THE FATS: A STORY OF NATURE'S ART*

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The fatty acids that constitute the glycerides of natural fats are, over wide ranges of biological species, remarkably uniform in kind, although the particular acids vary in fats of different biological origin, e.g. those of land flora, those of land fauna and those of aquatic origin. Concurrently with this very marked regularity in qualitative composition, striking although rare exceptions occur, in all sections of natural fats, in which the fat of a single family, and even of a single species of a family, consists largely of an acid which is uniquely different from any of the more prevalent and characteristic acids.

The overwhelming preponderance of natural fats that contain only a few specific mixtures of component acids is contrasted with the much less frequent and apparently erratic instances in which quite exceptional acids are to be observed.

The composition of the mixed glycerides, as shown by many examples in which a natural fat has been studied by the modern method of preliminary segregation into relatively simple mixtures of mixed glycerides by intensive crystallization from solvents at low temperatures, is much more regular. Each acid behaves in exactly the same way and as an individual, with the result that it tends to be distributed as evenly or widely as possible among all the glyceride molecules. Exceptions to this generalization occur only in a very few seed fats, and in certain animal body and milk fats (chiefly those of ruminant animals).

EXPERIMENTAL investigation of the constitution of natural products, of whatever kind, has extended so widely that, in many fields, it is easy to lose sight of broad regularities among the multitude of recorded observations: it is difficult to see the wood for the trees. At the same time, a large number of observations are requisite before it is possible to frame any valid generalizations. These remarks apply cogently to the fats (glycerides) produced in Nature, of which a sufficiently large number have been studied in the past twenty or thirty years to give ample opportunity to consider the various kinds of fatty acids that are produced by different organisms and the manner in which these fatty acids are assembled into the natural triglycerides. It is, however, proposed merely to discuss the similarities and differences that can be seen in the wide range of fats of diverse biological origin which have now been examined by many different workers in all parts of the world, with little or no attempt to explain them. Speculation as to the possible reasons for the presence of any fatty acids or glycerides in particular instances will be found absent or, at least, almost so. Clear insight into the general facts is essential before it is practicable to develop logical theories to explain them, and this paper will give a merely factual account of our present knowledge.

Component acids of natural fats

The kinds and proportions of the various fatty acids that make up the glycerides in a natural fat vary much more widely than the manner in which they are assembled into triglycerides, and this aspect will therefore receive most attention. Broadly speaking—very broadly—there are great similarities in the component acids in fats of related biological origin; and in this way fats of, for example, marine life, land flora, land-animal body fats and animal milk fats are found in any one group to possess many broad similarities, while each group differs widely from the others. Considerable regularity is thus at once evident in the fatty acids of fats of many biological groups, and yet, within any one group, striking exceptions or irregularities are to be found which (although the exceptions are not numerous) often appear extraordinary or, at all events, extraordinarily intriguing. The general picture is one of great regularity in the kinds, and often even in the proportions, of fatty acids present in fats from biologically related groups of plants or animals; but this regularity is broken by the occasional appearance of an unusual acid or acids in the fats of a particular biological family, and, more strikingly, by the quite exceptional irruption of one wholly unusual acid into the fat of a species belonging to a biological group, the fats of which otherwise conform to one of the regular types mentioned.

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Component acids of seed fats.—These dual features of predominant regularity and occasional marked exceptions are best seen, probably, in the seed fats of land vegetation, although they are to be found again in land-animal fats and in fats of aquatic organisms.

It has been estimated[1] that, in about a dozen commercial vegetable fats, oleic and linoleic acid together make up over 60% of the total fatty acids, and palmitic acid about 11%. The fats concerned included, however, a greater proportion of more saturated types than the average; consideration of all the seed-fat analyses now available suggests that oleic and linoleic acid together form at least 80% of all the fatty acids produced in seeds, and palmitic acid probably accounts for perhaps 7 or 8%. This means that all the other natural seed fatty acids—saturated (lauric, myristic, stearic, arachidic) or unsaturated (linolenic, elaeostearic, ricinoleic, erucic, petroselinic and a number of other still rarer acids)—together form only 12–15% at most of the annual world production of seed fatty acids in Nature. In the vast majority of seed fats only linoleic, oleic and palmitic acids are found as major components (and in many instances palmitic acid, although always present, forms less than 10% of the total acids). Fats of this general type are seen in the seeds of many trees and shrubs (e.g. conifers, walnut, beech, oak, hawthorn, *Prunus* spp., and many others), and in those of many of the largest and most familiar herbaceous families, such as Compositae, Labiatae, Papaveraceae, Rosaceae, Euphorbiaceae, Cucurbitaceae, Leguminosae, Cruciferae, Umbelliferae, Gramineae, and very many others. In some families, Malvaceae (including cottonseed) and *Citrus* spp. among others, palmitic acid becomes somewhat more prominent, and in a few tropical families (e.g. Sterculiaceae, Guttiferae, Sapotaceae) stearic acid also accompanies it as a major component. In all these families (of which only a few have been mentioned by name) the same *qualitative* mixture of linoleic, oleic and palmitic acids predominates in the seed fats. The relative proportions of the acids vary considerably from one seed fat to another, or, rather, from the seed fats of one family to those of another: for here again it is notable that, within a given family, the proportions of the three acids in the seed fats show considerable similarity, and their *quantitative* relations are to a considerable extent characteristic for the particular botanical family concerned. As data appear for seed fats of species previously not recorded, the regularity with which the results fall into place in accordance with the plant families is remarkable, and even tends towards monotony.

In contrast with this prevailing regularity we find the occurrence of one or more of the less usual acids. As mentioned above, this happens in two ways: (*a*) another acid (or acids) accompanies the common linoleic–oleic–palmitic mixture in the seed fats not of a solitary species, but of a whole plant family; (*b*) a specific acid appears in the seed fat of a single species (belonging to a family whose other seed fats are all of the conventional type), and usually forms the bulk of the fatty acids present (almost to the exclusion of oleic, linoleic and palmitic acids).

(*a*) *Acids specific to an entire plant family.*—A few instances (by no means complete) will serve to illustrate this type. In seed fats of the palm family, lauric and myristic acid together account for about 70% of the total fatty acids; lauric acid is predominant in many Lauraceae seed fats, and myristic acid in many of those of the Myristicaceae. (In these three families oleic acid sinks to a minor component, and linoleic acid is usually almost absent.) As mentioned above, stearic acid reaches major proportions only in the seed fats of a few tropical families (Sterculiaceae, Guttiferae, Sapotaceae): cacao butter, kokum butter, Borneo tallow, shea butter are well-known instances; in these families, oleic and palmitic acid are also prominent, but linoleic acid is usually a minor component.

In some other families, the usual prominence of linoleic and oleic acids is maintained at a somewhat lower level, but another acid (or acids) appears in major proportions. In the pea family (Leguminosae) arachidic, behenic and/or lignoceric acid are frequently found in significant amounts, and one or other of these occasionally (but rarely) forms over 20% of the seed fatty acids of species in this family or in the Sapindaceae. The two very large families Cruciferae and Umbelliferae are distinguished by the presence in their seed fats of another monoethenoid acid in addition to oleic: Cruciferous seed fats contain variable amounts (up to 40% or more) of erucic (docos-13-enoic) acid and Umbelliferous seed fats similar proportions of petroselinic (octadec-6-enoic) acid, a structural isomer of oleic acid.

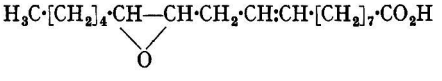
Linolenic acid is a major component of most Labiatae seed fats, and also of linseed (Linaceae); its occurrence elsewhere seems, however, to be limited to particular species or

genera of a family that otherwise belongs to the predominant linoleic–oleic–palmitic category, e.g. hemp seed (*Cannabis*, Moraceae), *Rosa* spp., *Spiraea* spp. (Rosaceae), *Aleurites moluccana*, *Euphorbia calycina*, *E. erythrea*, *Hevea* spp., *Mercurialis* spp., *Sapium* spp., *Tetracarpidium* spp. (Euphorbiaceae).

(*b*) *Acids specific to a single species.*—These are the outstanding exceptions to Nature's general design of seed fatty acids. Unlike the preceding class where an acid other than linoleic, oleic and palmitic accompanies the latter acids as a major component in the seed fats of an entire botanical family, these unusual acids are almost always typical of one or two species only, in whose seed fats, moreover, they form a very large proportion (e.g. 70–90%) of the total fatty acids. It is as though Nature, while on the whole conforming to one well-marked ' plan ' in the best traditions of a ' welfare state ', offers reminders that freedom is retained to do quite otherwise if desired—and for no (at present) apparent reason.

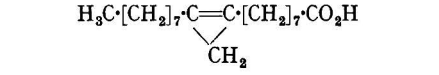
A very interesting case is that of the elm. Whereas the seed fats of most of our familiar trees (e.g. oak, beech, chestnut, pines and firs) are conventionally composed mainly of linoleic and oleic acids, that of the elm contains only about 20% of these two acids, the main constituent being over 60% of the saturated capric (decanoic) acid $C_{10}H_{20}O_2$, with minor amounts of octanoic, lauric and myristic acids.

Again, many seed fats of the large family Compositae which have been examined (e.g. sunflower, safflower, thistles, chicory, lettuce, ragweed) contain only the usual linoleic–oleic–palmitic mixture, yet in a single species (*Vernonia anthelmintica*, an Indian fleabane) the seed fat contains over 70% of an acid which is at present unique, namely 12 : 13-epoxyoctadec-9-enoic acid :[2]



It will be seen that in this acid the epoxy group occupies the same position as the second ethenoid bond in ordinary linoleic acid.

Another most exceptional acid forms over 70% of the seed fatty acids of two species of *Sterculia*. Other species of this genus resemble the cacao plant (which belongs to the same family) in producing fats of fairly high melting point, which have palmitic, stearic and oleic acids as the only major components. The seed fats of *Sterculia foetida* and of *S. parviflora*, however, are liquid oils, which decompose exothermally when heated to about 250°, this being due to the presence of over 70% of a peculiar fatty acid which contains a *cyclo*propene group[3] in place of the ethenoid bond in oleic acid :



This is another unique natural fatty acid, but its saturated analogue has been found in certain bacillus fats[4] (*Lactobacillus*).

On the other hand, a familiar acid, ricinoleic (12-hydroxyoleic) acid, of castor oil, is apparently equally specifically confined to the genus *Ricinus* (Euphorbiaceae), where it forms about 90% of the seed fatty acids ; the corresponding 9-hydroxyoctadec-12-enoic acid has been observed[5] to form up to about 10% of the seed fatty acids of *Strophanthus* spp. (Apocynaceae), but not elsewhere up to the present.

The seed fats of many species of the Rosaceae and Euphorbiaceae families belong to the conventional (usually linoleic-rich) type, but some of their species, on the other hand, ' specialize ' in seed fats that contain some unusually highly unsaturated acids. The familiar conjugated triene elaeostearic acid of tung oils forms 70–80% of the seed fatty acids in four species of *Aleurites* (notably *A. fordii*) and in *Garcia nutans* ; it is present to a less extent (30–50%) in some other Euphorbiates (e.g. *Ricinodendron*) and in some tropical species of the rose family. In the latter, two genera, *Parinarium* and *Licania*, are remarkable. *P. laurinum* seed fat contains, as well as some elaeostearic acid, about 50% of the conjugated tetraene octadeca-9 : 11 : 13 : 15-tetraenoic acid, which has not been observed elsewhere ; but the seed fats of *P. corymbosum*,

P. glaberrimum and *P. sherbroense* include, with some elaeostearic acid, 50–70% of the only natural keto-fatty acid yet noted, 4-ketoelaeostearic or licanic acid. Licanic acid is also prominent (60–70%) in the seed fats of *Licania rigida* (from which it takes its name) and some other species of this genus.

The cyclic chaulmoogric and hydnocarpic acids together form 60–75% of the seed fatty acids of some species of *Hydnocarpus* and other genera of the Flacourtiaceae, but are absent from those of other species of the family, which conform to the usual linoleic–oleic–palmitic type.

