', even though it is now known that part of it must have derived from cinerin I. In addition, there is the possibility that some of the acid may have pre-existed in the free state, or been derived from polymerized pyrethrins or other non-insecticidal compounds. It would, however, be remarkable if the barium sulphate precipitate were selectively to retain only that part of the chrysanthemic acid representing originally inert material! Further, experience in this Laboratory has revealed no reason for suspecting lack of correlation between the results obtained on flowers or extracts by the A.O.A.C. 7th Edition (Amended) assay and the corresponding biological tests on houseflies, using the modified Peet–Grady procedure previously described.[5] On the contrary, from experience in this Laboratory there is reason to suppose that the amended method gives more consistent, reliable and reproducible results. It is therefore suggested that those officially responsible for this method re-examine the matter, and especially the validity of the objections resulting in the rescinding of the modifications.

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London, N.1

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SOIL STERILIZATION. II.*—Ammonia and Nitrate Production in a Glasshouse Soil Steam-Sterilized *in situ*

By J. N. DAVIES and O. OWEN

Where soil in a glasshouse border was left undisturbed after steam-sterilization, high ammonia concentrations were maintained for a much longer period than where the soil was dug at regular intervals. Depth sampling of undisturbed soil in the house revealed high concentrations of nitrate (and other water-soluble salts) in the top half-inch, with decreasing concentrations at increasing depths, and ammonia was uniformly distributed. This nitrate accumulation is largely due apparently to an upward moisture movement concentrating soluble salts near the surface, and is not to be attributed to renewed nitrification in the surface soil as a consequence of aerial contamination by nitrifying organisms. Evidence is presented which suggests that contamination of undisturbed soil in the glasshouse by nitrifying organisms arises mainly from the subsoil and to a lesser extent from chance surface-infections.

Introduction

It has been shown that where a glasshouse soil is steamed and sampled *in situ* ammonia production is stimulated, increased concentrations are maintained, and no conversion into nitrate occurs for long periods.[1] In contrast, if the steamed soil is removed from the glasshouse immediately after steaming, incubated at 23·5° in the laboratory, and sampled by turning out and mixing, ammonia production reaches a maximum quickly and then falls with concurrent rapid nitrate-production. It was concluded that aeration and contamination by nitrifying organisms were the principal factors contributing to this difference.

The present paper deals with an attempt to simulate in the glasshouse the aeration and mixing inevitable in laboratory sampling.

Experimental methods

In this investigation an old tomato-soil having the following analysis was used:

Total nitrogen (Kjeldahl)	0·35%
Organic carbon (Walkley & Black)	3·18%
Potash (K_2O) (soluble in 0·5N-acetic acid)	0·10%
Phosphoric acid (P_2O_5) (soluble in 0·5N-acetic acid)	0·42%
Total carbonates (as $CaCO_3$)	1·99%

* Part I: *J. Sci. Fd Agric.*, 1951, **2**, 268

This soil has been cropped annually with tomatoes for 27 years and has been steamed frequently. Three adjacent plots were isolated from each other by means of asbestos sheeting buried vertically in the soil to a depth of 15 in. at the time of steaming. One of the plots was left unsteamed, and the other two were steamed *in situ* by the Hoddesdon pipe system.[2] The pipes were buried to a depth of approximately 9 in. and steam was passed for a total time of 20 minutes. Subsequently the unsteamed plot and one of the steamed plots were left completely undisturbed. The other steamed plot was sampled on the second day after steaming and then dug over to a depth of approximately 9 in. This procedure was repeated weekly until the 107th day.

A bulk sample (0–9 in.) of steamed soil was taken shortly after steaming, passed through an $\frac{1}{8}$-in. sieve while still too hot to handle in comfort, and incubated and sampled in the laboratory. Details of the sampling procedures and the determination of ammonia- and nitrate-nitrogen have been described previously.[1]

Determinations of pH were made with a glass electrode in soil suspensions prepared by shaking 24 g. of moist soil with 60 ml. of distilled water for 15 minutes.

Water-soluble salts were estimated in 100-ml. aliquots of 1 : 5 soil : water extracts prepared by shaking in an end-over-end shaker for 1 hour as recommended by Piper.[3]

All concentrations of ammonia- and nitrate-nitrogen, and water-soluble salts are given as parts per million expressed on the basis of oven-dry soil.