Only two other illustrations of acute departure from conventional type in a seed fatty acid will be referred to here. Hexadec-9-enoic or palmitoleic acid is a major constituent in most fats of aquatic origin, is a minor component of many land-animal fats, and it occurs in traces in probably most seed fats. However, in a single instance, the seed fat of an Australian shrub, *Macadamia ternifolia*, it forms as much as 20% of the total fatty acids.[6] The corresponding tetradec-9-enoic (myristoleic) acid is normally absent from seed fats, but in that of a single species (*Pycnanthus kombo*) belonging to the Myristicaceae family (whose seed fats are normally rich in myristic acid) it is present to the extent of 24% of the total fatty acids.[7]

Component acids of land-animal body fats.—The fats laid down for storage by animals are made up partly of fat synthesized by the animal and partly, in many cases, of dietary fat utilized by the animal for its fat reserves after, as a rule, some modification. There is great similarity between most animal depot fats, perhaps the most notable being the relatively large and constant proportions of palmitic acid: this, over a very wide range of species, approximates (within a very few units per cent.) to 30% of palmitic acid in the total fatty acids. In many animal fats the only other major component is oleic acid, but small proportions of myristic, hexadecenoic and linoleic (or other diene C_{18}) acids are often present, and in a few instances the amount of linoleic or hexadecenoic acid has been observed to reach 10% or somewhat higher. Stearic acid is also a minor component (less than 10%) in many animal fats, but in certain groups (notably ruminants and some anatomically related species, and in carnivora that feed on ruminants) the proportion of stearic acid may rise—at the expense of the *oleic* acid content—to as much as 30% of the total acids. The glyceride structure of these 'stearic-rich' fats suggests that they may well result from biohydrogenation of palmito-oleo-*glycerides* already present in the lipids of the animal (see below).

As in seed fats (although the range of animal fats that have been studied is not so great as in the vegetable fats) this marked uniformity is broken by a few outstanding exceptions. The palmitic acid content of the only elephant fat which has been examined was extraordinarily high[8]—45%—but whether this is normal for the elephant or due to some abnormal (pathological) condition remains to be seen. On the other hand there is a well-authenticated peculiarity in the body fats of the horse: the body fats of oxen, sheep, goats, rabbits and other animals feeding on pasture have never been found to contain linolenic acid (and in ruminant species the proportion of linoleic or other diene acid is quite small), but a horse feeding on similar pasture invariably lays down 15–18% of linolenic acid in its body (and also in the mare's milk) fat.[9]

Thus, in land-animal as in vegetable fats, predominating uniformity in general type is relieved by occasional abrupt departure, in one detail or another, from the prevailing regularity.

Component acids of fats of aquatic animals.—Here the characteristic fatty acids differ entirely from those of land flora and fauna. Although oleic acid is still almost always the largest individual contributor to the fatty acid mixture, saturated acids (mainly palmitic) rarely exceed 15–20%, and the rest of the fats consists of a complex mixture of unsaturated acids containing 16–24 carbon atoms in the molecule. The C_{16} acids (mainly monoethenoid) may form from 10 to 20% of the total acids, some polyethenoid (mainly tetraene) C_{18} acids are present, and often fairly large proportions of tetra- and penta-ene C_{20} acids and penta- and hexa-ene C_{22} acids. This general type of fatty acid mixture is characteristic of the liver and body fats of all kinds of fish and also of liver, body and milk fats of marine mammalia such as whales, seals, sea lions, sea elephants etc. The proportions of the various groups of fatty acids vary, however, from one group of aquatic animals to another. For instance, the flesh fats of the herring family are especially rich in polyethenoid C_{20} and C_{22} acids, the liver fats of cartilaginous (Elasmobranch) fish are frequently less unsaturated than those of the Teleostids, and often contain some monoethenoid C_{24} acid, fats of freshwater fish contain more unsaturated C_{18} and C_{16} acids and less

polyethenoid C_{20} and C_{22} acids (the latter often being very small in amount) than those of sea fish, and so on. The large marine mammals also show in their fats characteristic proportions of these various acids, which again differ in detail from those of the various groups of the fish kingdom. Nevertheless, these quantitative differences are all based upon the same qualitative mixture of fatty acids that is so characteristic for fats of aquatic origin.

These regularities, which prevail throughout the fats of aquatic fauna, are known to be subject to the usual striking departures from normality in a few instances. In one Elasmobranch family (Carcharinidae) of tropical sharks [10] the liver fats are unique (for a fish oil) in containing from 40 to 50% of saturated acids, which consist mainly of palmitic and stearic acid in the ratio of about 2 : 1. Even more curious exceptions occur in two families of marine mammals. The toothed or sperm whales (Physeteridae) store their fatty acids mainly in the form of esters of long-chain alcohols (chiefly oleyl and cetyl alcohols), glycerides forming only 25–30% of the body fats; moreover, the fatty acids are peculiar in that the unsaturated members are predominantly monoethenoid, and that saturated acids down to lauric (C_{12}) and capric (C_{10}) accompany the more usual palmitic acid. The fats deposited by dolphins and porpoises (Delphinidae) are perhaps the most exceptional of all, for in addition to the usual higher fatty acids they include substantial amounts of *iso*valeric acid (which is nevertheless in the form of mixed glycerides in which the usual 'aquatic' types of fatty acid are also present): in these marine animals Nature departs from the otherwise almost invariable rule that natural glycerides contain only acids with an even number of carbon atoms in the molecule and a linear, unbranched chain.

Thus it is seen that in each of the three main groups of natural fats the kinds of fatty acids are confined to a relatively small number, the three different types of mixture being qualitatively similar over wide ranges of the respective divisions of aquatic life, land fauna and land flora. Subordinate to these well-marked regularities, however, instances are encountered in each division of more unusual acids that are often common to a whole family or group of related biological species; finally there are a number of natural fatty acids which up to the present have been observed only in single species—and such species usually belong to families whose other members all produce fats of quite normal composition.

It was stated at the outset that no explanation of these curious phenomena would be attempted, nor does it appear profitable to attempt much speculation on this matter at the present time. Yet it is possible to deduce, from consideration of some of the seed fats in which the prevalent mixtures of much linoleic and oleic with but little palmitic and other saturated acids occur, that the unsaturated C_{18} acids are synthesized in the plant quite independently of the saturated acids.[11] The unsaturated acids in these fats belong to one series (C_{18}) only, and in a number of instances (e.g. sunflower, safflower, poppy, tobacco, linseed) it is well established that seeds of these species when grown in cooler climates contain more of the more unsaturated acid, and less oleic acid, than when grown in warmer regions; thus in sunflower-seed oils from temperate regions linoleic acid may form over 70%, and oleic acid only about 15%, of the total acids, whereas from tropical sources linoleic acid may form only 30% or less, and oleic acid about 55%, of the component acids. Moreover, these variations are not accompanied by the production of any extra stearic acid in seed fats grown in the warmer climates; on the contrary, the proportion of saturated acids is actually very slightly greater in the oils produced in colder regions, when linoleic (or, in linseed oil, linolenic) acid is at a maximum. This suggests that the unsaturated C_{18} acids are produced by a mechanism which proceeds from the direction of the more unsaturated towards the monoethenoid oleic acid (the latter being the final stage). The structures of linolenic, linoleic and oleic acids suggest further that C_3 units may well be involved in the biosynthesis.

On the other hand, although palmitic (C_{16}) acid is the predominant saturated acid, it is accompanied by small proportions of myristic (C_{14}) and stearic (C_{18}) acids; in the exceptional cases where another acid (e.g. lauric or stearic) is the major saturated component it is also accompanied by usually smaller amounts of acids with 2 carbon atoms less and with 2 carbon atoms more than the major component. In the Palmae seed fats a range of several saturated acids (from C_8 to C_{18}) is found, with lauric (C_{12}) as the main component. Seed-fat saturated acids may thus well be the result (as was suggested many years ago) of successive condensation of C_2 units; but the older view—unlikely on general grounds—that unsaturated acids were

produced by ' desaturation ' of pre-synthesized stearic acid is finally ruled out by the evidence referred to above.

Component glycerides of natural fats

It has been established that, in the great majority of natural fats, the fatty acids are distributed among the triglyceride molecules in a manner that tends towards the most ' even ' or wide distribution possible, and is far from the ' random ' distribution calculated arithmetically from considerations of probability. This conclusion was reached, from 1927 onwards, as the result of quantitative studies of the fully saturated glycerides present in the more saturated kinds of fat. The chemical isolation and determination of trisaturated glycerides limits experimental study to detailed consideration of the saturated acids present and only permits discussion of the two general types of fatty acid, saturated and unsaturated, without reference to individual unsaturated acids. The results obtained by the chemical method of approach, while, as stated, serving to point to the main features of glycerides structure, have been much extended since it became possible, by the use of appropriate solvents at temperatures down to — 60°, to segregate natural fats (liquid as well as solid) into a series of glyceride fractions sufficiently simple, although not individual, in character for the component glycerides in each fraction to be estimated directly from their fatty acid compositions.

This procedure has permitted the amounts of individual acids (e.g. oleic, linoleic etc.) that are present once, twice, or three times in a triglyceride molecule to be estimated. By plotting the amount of each glyceride type against the proportion of the acid concerned in the total acids of a fat it is therefore now possible to relate this proportion not only to the content of simple triglycerides (e.g. triolein) of the acid but also to the amounts of glycerides containing two (e.g. dioleo-) groups or one (e.g. mono-oleo-) group of the acid in question. Figs. 1, 2 and 3 illustrate results of this procedure for a large number of fats with reference to their contents of

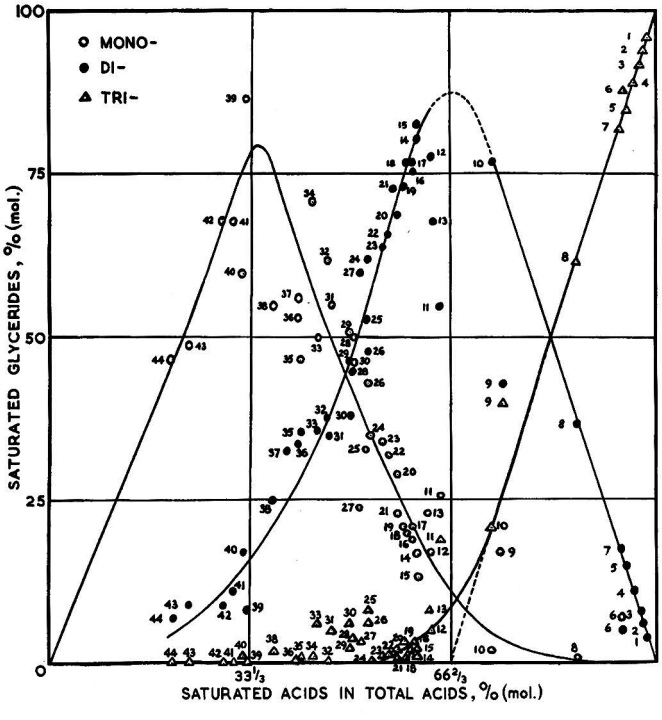


FIG. 1.—*Mono-, di- and tri-saturated glycerides in vegetable fats*

glycerides containing 3, 2 or 1 groups respectively of saturated, oleic or linoleic acids. The graphs in all three cases are of exactly similar form (and are indeed superimposable), and results for other less common acids (e.g. linolenic, elaeostearic, erucic, ricinoleic) which are not illustrated fall on the same general curves. The numbers given beside the points plotted represent individual specific fats so investigated.

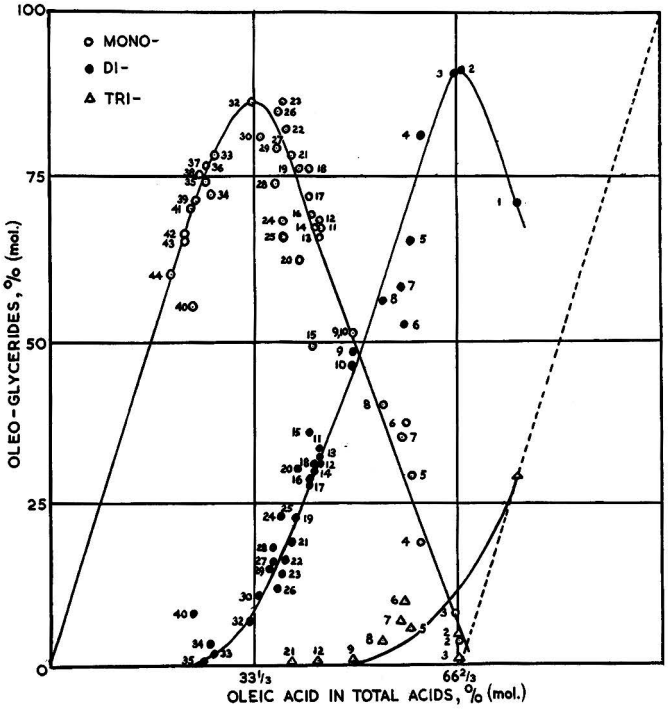


FIG. 2.—*Mono-, di- and tri-oleo-glycerides in vegetable fats*

The proportions of trisaturated glycerides determined by the crystallization method are the same, for any given fat, as those determined by their chemical isolation,[12] and those of simple triglycerides follow the same course, whether the acid is saturated, oleic, linoleic, linolenic, ricinoleic or elaeostearic. The approximate data for glycerides containing *one* or *two* identical acyl groups, made available by the crystallization procedure, enable the rules governing the distribution of fatty acids in natural glycerides to be defined more clearly. It will be seen that in all cases the content of glycerides containing one group of a particular acid reaches a maximum when that acid forms exactly one-third of the total acids; similarly the content of glycerides containing two groups of that acid is a maximum when the acid forms exactly two-thirds of the total acids. At these maxima about 85–90% of the whole fat consists of glycerides with respectively one or two groups of the particular fatty acid; but glycerides containing one group of a particular acid persist in small and diminishing amounts until that acid forms about 75% of the total acids, whereas those containing two groups of the acid begin to appear in small quantities when the acid forms about 20–25% of the total acids. Similarly, simple triglycerides containing three groups of the same acid begin to appear in small proportions when the acid forms from about 60% upwards of the total acids.

This is perhaps as complete a description as can be given of the operation in natural fats of what has come to be called the 'rule of even distribution'. Employment of the crystallization procedure (which has now been applied to many land- and some marine-animal fats as well as to

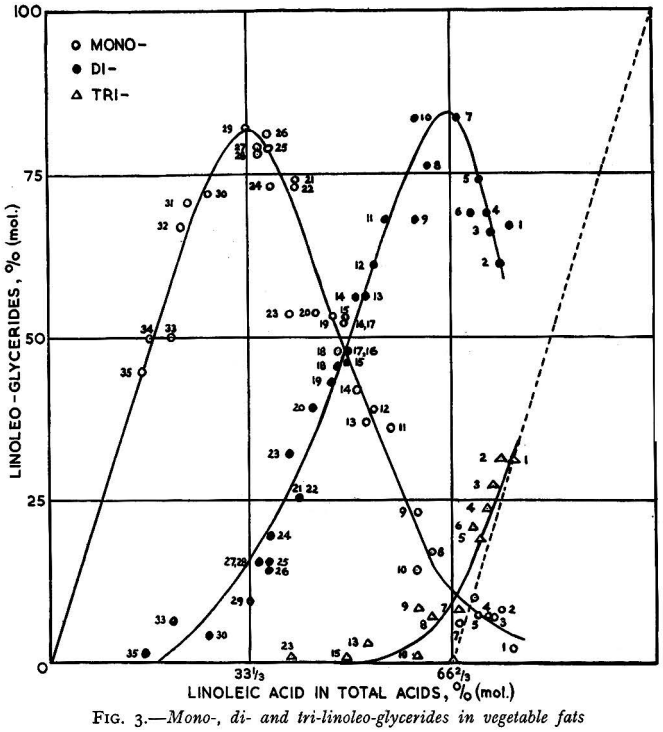


FIG. 3.—*Mono-, di- and tri-linoleo-glycerides in vegetable fats*

vegetable fats) has emphasized that each acid behaves individually in its glyceride distribution and that consequently the study of groups of acids (e.g. saturated, unsaturated) may not always lead to complete knowledge of glyceride structure. Thus in certain fats (e.g. cottonseed oils or palm oils) the presence of small proportions of myristic and stearic acids accompanying relatively large proportions of palmitic acids results in the appearance of significantly greater proportions of ' disaturated ' glycerides than would be expected from the total proportion of saturated acids in the fats; palmitic acid in such cases may contribute one acyl group to most or all of the glyceride molecules, but occasionally a stearo or myristo group will also be found in the same molecule.

In glyceride structure, as in the occurrence of specific component fatty acids, apparent exceptions to the general statements given above are to be observed, but such irregularities seem to be less erratic, or more explicable, in the case of glyceride structure. Three main kinds of exception have been noted:

(i) In a very few seed fats the presence of much more of a simple triglyceride has been reported than is consistent with the proportion of the fatty acid concerned in the total fatty acids. Instances are: trilaurin in laurel oil, trimyristin in the oils of *Myristica malabarica* and *Pycnanthus kombo*, tripalmitin in bacury fat, and triparinarin in *Parinarium laurinum*. In all these cases the abnormality is confined to the appearance of the simple triglyceride mentioned, the rest of the fats consisting of mixtures of mixed glycerides assembled on the usual pattern; it would therefore be explicable if, at some stage, the acid in question had been synthesized in the seed in much greater proportion than its companions. There is of course no evidence on this point; but conversely and somewhat strikingly, it is clear that the evenly distributed glyceride structure so widely observed in natural fats could hardly obtain unless, within fairly narrow limits, a very similar mixture of fatty acids was being synthesized by the organism throughout the entire development of the total fat.

The other marked divergence from ' even distribution ' is seen in the depot fats and milk fats of ruminant land animals.

(ii) *Ruminant depot fats.*—These belong to the ' stearic-rich ' group of animal body fats in which stearic may form 20–25% or more of the total acids (in addition to about 30% of palmitic acid), and their content of trisaturated glycerides is abnormally high for the total amount of saturated acids present. The amounts of trisaturated glycerides and their composition (palmito-stearins) are consistent with a ' random ' biohydrogenation of preformed ' evenly-distributed ' palmito-oleo-glycerides having taken place during the elaboration of these particular fats in the animal. The corresponding graph (Fig. 4) for the glycerides of animal body fats containing *one, two* or *three* groups of saturated acids supports this hypothesis in that, for saturated acid contents of less than 30% (non-stearic-rich fats), the curves for *mono-* and *di-*saturated glycerides follow those in Figs. 1, 2 and 3, whereas there is little or no regularity when stearic acid is present in major proportions.

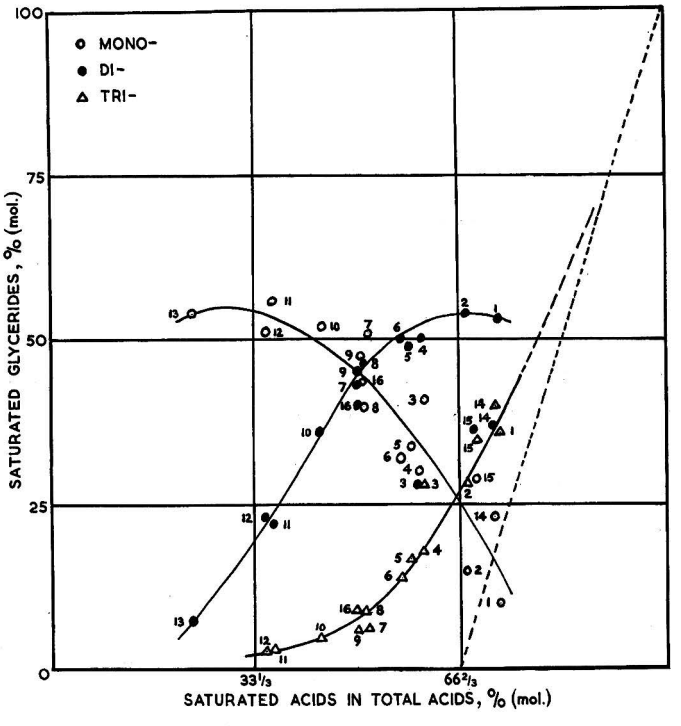


FIG. 4.—*Mono-, di- and tri-saturated glycerides in animal fats*

(iii) *Ruminant milk fats.*—These also are characterized by unusually high proportions of trisaturated glycerides, whereas their component acids are distinguished by the presence of lower saturated fatty acids (butyric to decanoic forming together about 20 out of 100 molecules of total fatty acids). These acids have been shown[13] to be synthesized in the lactating gland of the ruminant, and the typical structure of these glycerides can be satisfactorily explained[14] by acyl interchange on a random basis of the synthesized lower fatty acids with oleo groups in preformed ' evenly-distributed ' palmito-oleo-glycerides entering the lactating gland of the ruminant from the blood stream.

On the whole, therefore, the mode of assembly of constituent fatty acids into natural fat glycerides is more uniform and less subject to exceptional circumstances than the occurrence of

specific fatty acids in some divisions of natural fats, and the divergences from the general pattern in glyceride structure are perhaps more easily explained than the appearance of some of the less common fatty acids.

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POST-MORTEM CHANGES IN THE LENSES OF FISH EYES: ASSESSMENT OF STORAGE TIME AND FISH QUALITY

By R. M. LOVE

The eye lenses of fish stored for increasing lengths of time in ice become progressively turbid. The cause of this turbidity is shown to be the diffusion of water through the corneas into the intra-ocular fluid, followed by gradual hydration of the lens. In iced fish kept in a room of fairly constant air temperature, it was found possible by examination of 20 or more lenses to assess the storage time with an error of not more than 1 day. The limitations of the method are discussed.

It is well known that the appearance of the eyes of white fish after storage in ice is some guide in assessing the degree of spoilage.[1] Freshly caught fish normally have clear black pupils, but fish that have been kept for a few days in ice show varying degrees of milkiness.

It was the object of the present work to examine the changes in appearance of the eyes of white fish, and investigate the possibility of basing upon them a rapid objective method for assessing storage time and fish quality, since the need for such a method has not yet been met in an entirely satisfactory manner.

Experimental

Preliminary observations

The turbidity seen in the pupil of cod and haddock eyes was found to be in the crystalline lens. The following observations were made on lenses from the eyes of cod and haddock kept for varying lengths of time in ice: (1) The turbidity was essentially a surface effect, so that the lenses, especially in the early stages, appeared to be whiter round the edge than in the centre. (2) Differences in turbidity were much more easily detected when the lenses were observed against

a dark background after immersion in liquid, e.g. physiological saline, than if they were examined dry on the bench. (3) A dense patch of whiteness often formed on the lens, covering up to half the surface area. This patch was much more opaque than the rest of the lens, and was always located at the point nearest to the cornea. It is this patch, therefore, that an observer sees when examining intact eyes. A further property of the patch was a lower specific gravity than the rest of the lens, so that a patchy lens placed in saline always orientated itself with patch uppermost. (4) As the fish were kept longer in ice, so the size and the fragility of the lens increased. Fish lenses are spherical, and are made up of a hard medulla surrounded by a much softer cortex. It was the cortex that swelled and in which all the turbidity changes described took place. With fish of 12 or more days in ice, care was needed to excise the lens without bursting it. A burst lens was useless for examination.

Materials and method

Owing to the differing extent of the patchiness and size of the lenses, it was concluded that the method likely to give the best results would be a visual comparison of the lenses with a set of artificial standards of graded cloudiness.

A number of glass bulbs about the size of fresh cod lenses were accordingly made from 4-mm. (outside diameter) glass tubing, and filled with 'light' magnesium carbonate suspended in 0·3% agar dissolved in 50–50% glycerol–water. The glycerol–water mixture was found by experiment to be excellent for this purpose, since the agar gel dissolved in it was much clearer on setting than an agar–water gel, and also it prevented the magnesium carbonate from flocculating. The series (Fig. 1) consisted of 22 standards and the amount of magnesium carbonate in

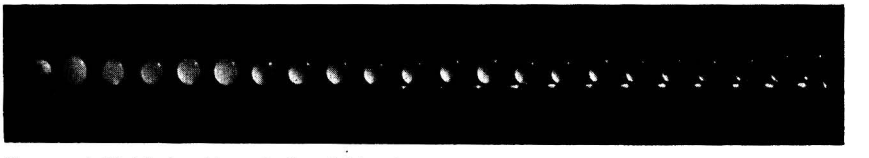

FIG. 1.—*Artificial glass 'lenses' of graded opacity, against which the haddock or cod lenses are matched*

each (Table I) was such that an observer could detect a definite difference in the turbidity of two adjacent standards placed side by side.

The artificial lenses, shown mounted in a wooden stand in Fig. 1, were placed in a row in a wide Petri dish on a dark background, and covered with physiological saline. The fish eyelids were cut away with fine-pointed scissors and forceps and a semi-circular cut was made in the side of the eye-ball, care being taken not to puncture the lens, which was pushed out through the incision. The ring of vitreous humour adhering to the lens was then cut away, and the lens placed in the dish of saline with the standards. If a patch of intense whiteness was present, the lens was turned with wide-ended forceps until the clearer part was visible, and it was firmly held in this position throughout the matching up, which was done in good diffused daylight, and, in cases of uneven cloudiness, through half-closed eyes. Half units were recognized, i.e. opacities which appeared to be halfway between adjacent standards, and good agreement was almost always reached between independent observers.