Results

Ammonia and nitrate production in the bulk samples of steamed and unsteamed soil incubated in the laboratory at 23·5° are shown in Table I.

Table I

Ammonia and nitrate production in bulk samples of steamed and unsteamed soil incubated at 23·5°

Time in days	Ammonia-nitrogen, p.p.m.		Nitrate-nitrogen, p.p.m.	
	Unsteamed	Steamed	Unsteamed	Steamed
2	2·0	14·2	64·9	80·8
9	1·9	3·8	71·6	100·6
16	1·4	3·1	76·1	105·9
23	1·9	3·5	80·9	106·3

A very short period of time elapsed between steaming and the recommencement of nitrification. Thus, by the 9th day the ammonia concentration in the steamed soil had fallen to a low value and nitrate was 19·8 p.p.m. higher than at the time of steaming.

Results for the steamed plots sampled *in situ* are, in contrast, markedly different. Fig. 1 shows the ammonia and nitrate fluctuations in the steamed undisturbed plot. It will be seen that two days after steaming the ammonia concentration had risen to 18 p.p.m. and increased slowly to 26 p.p.m. by the 37th day. Subsequently, concentrations fluctuated between 25 and 34 p.p.m., and 149 days after steaming ammonia was still high at 25 p.p.m. During this period fluctuations in nitrate concentration were wide, varying between the limits 32 and 92 p.p.m. Even after 10 months a sample from this plot contained 25 p.p.m. of ammonia-nitrogen but another sample taken a few days later contained only a few p.p.m. This is referred to later.

The changes in the steamed dug plot are shown in Fig. 2. Here, after a rapid initial rise ammonia-nitrogen increased steadily to 32 p.p.m. on the 65th day, after which it fell slowly to 3 p.p.m. by the 107th day. During the first 65 days after steaming, nitrates which were low at first increased slowly with only minor fluctuations. Thereafter, as the ammonia concentration fell, nitrate values increased more rapidly, reaching 97 p.p.m. on the 149th day.

Changes in ammonia and nitrate in the unsteamed plot are depicted in Fig. 3. Ammonia concentrations were very low throughout the sampling period, and nitrates fluctuated between the limits of 56 and 124 p.p.m.

Variations in soil pH during the sampling period were small, limits being 7·3 and 7·7 in the unsteamed plot, 7·3 and 7·6 in the steamed plot, and 7·4 and 7·7 in the steamed dug plot; there were no significant trends.

Depth sampling

In view of the possible contamination of the surface soil in the glasshouse by nitrifying organisms, the ammonia and nitrate contents of the surface $\frac{1}{2}$ in. of a small area of the steamed

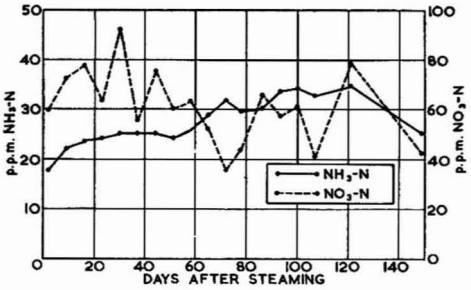

FIG. 1.—*Ammonia and nitrate production in the steamed, undisturbed plot (general samples 0–5 in.)*

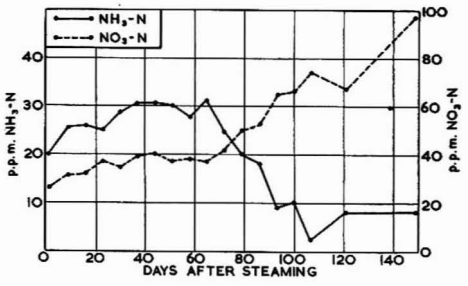

FIG. 2.—*Ammonia and nitrate production in the steamed plot, dug weekly from the 2nd to the 107th day (general samples 0–5 in.)*

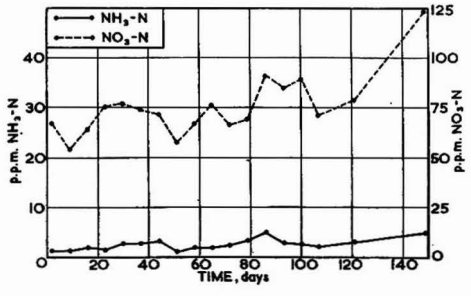

FIG. 3.—*Ammonia and nitrate production in the unsteamed, undisturbed plot (general samples 0–5 in.)*

undisturbed plot were compared with a general sample (0–5 in.) 28 days after steaming. If any surface contamination had occurred it would be expected that the ammonia concentration would be lower in the surface-soil sample.

Table II shows that ammonia-nitrogen was high in both samples. Nitrate-nitrogen was, however, some 90 p.p.m. higher in the surface sample than in the general one. A similar determination 14 days later again showed much higher concentrations of nitrate in the surface layer, and ammonia-nitrogen was equally high in both surface and general samples. Further investigations were begun to determine the reasons for this marked accumulation of nitrate at the surface.

Table II

Ammonia and nitrate concentrations in surface and general samples of the steamed undisturbed plot 28 days after steaming

	Ammonia-nitrogen, p.p.m.	Nitrate-nitrogen, p.p.m.	Moisture, %
Surface ½ in.	33·2	182·1	14·0
General sample 0–5 in.	25·7	92·4	19·9

Forty-eight days after steaming, soil was removed from the steamed undisturbed plot in layers down to a depth of 9 in. Analyses for ammonia- and nitrate-nitrogen and moisture content are shown in Fig. 4. The results show that the nitrate content fell rapidly with increasing distance from the surface in the top 3 in. of soil. Further increase in depth was accompanied by little change in nitrate concentration. Ammonia concentration, on the other hand, was uniformly high. Soil moisture increased progressively with depth, rising from 10·2% in the surface ½ in. to approximately 20% in layers at depths greater than 2 in.

Sampling of the unsteamed plot in depth gave similar results. From Fig. 5 it will be seen that there was a marked nitrate-concentration gradient in the top 2 in. of soil. Below this

depth nitrate decreased slowly with increasing distance from the soil surface. Ammonia concentrations were low at all depths sampled, and again soil moisture increased with increasing depth.

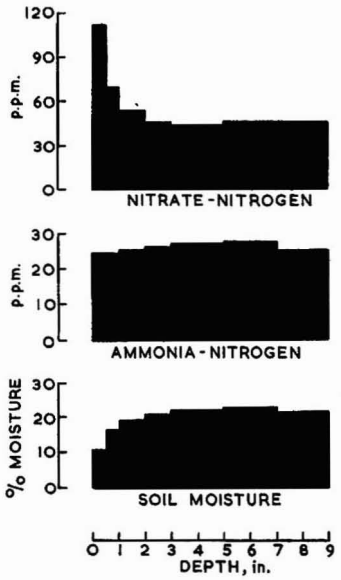

FIG. 4.—*Nitrate- and ammonia-nitrogen and moisture content of soil from increasing depths of the steamed, undisturbed plot 48 days after steaming*

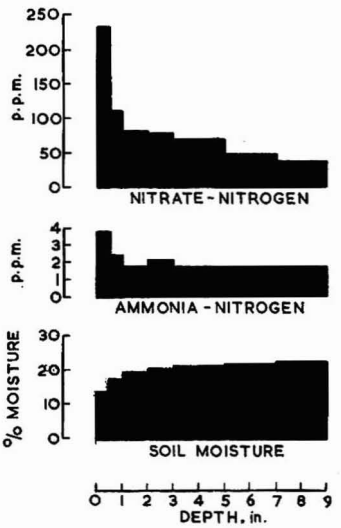

FIG. 5.—*Nitrate- and ammonia-nitrogen and moisture content of soil from the unsteamed, undisturbed plot*

In contrast with the foregoing results for the two undisturbed plots, a depth sampling of the steamed dug plot 51 days after steaming and 7 days after having been dug gave the results shown in Fig. 6. Here the effect of diminishing nitrate-content with increasing depth was not pronounced; there was 33 p.p.m. in the surface ½ in. and 21 p.p.m. in the 7–9-in. layer, and intermediate depths contained approximately 29 p.p.m. As in the steamed undisturbed plot the ammonia concentration was substantially constant at all depths sampled. The moisture gradient was not so pronounced as in the undisturbed plots, being 14·6% in the surface layer and increasing to 21·9% in the 7–9-in. layer.