Table I

Weight of magnesium carbonate suspended in 5 ml. of 0·3% agar in artificial fish-eye lenses of standard turbidity

Lens. no	Wt. of $MgCO_3$, mg.	Lens no.	Wt. of $MgCO_3$, mg.	Lens no.	Wt. of $MgCO_3$, mg.
1	100·0	8	15·0	15	5·0
2	50·0	9	12·5	16	4·0
3	40·0	10	10·0	17	3·0
4	30·0	11	9·0	18	2·0
5	25·0	12	8·0	19	1·5
6	20·0	13	7·0	20	1·0
7	17·5	14	6·0	21	0·0

Lens 21 contained 0·3% agar only; Lens 22 contained glycerol only.

Results

All data, unless otherwise stated, refer to haddock eyes. From some limited data, cod lenses appear to behave in an identical way.

Increase in turbidity with storage in ice

Haddock of various sizes were packed haphazardly in crushed ice in standard fish boxes, which were kept in an insulated container. The air temperature inside the latter was usually about 4°, but it was to some extent influenced by the atmospheric air temperature.

The results of the examination of over 500 haddock are summarized in Fig. 2, and standard deviations are shown in Fig. 3, the scale used being the arbitrary numbers assigned to the set of standards (Table I). It will be noted that the results are linear over the whole range studied, in contrast e.g. with the spoilage phenomena caused by bacterial action. There was as much

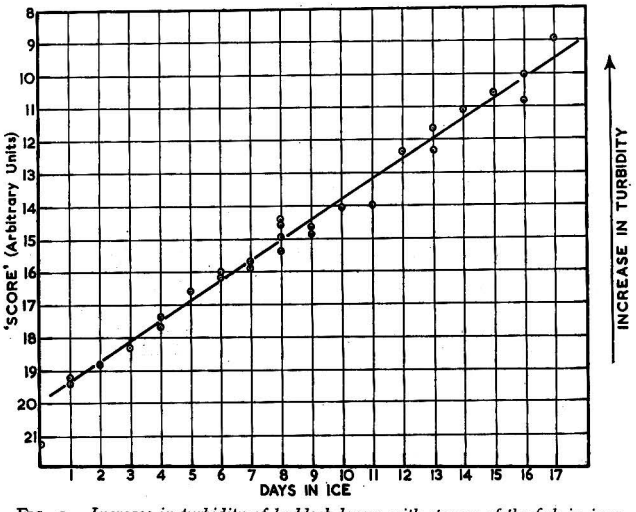

FIG. 2.—*Increase in turbidity of haddock lenses with storage of the fish in ice; each point is the mean of 30–40 lenses.*

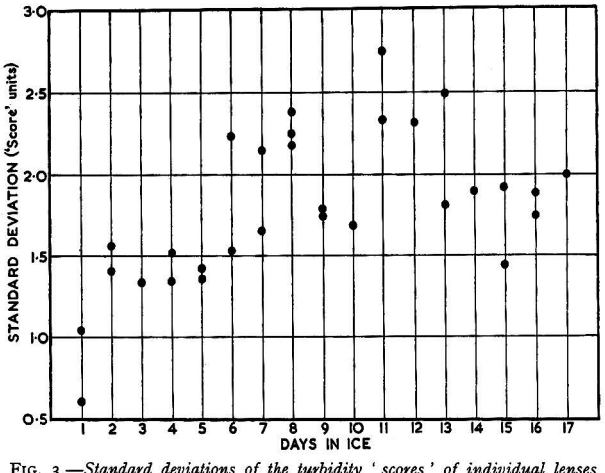

FIG. 3.—*Standard deviations of the turbidity 'scores' of individual lenses after different storage times*

variation between the two lenses of a single fish as there was between lenses selected at random from a batch.

Estimation of storage time

Three groups of 12 haddock stored in the insulated container for different lengths of time were examined under code. The lenses were removed and matched in the usual way, and the number of days in ice was calculated with the average ' score ' from the standard curve (Fig. 2) with the following results :

Group 1 : average score, 16·17 (standard deviation σ, 2·29) ; estimated storage time, 6 days ; actual time, revealed afterwards, 6 days
Group 2 : average score, 16·59 (σ, 1·36) ; estimated time, 5 days ; actual time, 5 days
Group 3 : average score, 15·74 (σ, 2·14) ; estimated time, 7 days ; actual time, 7 days

The fine degree of differentiation will be readily appreciated. In this case the results are exactly right in all groups, but it can be seen from the points on Fig. 2 that an error of 1 day can be made under these conditions. It was noted that the entire operation of dissection and matching of each group of 12 fish took about 20 minutes.

Changes in lenses suspended in different media

Fresh haddock lenses were suspended in (*a*) distilled water, (*b*) physiological saline (0·9% w/v NaCl), (*c*) sea-water, (*d*) absolute alcohol, and examined 10 minutes afterwards. Sea-water and alcohol caused considerable shrinkage and formation of a dense white material on the surface. Physiological saline caused practically no change, but in distilled water the lenses became turbid and similar in appearance to the lenses taken from iced fish. It was observed that the lenses were appreciably cloudy after 10 minutes in sodium chloride solutions of 1·1% concentration or above, or 0·5% or below, but remained virtually unchanged between the concentrations of 0·6 and 1·0% inclusive.

Fifteen haddock which had been stored for 24 hours in ice were used for the next experiment. Five fish were immersed in saline, distilled water and sea-water respectively for 48 hours at 0°.

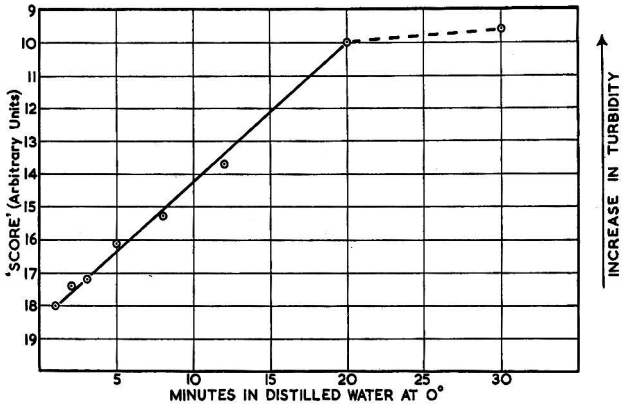

FIG. 4.—*Increase in turbidity shown by fresh lenses placed in distilled water at* 0° ; *each point is the mean of* 5 *lenses.*

The lenses were then dissected out and matched with the standards in the normal way. The resulting ' scores ', each the average of 10 lenses, were : saline, 16·25 (σ, 0·45) ; distilled water, 13·25 (σ, 0·25) ; sea-water, 1·0–2·0 (much shrunk). When these results are compared with those in Figs. 2 and 3, it will be seen that the lenses become more cloudy in fish kept in water at 0° than in fish packed in ice for a similar time, and also that the standard deviation among individual lenses is much lower.

As a final experiment under this heading, the rate of development of cloudiness in lenses

placed in distilled water at 0° was studied. Lenses from haddock stored for 24 hours in ice were used. Results are shown in Fig. 4. The general picture is the same as in iced whole fish, but the process is much more rapid. Tests showed that turbidity initiated by suspension of fish lenses in water could be arrested by transferring them to normal saline. It was never found possible to restore the clarity of lenses which had become turbid.

Water content of lenses

Lenses, from cod stored in ice in the insulated container, were placed in tared basins and dried to constant weight at 100°. Results are summarized in Table II.

Table II

Hydration of cod lenses with storage of whole fish in ice

No. of days in ice	No. of lenses used	Average wt. of one lens, g.	Water content, %
1	10	0·469	51·0
2	8	0·401	53·9
7	6	0·370	54·8
10	3	0·547	56·2
14	4	0·618	57·6
18	4	0·527	60·5
22	1	0·701	65·1

The inconsistent results for the average weight of a lens were due to a large variation in the sizes of the fish. The effect would have been lessened by taking a large number of eyes. However, the steady increase in water content as the fish are stored in ice is convincingly shown, confirming that the progressive turbidity observed is related to water uptake.

Effect of air temperature on fish stored in ice

It seems certain that the opacity which develops in the lenses of iced fish is caused by the action of water derived from the melting ice. The rate of melting of ice and thus the flow of water over the fish should therefore affect the rate at which opacity develops.

This factor was checked by holding well-iced fish at different air temperatures. A batch of haddocks, caught the same day, was divided into four lots, each comprising 30 fish, which were packed into standard wooden fish boxes with a large excess of ice. The boxes had adequate spacing of the bottom boards to allow melted ice to drain away. Lot 1 was placed in a room at 0°, lot 2 in a room at 2·5°, lot 3 in an insulated container with an internal temperature of 3–7°, except for two days at 1° during frosty weather, and lot 4 was kept in a shed at 9–16°. The ice level in the boxes was checked daily and the boxes were ' topped up ' when necessary.

After 4, 8 and 12 days, 10 fish were removed from each box, and the lenses matched against the standards. The results are shown in Fig. 5. The frosty weather occurred on the tenth and eleventh days, and the slowing effect on the turbidity developed in fish stored in the insulated container can be plainly seen in the curve marked with triangles.

The water contents of lenses from fish stored for 12 days in ice were found to be 60·5, 64·8, 67·5 and 72·2% respectively, for lots 1, 2, 3 and 4. Twenty lenses were used in each determination.

These results indicate clearly enough, despite irregularity of temperature control, that the ambient temperature has, as expected, a marked effect on the development of opacity in the eye lenses of well-iced fish ; the higher the temperature, the quicker opacity develops, with corresponding increased absorption of water.

Effect of freezing

Although this paper is concerned with fish stored in ice, incidental mention may be made of the observation that when fish were actually frozen and then thawed, the lenses presented a different appearance : in addition to more or less cloudiness in the lens cortex, the medulla became denatured also, and showed through as a dense white sphere. In a group of cod of previous history unknown to the author, it was possible within a few seconds to demonstrate correctly the ones that had been frozen and those that had been merely kept in ice.

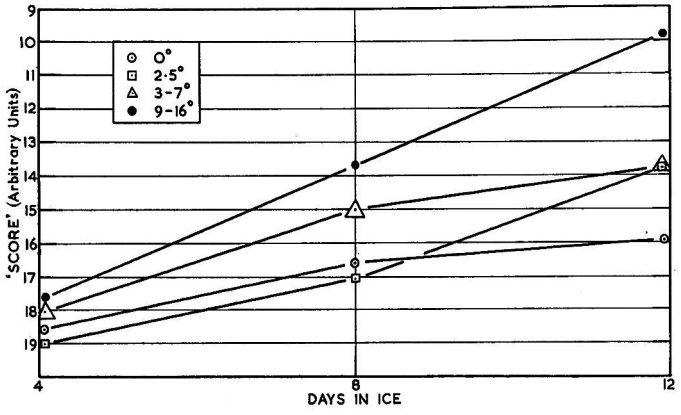

FIG. 5.—*Development of turbidity in lenses of iced haddock stored at different air temperatures*

Discussion

It has previously been reported that concentrated salt solutions shrink and cloud eye lenses. Bellows & Chinn[2] were, in fact, able to produce cataracts and shrinkage in the eyes of living dogs and rats by continuous intravenous injection of strong salt solutions for prolonged periods. Fischer[3] reported that electrolytes inhibited the swelling of lenses that had been induced by solutions of non-electrolytes, although, contrary to the results described here, there was no relation between swelling and transparency.

A relation between cloudiness and water content was shown by Salit,[4] who found that normal and ' highly sclerosed ' human lenses contained respectively 67·6 and 75·4% of water. This is in agreement with the results in Table II, although the lens of a fresh fish has a lower water content than that of a human lens.

It has been shown in the present paper that when water is accessible to fish's eyes or directly to their lenses, the lenses absorb water and develop an opacity that increases with the amount of water absorbed. Maximum access of water through direct immersion of the actual lens speeds up development of opacity enormously, as compared with immersion of the fish itself. Stowage of the fish in ice affords considerably less and more variable access of water, and therefore a correspondingly slower and more variable development of opacity in the lenses, than immersion of the fish itself. The extent of ice-melting, which is dependent on heat uptake by the ice from the fish before it is cooled, and from the surroundings all the time, has been shown to be a major factor here. The fact that the opacity ' scores ' for fish submerged in water showed far less variation from the mean than those for iced fish indicates also that lack of uniformity in the relative disposition of fish and ice throughout the period of storage is also important. Thus some eyes will be longer in actual contact with ice than others, and the amount of water flowing over an eye will depend on the orientation of the fish and on the proximity of the neighbouring fish. Also, the difference usually found between the ' scores ' of the two lenses of the same fish is very significant.

The results in Fig. 2 show that there is fortunately a linear relationship between development of lens opacity measured on the arbitrarily numbered scale of turbidities and time of storage (under standard conditions). Thus an observer can distinguish 2-day from 4-day iced fish as reliably as, say, 12-day from 14-day iced fish. It will be noted that the whole range of the results is between artificial lenses 9 and 19, where, with the exception of the two extremes, the progression of concentrations of $MgCO_3$ in the lenses is arithmetic (Table I).

Had the data for Fig. 2 been obtained from fish stored under more rigidly standardized conditions, e.g. in a constant-temperature room instead of an insulated ice chest, the points on the curve would undoubtedly have been nearer linearity. To emphasize this, it was found that

the low values on the eleventh and sixteenth days were obtained after preceding frosty days, whereas the high value at 8 days occurred after a day of mild, humid weather.

In Fig. 3 no attempt was made to draw a line through the standard deviations, but there seems to be a tendency towards a greater error in the middle of the range (6–13 days). The deviation falls again in the 14–17-day section. This may perhaps be explained by the fact that the eye of the observer is less sensitive to changes in opacity in the very opaque lenses, so that a number which in fact differ in opacity may look the same and be given the same ' score '.

In Fig. 2 each point is the mean of from 30 to 40 lenses (from 15 to 20 fish) and scatter due to individual variation is then quite small, e.g. the average ' scores ' from two identically treated groups of 18 fish from the same box differed by only 0·15 unit. From all the evidence it is concluded that as a practical procedure it should be possible, by using samples of, say, 20–30 lenses, to estimate in the course of about 20 minutes the time fish (e.g. haddock or cod) have been stored in ice *under given standard conditions* with an error of not more than 1 day over the range 0–17 days. An advantage of this method is that since only the eyes have to be removed in sampling, the fish remains salable as headed, dressed fish or as fillets.

Clearly this accuracy of assessment could not be assumed to be applicable where fish had not been kept all the time in ice under the standard conditions. For instance, bulk stowage of fish with ice on a trawler may result in deviation from these conditions of box stowage in an insulated room. In non-insulated trawler holds it has been observed that fish stowed next to the side of the ship may become completely denuded of ice during the last few days of the trip, rise in temperature by as much as 8° and, of course, deteriorate correspondingly in quality, as compared with fish of the same haul stowed further inboard, that remain well-iced and cool all the time.[5] The former conditions may well considerably affect the rate of development of opacity in the eye lenses, and hence the accuracy of assessing duration of storage.

More work then requires to be done in examining the effects of aberrations from chosen standard conditions. If the latter are chosen to be as nearly as possible representative of average commercial conditions, the method of estimating the duration of storage in ice by measuring lens opacity may prove to have considerable value both in research and in practical inspection of market fish.

The method would, however, exhibit its fullest usefulness if it could be shown that it provides a consistently good objective index of ' freshness ' or general quality, as organoleptically assessed, and this remains to be investigated. Although it is known that fish of any given species can deteriorate in quality at a variable rate under identical conditions of storage in ice, according to such factors as size, fishing ground, season etc., it is nevertheless possible that measurement of lens opacity may afford a reasonably useful objective index of fish quality.

Manipulation and matching of the lenses may present difficulty at first and have to be done in good diffused daylight. The essential requirement, i.e. to match at least 20 lenses for each estimation, is still rather tedious for routine work. It is hoped to eliminate these disadvantages by provision of a photoelectric device designed to measure the light-scattering of 20–25 lenses in a flat glass cell. This work is in its initial stages.

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BEEF-CURING BRINES. I.—Bacterial and Chemical Changes Occurring in Rapidly Developing, Short-life Brines

By H. C. HORNSEY and J. H. MALLOWS

The changes taking place in this type of brine are shown to be: an increase of bacterial population (almost entirely a halophilic Gram-negative rod of the *Achromobacter* species), with a consequential lowering of the oxidation/reduction potential, hence a reduction of nitrate to nitrite, and ultimately, when the potential is low enough, to gaseous nitrogen and free alkali, resulting in an increased pH. This reduction occurs at the expense of the lactic acid derived from the meat, which is oxidized to carbon dioxide.

When the lactic acid has been used up, the large bacterial population, at the low potential and high pH then reached, produces 'off' odours by the decarboxylation of amino-acids.

No evidence of protein breakdown has been found.

Introduction

Haldane[1] first showed that the characteristic colour of cured meat was due to nitroso-haemoglobin, formed by combination of the haemoglobin (and myoglobin) with nitrite. Very little nitrite is actually needed for complete combination with all the available haemoglobin and myoglobin. Brooks *et al.*[2] have shown that 10 p.p.m. of sodium nitrite is sufficient theoretically for pork; beef, being richer in the muscle pigments, would presumably need about 20 p.p.m. for complete conversion.

It is well known that the nitrite in curing brines arises from the bacterial reduction of the added potassium nitrate. Ingram *et al.*[3] found a general connexion between the salt concentration and nitrite development in bacon-curing brines. The disappearance of nitrite that sometimes occurs was suggested by Brooks *et al.*[2] to be a re-oxidation to nitrate. Ingram[3] has suggested that possibly three mechanisms are concerned, i.e. absorption into the meat, oxidation by air, and further bacterial reduction. Little appears to have been published, however, on changes in other constituents of brine.

The bacterial flora of brines is large and variable. Brooks *et al.*[2] found (by direct microscopic count) that 10^7–10^8 cells/ml. were present in bacon-curing brines, but only 2% of these were viable under the best conditions of cultivation and dilution. They assumed that all the larger groups of organisms present were represented to some extent in the 2% that were viable on the plates.

Lochhead,[4] working with bacon pickles from one packing plant, found that the ratios of bacterial counts on nutrient agar containing 15, 10, 5 and 0% of sodium chloride, incubated at 20°, were 44·5, 100, 52·3 and 5·7 respectively. If the nutrient agar was incubated at 37°, only 1·3% of the count on 10%-salt agar at 20° was obtained. Gibbons[5] examined pickles from 16 plants and found that the ratios of the bacterial counts on media containing 10, 4 and 0% of sodium chloride at 20°, and 0% of sodium chloride at 37°, were 100, 50, 10 and 2·5 respectively.

Most interest has been attached to the curing of pork, i.e. bacon and ham, and not to beef. Many butchers, however, are concerned with the small-scale curing of beef joints and, as this was our main interest, it was decided to investigate beef brines. Many hundreds of samples of commercial brines, taken from small 9-gal. curing tanks at retail butchery branches, have been examined during the last few years. These could be broadly classified into two types: (1) short-life, good curing brines of bright colour, with high bacterial counts and fairly high pH values; (2) long-life brines, sometimes as much as three or four years old, of acid reaction, containing little or no nitrite, and with apparently low viable counts.

In order to explain and evaluate their condition, some experimental brines were made and the changes taking place in them were observed by regular and full analyses.

Experimental

Five experimental brines were made up in glass tanks (internal dimensions 15 in. × 8 in. × 6½ in.) as follows: 1790 g. of coarse salt (approx. 97% pure), 53 g. of commercial fused potassium nitrate (KNO_2 < 100 p.p.m.), 21 g. of mixed spice in a muslin bag, 21 g. of brown sugar and 1·5 gal. of tap-water.

After the composition of these brines had been checked by analysis, one piece of lean silverside, free from fatty tissue and about 4 lb. in weight, was completely immersed in each brine, and held submerged by a glass arm for a period of 4 days. The meat was then removed, and drained for 2 hours back into the tank. The brines were stirred and samples withdrawn for analysis, fresh meat was introduced and the cycle repeated. In all, ten or more batches of meat were cured in each brine, at the rate of one each week, until 'off' odours made the brines unusable.

The leanness of the meat and the comparatively small surface area of the brines would both tend to exaggerate the normal chemical and bacteriological changes, as indeed has been shown by their comparatively short life. It was thought, however, that this acceleration might emphasize the changes taking place, and perhaps show some effects which, though important, were too small (under normal conditions) to be very obvious.

The temperature of the brines remained reasonably constant over the whole 10-week period, i.e. 9–11°.

The readjustment of salt and nitrate was made after each cure by adding the requisite quantity of solids, as calculated from the analytical results, and stirring periodically until dissolved.

The extra liquid introduced by the meat compensated for the amount (approx. 100 ml.) of sample withdrawn each time for analysis. The volume of the brines therefore remained almost unaltered throughout the experiment.

Frothing became evident in each brine after a time, followed by reddening and some precipitation of organic matter. This stage was reached in brines I–V during the 12th, 9th, 7th, 6th and 6th cures respectively.

No special aseptic precautions were taken, although general hygiene was observed throughout when handling meat or sampling the brines. Hands were washed in soap and water, tanks were loosely covered with glass plates, glass submersion arms and stirring rods were well washed before use. The meat was not washed in any way and, although some atmospheric or handling contamination could have taken place, the main source of the bacterial flora in the brines was probably derived from the surface of the meat.

The brine samples were examined after each cure for: viable count, specific gravity, salt, nitrite, nitrate, pH, oxidation/reduction potential, heat-coagulable protein, total amino-nitrogen, ammonia-nitrogen, titratable acidity (or alkalinity) after heat clearing, and total lactate content in some later samples.

Analytical methods

(1) *Specific gravity* was determined at 20° in a Westphal balance.

(2) *Salt* was determined by direct titration with silver nitrate solution. Results were expressed as per cent. (w/v) of sodium chloride.

(3) *Nitrite* was determined colorimetrically by using Griess–Ilosvay reagent. Results were expressed as p.p.m. of sodium nitrite.

(4) *Nitrate.*—The combined nitrate- and nitrite-nitrogen was determined on the cleared solution by reduction with Devarda's alloy, followed by distillation and volumetric determination of the resultant ammonia. Results were expressed as nitrogen. Nitrite-nitrogen as separately determined was deducted, and the final results were expressed as per cent. (w/v) of potassium nitrate. These values were not corrected for the relatively small amounts of free ammonia present.

(5) *pH.*—The pH values were determined electrometrically at 20° by using a glass electrode in combination with a calomel half-cell and 3·5M-potassium chloride solution with a Muirhead type-D4.7 potentiometer.

(6) *Oxidation/reduction potential.*—The oxidation/reduction potential was determined electrometrically at 20°, as above, by using a platinum electrode in combination with the calomel half-cell and 3·5M-potassium chloride solution. The value due to the calomel half-cell, 0·255 v, was added to all readings. The platinum electrode was lightly rubbed with sand before each reading. Although steady readings were usually obtained after a few seconds, the very low values usually needed up to 10 minutes before a constant reading was obtained.

(7) *Heat-coagulable protein.*—Brine (2 ml.) was pipetted into a micro-Kjeldahl digestion flask, which was then immersed in a boiling-water bath for 5 minutes. Water (8 ml.) was added, the contents were thoroughly mixed, cooled, and filtered through a small paper into a dry test-tube. The filtrate was reserved for estimations (8), (9) and (10).

The precipitated organic matter was washed with water, rinsing out the Kjeldahl flask first. After being washed and drained, the filter paper and precipitate were returned to the original micro-Kjeldahl flask and digested ; the ammonia was estimated in a micro-Kjeldahl distillation apparatus. Results were expressed as mg. of nitrogen/100 ml. of brine.

(8) *Nitrogen as ammonia.*—A 1- or 2-ml. aliquot of the filtrate obtained in (7) was pipetted into a dry test-tube, 9 ml. of water was added, followed by 1 ml. of Nessler solution. After the contents were mixed and set aside for 5 minutes the absorption was measured in a photoelectric colorimeter, with a 1-cm. cell and a Chance OB1 filter. Values of nitrogen were then read from a previously prepared standard graph. Results were expressed as mg. of nitrogen/100 ml. of brine.

(9) *Amino-nitrogen.*—A semi-micro Sørenson formol titration was carried out with 5 ml. of the filtrate obtained in (7). The preliminary adjustment to pH 8·5 was recorded as a convenient measure of the titratable acidity.

The results were expressed as mg. of nitrogen/100 ml. of brine. Since this value was due to amino- plus ammonia-nitrogen, the previously determined value for ammonia-nitrogen was deducted, thus giving the mg. of nitrogen/100 ml. of brine due to amino-nitrogen.

(10) *Titratable acidity.*—The titration figures for the preliminary adjustment of brine filtrate to pH 8·5 in estimation (9) were used. Results were expressed as percentage of lactic acid for convenience. When the filtrate was more alkaline than pH 8·5 the alkalinity was also expressed as percentage of lactic acid but as a negative value.