High nitrate-concentrations in the top ½ in. of the undisturbed steamed plot are not likely to be due to activity by nitrifying organisms in view of the presence of considerable amounts of ammonia and the low moisture content. The explanation of these results may be a physical one. Evaporation of water from a moist soil-surface causes moisture to move upwards from lower levels to replace that lost, and in so doing transports soluble salts concentrating them in the surface layers. Nitrates will thus move freely towards the surface, and ammonia, which is held in the base-exchange complex of the soil, is not mobile to any extent.

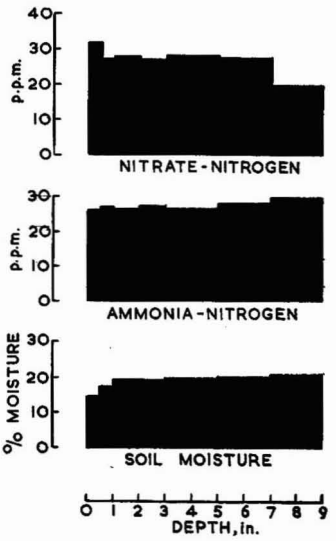

FIG. 6.—*Nitrate- and ammonia-nitrogen and moisture content of soil from increasing depths of the steamed dug plot (one week after being dug)*

Evidence in favour of this explanation was obtained in the following manner. Soil was removed around two 10-in. blocks of soil in the steamed undisturbed plot so that three vertical sides were exposed to the atmosphere. The fourth vertical side was continuous with the main body of the soil. The blocks of soil were left undisturbed for 53 days and then sampled, one in depth as described previously, and the other at increasing distances from the exposed vertical soil surfaces towards the centre of the soil block. The results of analyses for ammonia- and nitrate-nitrogen and moisture content are shown in Table III. It will be seen that nitrates were high near the surfaces from which moisture could evaporate and decreased rapidly with increasing distance from such surfaces. Thus, in the soil block sampled in the normal way the nitrate concentration in the surface ½ in. was 201·2 p.p.m., falling rapidly to about 50 p.p.m. at depths below 1 in.

Table III

Ammonia- and nitrate-nitrogen and soil moisture in two exposed blocks of steamed soil (a) *at increasing distances from the upper surface and* (b) *at increasing distances from the vertical exposed surfaces*

	(a)				(b)		
Depth, in.	Soil moisture, %	Ammonia-nitrogen, p.p.m.	Nitrate-nitrogen, p.p.m.	Distance from vertical exposed surfaces, in.	Soil moisture, %	Ammonia-nitrogen, p.p.m.	Nitrate-nitrogen, p.p.m.
0–½ ..	6·6	28·7	201·2	0–½ ..	11·7	28·6	295·2
½–1 ..	12·6	29·3	92·3	½–1 ..	14·7	27·8	96·0
1–2 ..	15·9	29·6	53·8	1–2 ..	17·5	27·4	70·2
2–3 ..	17·1	29·9	52·1	2–3 ..	18·5	27·4	68·4
3–5 ..	18·0	29·7	50·1	3–4 ..	19·5	26·3	73·0
5–7 ..	19·3	30·0	60·1	Centre ..	20·3	26·4	70·8
7–9 ..	20·3	22·4	53·3				

Nitrification of ammonium sulphate in soil from various depths

Ammonia concentrations in the steamed undisturbed plot were still high 127 days after steaming (Fig. 1), and it was decided to determine the nitrifying capacity of the soil from various depths with ammonium sulphate. In order to reduce contamination risks to a minimum during sampling, soil was removed from the plot in layers, sieved and weighed into flasks on the site. On removal to the laboratory the soil in the flasks was moistened as evenly as possible with 5 ml. of a solution of ammonium sulphate, equivalent to 7·5 mg. of nitrogen. Control samples were moistened with an equal volume of distilled water. The samples were then incubated at 23·5° and duplicate flasks withdrawn for analysis after 2, 5, and 8 days' incubation.

Ammonia and nitrate values for the control samples from different depths moistened with water and incubated are given in Table IV. In Table V data for the percentage recovery of added nitrogen (as nitrate), expressed as difference from control, are given for the various soil layers.

Table IV shows that in every soil layer with the exception of the 0–½ in., sampling, moistening and incubation induced nitrification of the soil ammonia. The rapidity with which the ammonia concentration fell with concomitant nitrate production was most marked in the

Table IV

Ammonia and nitrate production in soil samples from various depths of the steamed undisturbed plot (127 *days after steaming*) *on incubation at* 23·5°

Period of incubation, days	0–½ in.		½–1 in.		1–2 in.		2–3 in.	
	NH_3-N	NO_3-N	NH_3-N	NO_3-N	NH_3-N	NO_3-N	NH_3-N	NO_3-N
0	26·1	1024	23·4	307	24·3	158	27·9	100
2	27·7	1030	23·0	318	22·4	161	26·3	103
5	26·3	1026	2·5	347	3·7	184	12·0	122
8	20·6	1033	2·9	344	3·7	192	3·1	132

Period of incubation, days	3–5 in.		5–7 in.		7–9 in.	
	NH_3-N	NO_3-N	NH_3-N	NO_3-N	NH_3-N	NO_3-N
0	27·9	77	25·2	62	21·0	50
2	27·4	80	26·8	61	18·2	47
5	10·4	92	21·6	67	7·5	66
8	1·6	109	0·9	89	1·2	72

Table V

Percentage nitrification of added ammonium sulphate (expressed as difference from control) in incubated soil samples from increasing depths of the steamed undisturbed plot 127 days after steaming

Depth, in.	0–½	½–1	1–2	2–3	3–5	5–7	7–9
Period of incubation, days							
2	< 1	5·9	2·9	1·2	< 1	< 1	4·3
5	6·5	20·3	14·5	3·9	6·7	6·7	8·0
8	19·1	94·1	92·1	66·7	44·0	19·9	62·5

½–1-, 1–2-, and 7–9-in. layers. Soil from layers 2–7 in. deep was slower in its response, as shown by the figures for 5 days' incubation. After 8 days, ammonia concentrations in all the layers with the exception of the surface ½ in. had fallen to a low level. In the 0–½-in. layer, however, there was only a slight fall in ammonia after 8 days.

Table V shows that there were marked differences in the response of soil from different depths to the addition of ammonium sulphate. Ignoring the 0–½-in. layer, nitrification of added ammonium sulphate was slowest in the 5–7-in. layer and almost complete in the ½–1-in. and 1–2-in. layers after 8 days' incubation. It is of interest that soil from the 7–9-in. layer was capable of more rapid nitrification than the adjacent 5–7-in. layer. The lack of response shown by the soil from the 0–½-in. layer suggests that here the soil was almost sterile as a result of prolonged desiccation.

If the upward movement of soil moisture was responsible for the accumulation of nitrates near the surface, it would be expected that other water-soluble salts would also concentrate in the upper soil layers. This was confirmed by determining total water-soluble salts in the various soil layers at the time of sampling for the nitrification experiment. Detailed results are shown in Table VI.

Table VI

Moisture content, ammonia- and nitrate-nitrogen, water-soluble salts and pH in soil from various depths of the steamed undisturbed plot 127 days after steaming

Depth, in.	Moisture, %	Ammonia-nitrogen, p.p.m.	Nitrate-nitrogen, p.p.m.	Water-soluble salts, p.p.m.	pH
0–½	11·1	26·1	1024	18067	7·36
½–1	15·7	23·4	307	5993	7·48
1–2	17·7	24·3	158	4080	7·53
2–3	19·1	27·9	100	3747	7·57
3–5	20·3	27·9	77	3408	7·56
5–7	21·9	25·2	62	2855	7·49
7–9	22·9	21·0	50	2557	7·50

Concentrations of nitrates and water-soluble salts were very high in the 0–½-in. layer, and decreased with increasing depth. Ammonia was evenly distributed, although the concentration in the 7–9-in. layer was again lower than in the upper layers. Values of pH showed little variation, but soil from the surface ½ in. was less alkaline than the remainder.