(11) *Total lactate.*—The method was adapted from that of Davidson.[6] To a 2-ml. aliquot of the suitably diluted and cleared solution in a boiling-tube (6 in. × 1 in.) were added 2 drops of 5% $CuSO_4,5H_2O$ solution. While the tube was being cooled in a large beaker of water, 12 ml. of concentrated sulphuric acid was added, with thorough mixing and cooling, particularly at first.

The tube was immersed in a boiling-water bath for 10 minutes, cooled to 20° and 4 drops of a 1·5% solution of *p*-hydroxydiphenyl in 0·5% sodium hydroxide solution were added, with thorough mixing. The tube was maintained at 30° for 15 minutes, after which 2 further drops of reagent were added and incubation at 30° was continued for a further 15 minutes. The tube was then heated in a boiling-water bath for 1½ minutes to eliminate excess of reagent. The product was cooled and the absorption of the blue-mauve coloration was then measured in a 1-cm. cell with a Chance OG1 filter. Values were calculated from a standard graph and expressed as percentage of lactic acid. The small amount of brown sugar originally present in the brines was gradually lost during curing, and was not found to interfere with this estimation.

(12) *Viable counts.*—Heller[7] had shown that the highest viable counts were obtained by using a medium of the following composition : ' Lab. Lemco ' 5 g., peptone 10 g., sodium chloride 232 g., potassium nitrate 6 g., and agar strip 20 g., made up with tap-water to 1 litre, to which was added sterile citrated blood plasma immediately before use.

The agar was dissolved in 700 ml. of tap-water, and mixed with the Lemco and peptone dissolved in 200 ml. of tap-water. The pH was adjusted to 9·0, the mixture boiled for 15 minutes and then filtered through paper pulp. The pH was then adjusted to 6·0 and the volume made up to 1 litre.

A volume of 800 ml. of this nutrient agar was heated, and the salt and nitrate were added. When these had dissolved the volume was made up to 1 litre with more of the nutrient agar. This brine agar was distributed (19 ml.) into 1-oz. McCartney bottles and sterilized at 115° for 30 minutes.

Plates were prepared by melting the contents of the McCartney bottles, cooling to 45–50° and adding 1 ml. of sterile citrated blood plasma, mixing and pouring. After the medium had set, the plates were dried at 37° for 1 hour.

Counts were made by placing 0·02-ml. drops of the brine (and drops of series decimal dilutions in 22% salt) on to the plates. After the drops had been spread with a platinum wire, the plates were incubated at 22° for 3 days and the colonies counted.

In addition, drop surface counts on Lemco agar, presumptive coliform counts, and inoculations into Robertson's meat broth, were carried out at 37°. These served to estimate the cleanliness of the curing operations.

Counts on plasma brine agar were also determined at intervals anaerobically, but as the counts appeared to vary in accordance with the (plasma) aerobic counts, and were of similar magnitude, they were discontinued.

Results and discussion

Meat

If all the brines are considered generally, the first six cures were only fair as, although colour development in the meats on cooking was satisfactory, the penetration of colour was rather poor. Subsequent cures progressively improved in all respects (brine I, slow). In these last, more alkaline stages, the meat was considerably softer and 'juicier'. A swelling and increased volume was apparent and penetration was found to be greatly increased, at least for colour. This is contrary to the usually accepted view that curing salts can penetrate into the flesh more easily when the pH is low.[8] The meats, even from the last cures, were quite wholesome.

Viable counts

The counts of organisms growing at 37° on nutrient agar did not show any significant trends or smooth growth curves (Table I). The colonies were mainly micrococci, except for those giving the higher counts towards the end, when some small colonies of a Gram-negative rod were becoming evident.

Table I

Viable counts. Colonies/ml. of brine

	Agar, incubated at 37°				
Brine no. ..	I	II	III	IV	V
After 1 week	2,000	30,000	10,000	175,000	405,000
,, 2 weeks	35,000	69,000	29,000	190,000	390,000
,, 3 ,,	40,000	75,000	85,000	140,000	100,000
,, 4 ,,	400,000	700,000	450,000	500,000	250,000
,, 5 ,,	200,000	$1{\cdot}7 \times 10^6$	108,000	113,000	$1{\cdot}3 \times 10^6$
,, 6 ,,	150,000	800,000	135,000	250,000	88,000
,, 7 ,,	150,000	$1{\cdot}1 \times 10^6$	380,000	180,000	760,000
,, 8 ,,	90,000	95,000	225,000	185,000	475,000
,, 9 ,,	90,000	700,000	200,000	100,000	330,000
,, 10 ,,	120,000	400,000	750,000	410,000	650,000
,, 11 ,,	5,000				
,, 12 ,,	18,500				

	Plasma salt agar, incubated at 22°				
Brine no.	I	II	III	IV	V
After 1 week	9,000	180,000	70,000	80,000	600,000
,, 2 weeks	225,000	250,000	95,000	205.000	440,000
,, 3 ,,	$1{\cdot}25 \times 10^6$	550,000	850,000	700,000	240,000
,, 4 ,,	$5{\cdot}4 \times 10^6$	$1{\cdot}15 \times 10^6$	$3{\cdot}9 \times 10^6$	$3{\cdot}3 \times 10^6$	$2{\cdot}6 \times 10^6$
,, 5 ,,	$9{\cdot}0 \times 10^6$	26×10^6	34×10^6	22×10^6	75×10^6
,, 6 ,,	$9{\cdot}0 \times 10^6$	$21{\cdot}5 \times 10^6$	70×10^6	84×10^6	123×10^6
,, 7 ,,	$7{\cdot}9 \times 10^6$	45×10^6	102×10^6	123×10^6	n.d.
,, 8 ,,	$5{\cdot}5 \times 10^6$	90×10^6	175×10^6	235×10^6	250×10^6
,, 9 ,,	$9{\cdot}5 \times 10^6$	99×10^6	260×10^6	305×10^6	445×10^6
,, 10 ,,	14×10^6	375×10^6	960×10^6	1750×10^6	1025×10^6
,, 11 ,,	$37{\cdot}5 \times 10^6$				
,, 12 ,,	$54{\cdot}5 \times 10^6$				

n.d. = not determined

The viable counts at 22° on plasma salt agar, however, were very much larger, and gave fairly smooth growth curves (Table I, Fig. 1). All the brines gave a similar type of curve, though the growth in brine I was slower. The predominant organism was the same in each case, a Gram-negative rod, possibly an *Achromobacter* species. The predominance of this organism in the brines was also very evident from the direct examination of total cells present in the actual

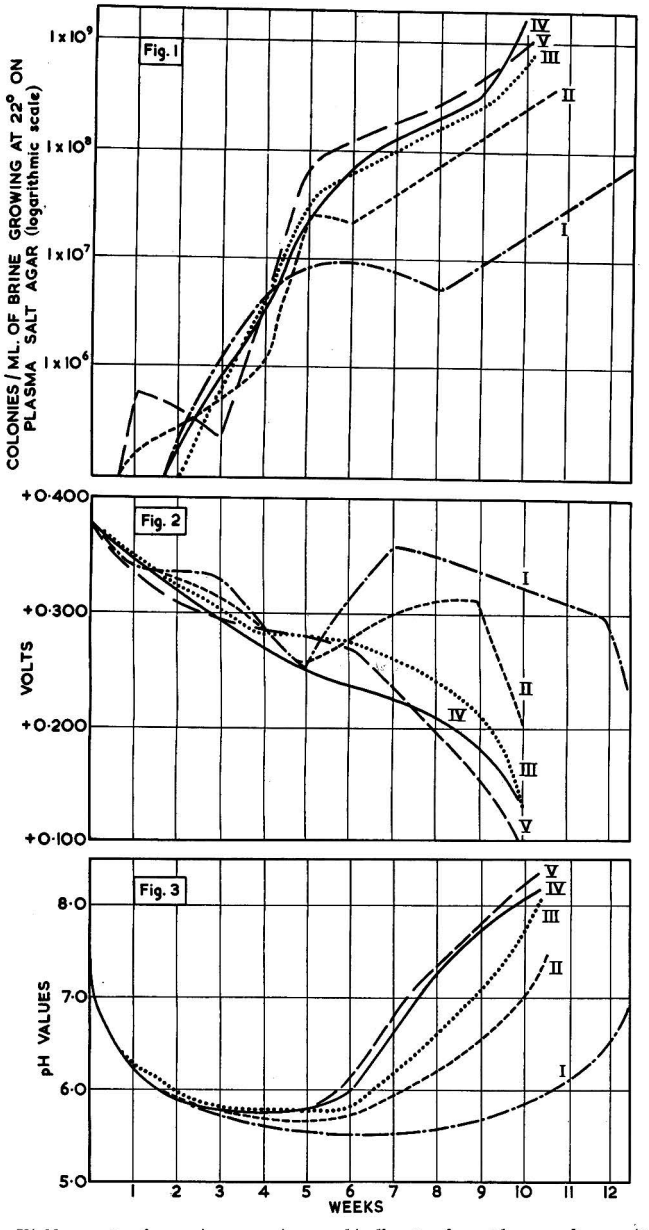

FIG. 1.—*Viable counts of organisms growing aerobically at 22° on plasma salt agar (type A)*
FIG. 2.—*Oxidation/reduction potentials*
FIG. 3.—*pH values*

brines. This organism is afterwards referred to as type A and had the following characteristics: short to medium-sized rods, fairly stout; non-motile; Gram-negative; facultative.

Only slight growth occurred on nutrient agar at 37° and 22°; fair growth on 20%-salt agar at 22°, and good growth on plasma salt agar at 22°. The colonies on plasma salt agar were

small, circular, convex, slightly olive-green, semi-transparent, and visible after 2–3 days. They reduced nitrate; had little or no fermentative ability in carbohydrates; gave a negative reaction towards hydrogen sulphide and indole; gave no liquefaction of gelatin and produced alkalinity in litmus milk.

Frothing was first noticed in each brine when a viable count of approximately 1×10^8 per ml. was reached. Reddening, with some precipitation of organic matter, soon followed and each brine developed ' off ' odours when a count of approximately 1×10^9 per ml. was found.

Oxidation/reduction potential

The curves obtained (Fig. 2), though not particularly smooth, did, in general, follow the viable-count curves. All five showed an initial drop, followed by either a short horizontal portion or a rise, then eventually a fall to much lower values. Again, the first appearance of frothing and reddening occurred in each case at approximately the same potential, + 0·280 v.

Correction of the oxidation/reduction potentials for the potential due to the pH of the system gave curves more similar to each other than were the uncorrected curves. Though this correction is probably not exact for the system, it does indicate that the potential of the brines was decreasing independently of the decrease due to the changes in hydrogen-ion concentration.

pH, titratable acidity and total lactate

The pH value for each brine fell regularly to a value of approximately 5·8, stayed constant for varying periods, and then increased to values of 7·5 and above. The resultant curves (Fig. 3) were similar for all brines, and differed only in the length of the horizontal portion. Frothing was again found to occur in each brine at about the same pH value, i.e. 6·25.

The titratable acidity values, expressed as lactic acid, all increased linearly up to values of 0·1% and over, then decreased to quite alkaline conditions, equivalent to more than — 0·1% in terms of lactic acid. The curves, shown in Fig. 4, were, as was to be expected, similar to those for pH. Only when the decrease of the titratable acidity became marked was it realized that

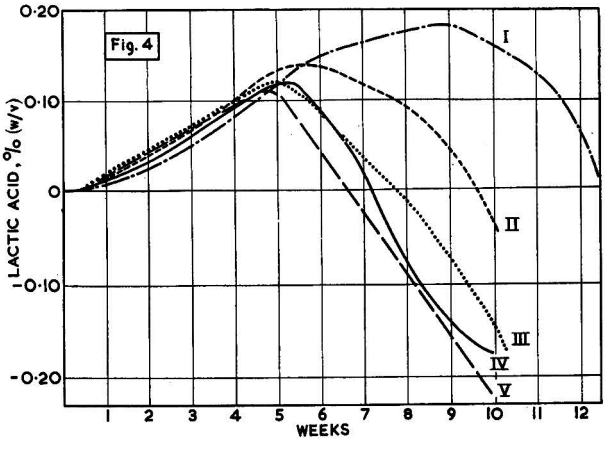

FIG. 4.—*Titratable acidity/alkalinity of cleared solutions*

total lactate estimations would be necessary to distinguish between neutralization and decomposition of lactic acid. A complete history of lactate concentration is therefore missing. The lactic acid content of muscle is, however, approximately 1·1%,[9] and it will be shown in Part II of this series that, if no destruction takes place, the lactic acid content accumulates in the brine at a rate of about 0·05% per week. The total lactate contents of the brines (Table II), after cure 6, are graduated in the same order as their respective pH and titratable acidity values,

indicating some destruction in the more advanced brines. Later determinations showed that the constant increments from the meat did not accumulate. In those cases where the oxidation/reduction potential was low, the rate of destruction easily exceeded the rate of addition. It must be added that the method used would have included the estimation of acetaldehyde, one of the possible decomposition products of lactate. The figures for lactic acid are, therefore, probably even lower than those actually recorded.