A nitrification experiment similar to that described above was carried out with soil from different depths of the unsteamed undisturbed plot. Ammonia and nitrate analyses of control samples after 2, 5, and 8 days' incubation are given in Table VII, and those for samples incubated with added ammonium sulphate in Table VIII. From Table VII it will be seen that moistening and incubating stimulated ammonification in soil from the 0–½-in. layer, ammonia increasing from an initial 8 p.p.m. to 17·1 p.p.m. in 5 days ; by the 8th day it had fallen again to 11·5 p.p.m. Nitrates meanwhile decreased slightly after 2 days, but subsequently increased by 14 p.p.m. by the 8th day. Some nitrification occurred in soil from the deeper layers on incubation, but ammonia was uniformly low. Water-soluble salts were determined in the various layers at the time of sampling, and again were found to be concentrated near the surface. Thus, there was 4849 p.p.m. of water-soluble salts in the top ½ in., decreasing to 2796 p.p.m. in the 7–9-in. layer.

Table VIII shows that, in contrast with the results for the steamed undisturbed plot (Table V), over 90% of the added nitrogen was nitrified after 8 days' incubation in all the soil layers with the exception of the 0–½-in. and ½–1-in. layers. Nitrification in the ½–1-in. layer was a little slower than in the deeper layers, and in the soil from the surface ½ in. only 3·7% of the added nitrogen was recovered as nitrate after 8 days' incubation. This shows that the

Table VII

Ammonia and nitrate production in soil from various depths of the unsteamed undisturbed plot on incubation at 23·5°

Period of incubation, days	0–½ in.		½–1 in.		1–2 in.		2–3 in.	
	NH_3-N	NO_3-N	NH_3-N	NO_3-N	NH_3-N	NO_3-N	NH_3-N	NO_3-N
0	8·1	180·8	2·9	141·5	3·1	75·1	2·7	62·4
2	10·5	179·2	1·8	139·6	0·5	73·8	—	62·3
5	17·1	186·8	—	148·9	0·5	81·4	1·0	63·4
8	11·5	194·8	1·3	152·2	—	82·5	0·2	67·4

Period of incubation, days	3–5 in.		5–7 in.		7–9 in.	
	NH_3-N	NO_3-N	NH_3-N	NO_3-N	NH_3-N	NO_3-N
0	3·3	60·7	1·4	53·1	1·4	39·0
2	—	59·6	1·1	52·5	—	38·1
5	0·9	61·4	—	61·0	—	41·3
8	0·5	64·6	0·8	57·7	—	41·0

Table VIII

Percentage nitrification of added ammonium sulphate (expressed as difference from control) in incubated soil samples from increasing depths of the unsteamed undisturbed plot

Depth, in.	0–½	½–1	1–2	2–3	3–5	5–7	7–9
Period of incubation, days							
2	—	2·0	5·1	1·6	3·9	3·5	1·9
5	—	18·9	30·7	30·9	36·1	36·0	20·8
8	3·7	83·7	93·3	92·0	91·2	92·3	92·4

surface soil of the unsteamed undisturbed plot was, like that of the steamed undisturbed plot, virtually sterile.

When soil in the steamed undisturbed plot was excavated for the nitrification experiment described above, the fresh soil surface at a depth of 9 in. was left exposed to the atmosphere. After 40 days depth-sampling was carried out below this fresh surface. Sampling was continued only for a further 7 in. (i.e. 16 in. below the original soil surface), as at greater depths the gravel in the subsoil became increasingly apparent. Analyses for soil moisture, ammonia- and nitrate-nitrogen, water-soluble salts and pH are given in Table IX.

Table IX

Moisture content, ammonia- and nitrate-nitrogen, water-soluble salts and pH in soil from the undisturbed steamed plot at increasing distances from a fresh soil surface exposed 40 days previously

Depth, in.	Moisture, %	Ammonia-nitrogen, p.p.m.	Nitrate-nitrogen, p.p.m.	Water-soluble salts, p.p.m.	pH
0–½ (9–9½)*	5·4	10·7	234·0	7748	7·41
½–1 (9½–10)	11·3	5·8	62·0	3543	7·61
1–2 (10–11)	15·3	3·3	29·1	2586	7·62
2–3 (11–12)	17·4	1·5	24·2	2017	7·65
3–5 (12–14)	18·0	< 1	25·2	2398	7·62
5–7 (14–16)	18·6	< 1	25·2	2003	7·58

* Figures in parentheses refer to distances from the original soil surface

It will be seen that 40 days after the exposure of the fresh soil surface, water-soluble salts had accumulated in the (new) upper soil layers and decreased with increasing depth. Ammonia-nitrogen also decreased with increasing depth, which is in contrast with the earlier results for the 0–9-in. layers where ammonia was evenly distributed. In addition, the concentrations in soil from depths greater than 10 in. were as low as those found in the unsteamed plot; this suggested that nitrifying organisms were active.

As mentioned earlier, the ammonia concentration in some parts of the steamed, undisturbed plot was still high 10 months after steaming. Accordingly, two further depth-samples were taken from different parts of the plot. Table X shows the ammonia-nitrogen in the various layers.

Table X

Ammonia-nitrogen in soil from increasing depths of two different parts of the steamed, undisturbed plot 10 *months after steaming*

Depth in in.	Ammonia-nitrogen, p.p.m. I	II
0–½	8·6	30·6
½–1	5·7	34·1
1–2	2·4	26·0
2–3	1·0	8·1
3–5	1·7	2·3
5–7	0·6	1·6
7–9	0·9	1·2

It will be seen that in one case ammonia was low throughout the 9 in. sampled, whereas, in the other, ammonia was high in the top 2 or 3 in., but low at greater depths. In both sets of samples, nitrates decreased with increasing depth.

Discussion

It has been shown that nitrification of the ammonia produced as a result of steaming began within a few days in the bulk sample of steamed soil, incubated and sampled in the laboratory. This is in agreement with work reported and discussed previously [1] and will not be further considered here.

Regular turning over of a steamed soil in the glasshouse had the effect of hastening the onset of nitrification when compared with undisturbed steamed soil. This is probably due to two effects: more effective contamination, and improved aeration. When the soil is regularly dug, the soil is well mixed, as is shown by the uniformity of the nitrate concentrations at different depths (Fig. 6) and by the regularity of the nitrate values in general samples (Fig. 2). This is in marked contrast with the results for the undisturbed plots, where nitrate values fluctuated considerably. The regular mixing ensures that any contamination of the surface soil by nitrifying organisms, and also any rising from below, is effectively introduced into the main body of the soil. Here, the moist conditions prevailing are conducive to bacterial multiplication, and, in addition, the improved aeration probably exercises a favourable influence on the spread of the infection. As a result nitrification in the dug plot recommences much earlier than in the undisturbed soil, and ammonia disappears. In this instance, the somewhat slow recolonization of the soil by the appropriate organisms is probably due to the low temperatures obtaining in the glasshouse for the first 90 days after steaming. During this period the moisture content of the dug plot fell only 3·5%, from an initial 21 to 17·5%, whereas, in the following 60 days when the plot was not dug but the glasshouse was heated, the moisture content fell much more rapidly, a value of 12·2% being observed on the 149th day.

In contrast with the dug plot, ammonia concentrations in the steamed undisturbed plot remained high for a long time. With no watering or other disturbance the top few millimetres of soil are air-dry within a few weeks of steaming, and any nitrifying organisms falling on the surface are not likely to proliferate. Any rapid contamination of even the top few inches is thus rendered unlikely. That under these conditions the surface soil does become virtually sterile is confirmed by the observation that soil from the surface ½ in. of both steamed and unsteamed plots was very slow in nitrifying ammonium sulphate. Soil from different depths of the steamed, undisturbed plot 127 days after steaming differed markedly in response to incubation with added nitrogen. Thus, nitrification was slowest in soil from a depth of 3–7 in. Soil from the 7–9-in. layer was more rapid in its response, being similar to that from the 2–3-in. layer. Nitrification of the added nitrogen was almost complete in soil from depths of ½–2 in. In contrast, unsteamed soil samples from depths below 1 in. were capable of nitrifying over 90% of the added nitrogen in 8 days. With the exception of the 0–½-in. layer, nitrification was stimulated by sampling, moistening and incubating soil from all the depths of the steamed undisturbed plot 127 days after steaming. As a result, the ammonia disappeared, but in the plot itself the ammonia level was still high several weeks later.