The maximum of the titratable acidity curve coincided with the end of the horizontal portion of the pH curve, for each of the brines. These results for the first half of each curve are

Table II

Total lactate as lactic acid, per cent. (w/v)

Brine no. ..	I	II	III	IV	V
Initial	Nil	Nil	Nil	Nil	Nil
After 1 week	n.d.	n.d.	n.d.	n.d.	n.d.
,, 2 weeks	,,	,,	,,	,,	,,
,, 3 ,,	,,	,,	,,	,,	,,
,, 4 ,,	,,	,,	,,	,,	,,
,, 5 ,,	,,	,,	,,	,,	,,
,, 6 ,,	0·20	0·07	0·05	0·05	0·05
,, 7 ,,	0·24	0·10	0·08	0·08	0·09
,, 8 ,,	n.d.	n.d.	n.d.	n.d.	n.d.
,, 9 ,,	,,	,,	,,	,,	,,
,, 10 ,,	0·21	0·11	0·03	Nil	Nil
,, 11 ,,	0·20	,,	,,	,,	,,
,, 12 ,,	0·20	,,	,,	,,	,,
,, 13 ,,	0·19	,,	,,	,,	,,

n.d. = not determined

attributed mainly to the gradual accumulation in the brines of lactic acid and mineral salts from the meats. Soluble proteins from the meat, also accumulating in the brines, led to increased buffering power, i.e. the pH curve tended to flatten out to a constant value, and the titratable acidity (of the cleared solution, i.e. unbuffered by protein) continued to increase. Afterwards, as the concentration of these meat-juice constituents in the brine approached the concentrations in the meats, i.e. towards equilibrium concentration, so the rate of increase fell.

The subsequent large increase of pH and decrease of titratable acidity must have been due to one or more of three possible causes:

(*a*) *Alteration of buffering power.*—Any protein breakdown was without significance, and no analytical evidence for it could be found, either as a loss of albumen, or as an increase of amino- and ammonia-decomposition products (Figs. 5, 6 and 7). Any small undetected change in the soluble proteins would not be expected to have had any influence on the total buffering power.

If it is assumed that buffering power is *decreased* at this stage, the pH curve would be expected to fall to, and then remain constant at, the pH of the meat (5·7–5·9) for the last half of the curve as well. The titratable acidity of the cleared brines should be independent of the protein buffering action and would be expected, therefore, to continue at the maximum value reached, and would not be expected to reach alkaline values.

Conversely, if it is assumed that buffering power is *increased* at this stage, the pH would be expected to stay approximately constant without affecting the titratable acidity of the cleared brines.

(*b*) *Decomposition of acid.*—The effect of gradually increasing decomposition of the organic acids present would be expected to show itself in increasing pH values, and also in decreasing titratable acidity values, both of which were found to occur. This destruction of lactic acid, however, would not be expected to give the alkaline values that were eventually reached. The high buffering power of the soluble proteins would also tend to make pH changes due to this cause very slight.

(*c*) *Production of alkali.*—The only addition, apart from salt and nitrate, were juices derived from the meat, which are known from the first part of the investigation to be acidic.

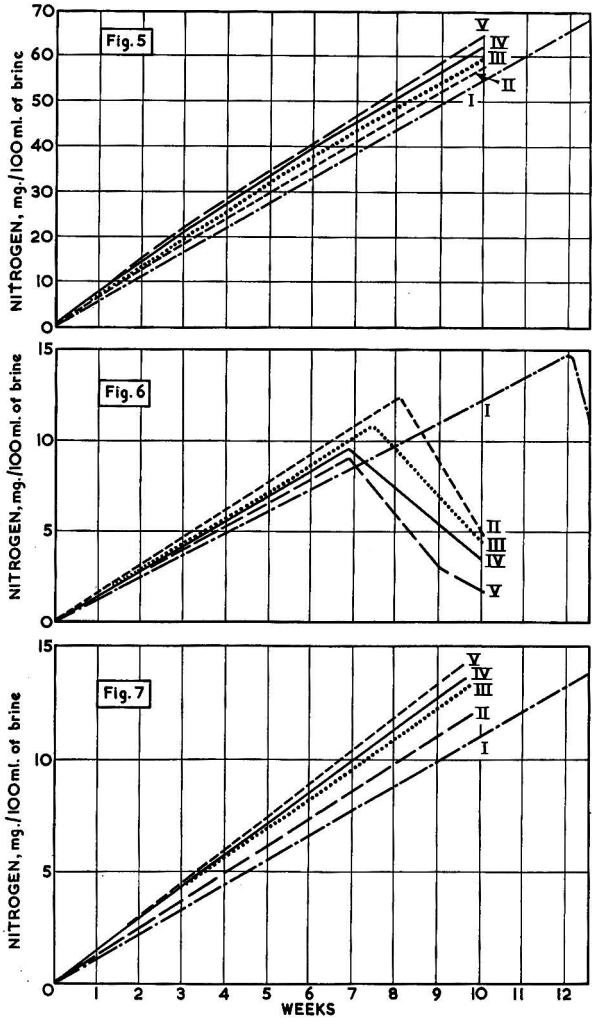

FIG. 5.—*Nitrogen present as heat-coagulable protein*
FIG. 6.—*Nitrogen present as amino-compounds*
FIG. 7.—*Nitrogen present as ammonia*

Any formation of alkali would entail either the production of ammonia, or the decomposition of alkali-metal salts. It has been shown that the ammonia values increased only gradually and approximately linearly, at a rate similar, proportionately, to those of both the albumen and the amino-nitrogen, i.e. the increases were evidently due only to incremental additions from the meat, and not to formation by protein breakdown in any appreciable or significant quantity.

The only alkali-metal salts present in sufficient quantities to merit consideration were sodium chloride, and potassium nitrate with its reduction compounds. It is difficult to reconcile any loss of chlorine, under the conditions considered, with the usual stability of the sodium and chloride ion system. Potassium nitrate breakdown, however, is in a different category, and

nitrate is reduced to nitrite under these conditions. Significant quantities of free alkali might be liberated by the further reduction of the nitrite.

It is therefore suggested that both alkali formation and lactic acid decomposition do take place. After the initial maturing of the brine and the near attainment of equilibrium in soluble meat extractives, a suitable oxidation/reduction potential appears to be established by the bacterial population, which has by now increased sufficiently for its effect to be noticeable, and the lactic acid is utilized by acting as hydrogen donor in the reduction of nitrate (and nitrite) by the bacterial enzyme reducing system. Supporting evidence for this redox decomposition is that the time when pH values start to increase, and when the titratable acidity decreases, coincides with the beginning of the very rapid rise in the production of nitrite for each of the brines.

The reduction of nitrate to gaseous nitrogen at the later stage of high bacterial growth, when there is a low oxidation/reduction potential, results in the formation of free alkali, and this proceeds rapidly, coincidental with any decrease of acidity due to the removal of lactic acid.

Nitrite concentration and loss of inorganic nitrogen compounds

As shown by Fig. 8, slow initial increases of nitrite were soon followed by a very rapid rise, then a decrease. Brine I, however, was slower and more prolonged in its initial rate of increase, but it eventually followed the pattern of the others. In brines II, III, IV and V (and I eventually) it became apparent after some time that the total inorganic nitrogen determined was less than could be explained by mere dilution. Values of potassium nitrate and sodium nitrite (expressed as nitrogen) present in each brine were calculated both before and after each charge of meat, and the total losses of nitrogen were obtained. By using the determined salt values as a basis

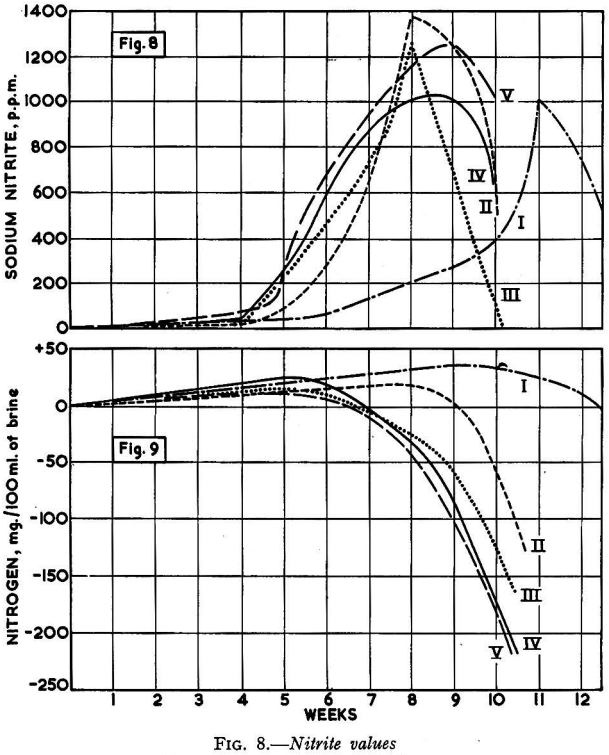

FIG. 8.—*Nitrite values*

FIG. 9.—*Loss of inorganic nitrogen*

These values (corrected for dilution) represent the sum of the increments of nitrogen added as potassium nitrate, minus the nitrate- and nitrite-nitrogen found by analysis after each cure.

Table III

Contents of sodium chloride and potassium nitrate

Brine no. ..	Sodium chloride, per cent. (w/v)					Potassium nitrate, per cent. (w/v)				
	I	II	III	IV	V	I	II	III	IV	V
Initial	22·3	22·2	22·6	22·1	22·2	0·69	0·68	0·69	0·69	0·69
After 1 week	21·8	21·6	21·8	21·3	21·6	0·69	0·70	0·69	0·67	0·68
,, 2 weeks	20·5	20·1	20·9	20·1	20·4	0·65	0·67	0·65	0·64	0·60
,, 3 ,,	19·9	19·9	20·3	19·9	20·1	0·70	0·72	0·69	0·69	0·69
,, 4 ,,	19·8	19·5	19·8	19·4	19·4	0·66	0·56	0·60	0·67	0·66
,, 5 ,,	18·8	18·9	18·9	18·9	19·1	0·62	0·58	0·59	0·58	0·55
,, 6 ,,	20·2	20·0	20·1	20·0	20·5	0·64	0·58	0·53	0·47	0·55
,, 7 ,,	20·1	20·1	20·1	19·5	20·1	0·72	0·59	0·48	0·45	0·49
,, 8 ,,	20·8	20·5	20·6	20·5	20·8	0·61	0·54	0·48	0·47	0·38
,, 9 ,,	20·5	20·5	20·6	20·7	21·3	0·69	0·58	0·54	0·31	0·44
,, 10 ,,	20·3	20·1	20·0	20·6	20·0	0·53	0·30	0·27	0·16	0·13
,, 11 ,,	20·9					0·55				
,, 12 ,,	19·3					0·62				
,, 13 ,,	19·5					0·60				

After each cure, the salt content was brought back to 22·5%, and the nitrate content back to 0·70%.

(Table III), the loss of nitrogen due to dilution etc. was calculated. This involved the assumption that, apart from the dilutions of salt and nitrate being identical, the amounts taken up and removed by the meats were also proportional, which, however, may not be exactly correct. These corrected values of the loss of nitrogen were then plotted (Fig. 9) and, as was expected, gave for the first portion almost a horizontal line. These lines had a very slight negative slope, i.e. values were slightly over-corrected, probably owing to salt dialysing faster than the nitrate into the meat. All then curved away to very high values, representing losses of the order of 2 g. of potassium nitrate per 100 ml. over the whole period. The change of slope for each curve coincided with the first appearance of frothing in the brine, suggesting that considerable quantities of nitrogen, free or combined, were being lost in a gaseous form.

Small amounts of each brine were kept for a few days in inverted boiling-tubes, fitted with a side tube, all air being displaced by brine. Gas formation did occur, and the collected gases were analysed in an Ambler's apparatus. The gas showed no absorption in strongly alkaline pyrogallol, i.e. indicating the absence of oxygen, air, carbon dioxide and oxides of nitrogen; no loss occurred on combustion either alone or mixed with air, thus indicating the absence of hydrogen and hydrocarbons. The gas was, therefore, assumed to be elementary nitrogen.