If nitrification of added ammonium sulphate in an arbitrary incubation period is interpreted as a measure of the extent of contamination by nitrifying organisms, these results suggest that the steamed undisturbed plot was contaminated at depths below ½ in. four months after steaming, but some additional stimulus was necessary for it to become effective. On several occasions ammonia concentrations in the 7–9-in. layer were found to be lower than in the upper layers, and, 127 days after steaming, soil from this layer nitrified ammonium sulphate more rapidly than did soil from the adjacent 5–7-in. layer. Ten months after steaming, ammonia was

high only in the top 2 or 3 in. of the undisturbed plot, lower levels containing only a few p.p.m. When a fresh soil surface 9 in. below the original soil surface was exposed 127 days after steaming and sampled 40 days later, ammonia concentrations decreased with increasing depth. These observations lend support to the view that contamination of undisturbed steamed soil by nitrifying organisms can be introduced from the subsoil. Even though the holes in the steaming pipes are slightly towards the underside, it is probable that when the pipes are buried at a depth of approximately 9 in., some nitrifying organisms below this depth survive the steaming, and subsequently recolonize the steamed soil. Unfortunately, owing to the lack of any microbiological investigations during this study, no positive evidence is available confirming this hypothesis. The results for depth samplings of the steamed undisturbed plot 10 months after steaming show that there was also considerable variation in the extent of the contamination, possibly owing to different degrees of compaction of the soil.

The relationships between soil moisture movement and nitrates and other soluble salts have been studied by many workers. Ammonium ions are adsorbed by the base-exchange complex of the soil and are immobile, but nitrate ions are free to move with any soil moisture movement. Thus, it has been shown, in leaching experiments with different forms of nitrogen, that most of the nitrate was lost whereas nearly all the ammonia was retained.[4, 5] Where water is evaporating from a soil surface a movement of capillary moisture towards the surface takes place, and in so doing concentrates soluble salts in the upper layers. Thus Krantze, Ohlrogge & Scarseth, in a study of the behaviour of nitrate- and ammonia-nitrogen in field soils in relation to rainfall and soil moisture movement, observed that, during prolonged drought, nitrates accumulated at the surface and returned to the root zone after any moderate rainfall.[6] Other investigators[7, 8] have demonstrated nitrate-accumulation in the surface layers of fallow soils during hot, dry periods. Salts have also been shown to concentrate in the outer layer of soil and in the adjacent walls of porous pots, because of the evaporation of water from the surface of the pots.[9]

Results similar to those reported by these workers were obtained in the glasshouse in the course of the present investigations. Depth sampling of undisturbed soil showed an accumulation of soluble salts near the surface. Ammonia, on the other hand, was evenly distributed in the steamed plots. This accumulation of soluble salts was independent of steaming, as it occurred in both steamed and unsteamed plots. With no watering or other disturbance moisture in the soil tends to move towards surfaces from which evaporation can take place, and in so doing concentrates salts near such surfaces.

It is probable that the widely fluctuating nitrate-values in general soil-samples from the glasshouse reported here and previously[1] are due to the inclusion of varying quantities of the surface soil in the samples. Where the soil was dug regularly there was no time for salts to accumulate at the surface to any marked extent in the intervals between sampling. As a result, nitrate concentrations in samples from the dug plot showed a general tendency to increase during the first 60 days after steaming, and only minor fluctuations were observed. If the nitrate concentrations in the steamed undisturbed plot at depths below 2 or 3 in. at different intervals after steaming are compared, there is considerably less variation than in the general samples. Thus, for example, in the 5–7-in. layer on the 48th, 105th and 127th days after steaming there were 46·1, 60·1 and 62·0 p.p.m. respectively, and concentrations in general samples fluctuated between the limits of 33 and 78 p.p.m. These results emphasize the desirability of soil-profile examination in order to avoid misleading results. This point has been recently stressed in connexion with the correct sampling of grassland, where previous treatment may affect the vertical distribution of phosphate and potash.[10]

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