Many organisms are known to reduce nitrate to nitrous oxide, nitrogen or ammonia. These nitrate reductions have been critically considered by Kluyver,[10] who regards them as *dissimilatory nitrate reductions*, as distinct from the *assimilatory nitrate reductions* to cell proteins etc., the nitrate (and nitrite) apparently replacing oxygen as hydrogen acceptor in the respiration process.

Denitrification of nitrite by an *Achromobacter* species, with lactate as substrate, has recently been demonstrated by Youatt.[11]

The carbon dioxide content of brine V was determined after the 10th week, and contained 0·8%. This is of the right magnitude for the amount of lactate (about 0·5%) that should have accumulated during this period, and also is of the right order for the value of 1·5% of potassium nitrate destroyed in this period.

It appears evident, therefore, that when the population of type-A organisms has increased sufficiently, oxidation of lactic acid and reduction of nitrate to nitrite occur. As growth proceeds, probably accelerated by the resultant pH changes, the redox potential falls and the nitrite is itself reduced (probably *via* hyponitrite or nitramide)[10] to elementary nitrogen:

$$KNO_3 \xrightarrow{-O} KNO_2 \xrightarrow{-O} \tfrac{1}{2}K_2N_2O_2 \xrightarrow{-\frac{1}{2}O} \tfrac{1}{2}N_2 + \tfrac{1}{2}(K_2O) \xrightarrow{+\frac{1}{2}CO_2} \tfrac{1}{2}K_2CO_3$$

The nitrite concentration then depends on the relative rates of the reduction stages. As can be seen from the nitrite curves, the major increases of slope (rate of production) coincide for all the brines, with a redox value of approximately + 0·275 v. These increases also occur at the same ordinate (10×10^6) on the viable-count graph.

The subsequent reduction in nitrite concentration, i.e. nitrite reduced as fast or even faster

than it is formed, also coincides, at the appropriate ordinate, with population values of approximately 100×10^6 per ml., and a redox potential of + 0·225 v, for each of the brines.

It must, however, be stressed that the values of redox potential recorded are only for the mixed brine after removal of the meat. When the meat is actually in the brine, appreciably lower values of potential are probably reached in small local areas, close to, and inside, the meat. The gradual build-up of small amounts of nitrite to appreciable values, at potentials of the mixed brines not as low as those stated above, is therefore not precluded, and does indeed take place, as shown by the slow initial formation of nitrite

Conclusions

The changes taking place in a rapidly developing, short-life meat-curing brine may conveniently be considered as taking place in three successive stages.

(1) A gradual build-up of soluble meat-juice constituents occurs, with attainment of a fairly stable pH with an increasing bacterial flora. The small amounts of nitrite developed and accumulated are amply sufficient in theory for the necessary colour development. Only fair curves, however, are obtained in this initial stage, and penetration into the meat is slow. This is mitigated in commercial practice by prior pumping of the meat with brine. The natural attainment of the next stage follows inevitably with the increase in bacterial numbers.

(2) In the second stage, full maturity is reached, and good cures are obtained. Greater penetration of colour into the meat occurs and the nitrite production is maintained, but the oxidation/reduction potential and bacterial growth have not yet reached values giving appreciable destruction of nitrate and decreased acidity.

(3) In this last stage the bacterial population has increased to the point where nitrite is lost by reduction to nitrogen faster than it is formed. As a result of the loss of gaseous nitrogen, acidity decreases, organic matter is precipitated, and potassium nitrate is wastefully consumed. Although very good cures can be obtained, bacterial growth is so vigorous that the brine uncontrollably progresses to an ' off ' condition. Conditions are near to those required by the bacterial proteolytic enzymes, and although no significant breakdown of actual protein is observed, it can be seen that, at this stage, after complete oxidation of the lactic acid present has been achieved, decomposition of some of the amino-acids present has just begun. This decarboxylation results in the production of free amines, which impart an ' off ' odour to the brine, making it unusable. The countryman's adage ' soon ripe, soon rotten ' appears to be applicable to this type of brine.

It was stated earlier in this paper that these results represent brines that have undergone rather accelerated development. Analyses of many samples of brine have shown that the cycle of events described does take place in commercial practice, although of course the rate and extent of the changes are less. The same predominant organism has been frequently found. When other halophilic organisms predominate, however, the types of brines then obtained are completely different; these will form the subject of later papers.

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THE INFLUENCE OF THE METHOD OF DETERMINATION OF LIGNIN ON THE LIGNIN-RATIO TECHNIQUE FOR DIGESTIBILITY IN THE COW

By D. A. BALCH, C. C. BALCH and S. J. ROWLAND

Three methods for the determination of lignin (Norman & Jenkins, Ellis *et al.* and Armitage *et al.*) have been compared on samples of food and faeces from eight digestibility trials with lactating cows receiving a diet of hay and concentrates. Details are given of the different values obtained for crude lignin, the nitrogen content of the crude lignin, and lignin corrected for its nitrogen content. The method of Armitage *et al.* gave the lowest values. The mean digestibility coefficients for crude lignin and corrected lignin respectively, for the three methods in the above-mentioned order, were 9·3 ± 0·7 and 8·3 ± 0·5, 4·7 ± 0·6 and 9·1 ± 0·7, 1·3 ± 0·8 and 4·4 ± 1·0. Thus lignin as determined by the method of Armitage *et al.*, and expressed as crude lignin, showed a negligible digestibility, which should be acceptable as the basis of a ratio technique for the dairy cow. In a subsequent series of ten digestibility trials with cows on hay–concentrate diets, and when using this method, the mean digestibility coefficient for crude lignin was − 0·72 ± 0·7.

Introduction

In recent years there has been an increasing interest in the ' ratio technique ' for determining the digestibility of the constituents of feeding-stuffs from the ratio of the amount of an indigestible reference substance to that of the nutrient constituents in the diet and in the corresponding faeces, without the need to measure, as in the conventional method for digestibility coefficients, the actual weights of diet ingested and faeces excreted. The reference substances used have included ferric oxide and chromic oxide added to the diet for the purpose, natural lignin, and latterly the chromogen[1] contents of the diet. The possibilities of the lignin-ratio technique for studies with ruminants have attracted a number of investigators : some have found the lignin to be virtually indigestible, which is essential for its successful use, but others have reported an appreciable apparent digestibility of lignin, leading to low values, by the ratio technique, for the digestibility of the other dietary constituents.

Comparison of these different results is complicated by the variety of methods that have been used to determine the lignin. The full constitution of lignin has not been elucidated, and ' lignin ' as measured in feeding-stuffs is not a chemical entity but a variable fraction isolated by empirical, and lengthy, procedures that are normally based on the insolubility of lignin in 72% (w/w) sulphuric acid. A review of the early literature on these methods, and a critical examination of the effect of the various stages in the treatments involved, are given in a paper by Thomas & Armstrong,[2] and in a series of papers by Moon & Abou-Raya[3] that have appeared since the investigation now to be described was carried out.

By using the method of Norman & Jenkins[4] for the determination of lignin in digestibility trials with sheep, Louw[5] found up to 38·3% of the lignin of lucerne hay, and Ferguson[6] from 4·3 to 16·6% (average 10·2%) of the lignin of wheat straw, to be digested, whereas Gray[7] obtained acceptable recoveries of from 96 to 101% of the lignin from straw and lucerne hay.

The method of Crampton & Maynard[8] for lignin has usually given appreciable lignin digestibilities ; Crampton[9] reported an average value of 34% for steers fed on dried herbage, and Hale *et al.*[10, 11] an average of 21·5% for cows fed on lucerne hay. The method of Ellis *et al.*[12] has been found by some workers to give a satisfactory recovery of lignin : with this method, Ellis *et al.*[12] obtained an average recovery of 102·6% for cows fed on a mixed feed, Forbes *et al.*[13] of 101·0% for sheep fed on hay, and Forbes & Garrigus[14] of 102% for steers fed on freshly cut grass. In later trials with the same method, however, Forbes & Garrigus[15, 16] found significant negative digestibility coefficients when herbage was fed to steers and sheep, including an average recovery of 114% of the lignin in one trial with steers. Recently, when investigating the composition, and digestibility by the dairy cow, of lignin in hay cut at four stages of maturity, Ely *et al.*,[17] by using the method of Ellis *et al.*,[12] found the digestibility coefficients of crude lignin to range from 3·8 to 16·0% and of lignin corrected for its nitrogen content to range from 7·5 to 19·8%, and concluded that ' the lignin recoveries all were somewhat lower than is desirable for an inert tracer material '.

We wished to use the lignin-ratio technique in studies of the extent of digestion of various nutrients in the reticulo-rumen of the lactating cow, but in view of the number of methods available for the determination of lignin, and the concomitant variation in its published digestibility coefficients, we decided to make a preliminary investigation. Three methods for lignin, two already commonly used methods and a third, promising method then only recently published, were applied to the materials that had just previously been collected in conventional full digestibility trials with lactating cows fed on typical hay–concentrate diets. The results, of which an outline has been published,[18] are here recorded for the guidance of other workers who may be interested in the use of the lignin-ratio technique.

Experimental

Digestibility trials and samples

The samples of food and corresponding faeces were obtained from an experiment carried out with four lactating Shorthorn cows to observe the effect of the administration of L-thyroxine on the digestibility of a hay–concentrate diet. The hay was mainly Italian rye-grass (*Lolium italicum*), with a little clover, and the concentrate mixture consisted of dredge corn (oats and beans), wheat bran, coconut meal, palm-kernel cake, decorticated cottonseed cake, decorticated groundnut cake and a mineral mixture.

The experiment was divided into two parts (I and II), the hay–concentrate diet being fed throughout. During the second part, each cow received 100 mg. of L-thyroxine daily, but this caused no significant change in the digestibility of the dietary constituents (see Balch *et al.*[19]). Digestibility trials lasting 14 days were carried out in each part of the experiment. The bulk samples of food and faeces were dried at 90°, ground and sub-sampled for analysis.

Analytical methods

The analyses were carried out on air-dry material reground to pass a fine screen (0·4 mm.), and the results reported as a percentage of the dry matter, which was determined concurrently. The following three methods were used for the determination of lignin:

Method A, the method used by Gray,[7] which is essentially that of Norman & Jenkins[4] with the exception that, subsequent to the refluxing with 5% (w/w) sulphuric acid, the residue is separated from the acid by centrifuging and washed in the centrifuge tube twice with 95% ethyl alcohol and once with ether.

Method B, the method of Ellis *et al.*,[12] except that the filtrations were not done with a filter stick fitted with a fritted-glass disk, but through linen stretched over a Buchner funnel. Solid matter was retained on the linen and readily removed with a spatula.

Method C, the method of Armitage *et al.*,[20] which was then recently published.

The determinations of lignin were made in triplicate by each method; two of the residues were incinerated to give the percentage of ash-free lignin, and the third was used to determine the nitrogen content of this lignin. The crude lignin value was then adjusted for its nitrogen content, on the usual assumption that the nitrogen was present as contaminating protein, to give a value for 'corrected lignin', i.e. crude lignin minus (nitrogen × 6·25).

Results

The effect of the method of determination on the apparent lignin content

Values for the crude lignin content of each sample, the nitrogen content of the lignin, and corrected lignin are given in Table I. For all samples there was a consistent difference between the lignin results by the different methods, method A giving the highest and method C the lowest values. The methods that gave the higher lignin values gave lignins with a higher nitrogen content. Although methods A and B gave corrected values of the same order, method C regularly gave lower values. A further point of interest is that the nitrogen contents of lignins isolated by method A from food and the corresponding faeces were of the same order, whereas both methods B and C yielded food lignin of a lower nitrogen content than the corresponding faecal lignin.

Table I

The crude lignin and corrected lignin contents of food and faeces as determined by three methods, and the nitrogen contents of the isolated crude lignins*

	Method A			Method B			Method C		
	Crude lignin (% of dry matter)	N in crude lignin (% of crude lignin)	Corr. lignin (% of dry matter)	Crude lignin (% of dry matter)	N in crude lignin (% of crude lignin)	Corr. lignin (% of dry matter)	Crude lignin (% of dry matter)	N in crude lignin (% of crude lignin)	Corr. lignin (% of dry matter)
				Part I					
Hay	11·33	2·89	9·28	10·43	2·19	9·00	8·29	1·70	7·41
Concentrates	7·13	3·21	5·70	6·23	2·14	5·40	5·35	1·77	4·76
Faeces									
Cow A	23·40	2·88	19·19	21·84	2·83	17·98	18·51	2·41	15·72
Cow B	22·06	2·99	17·94	21·04	2·74	17·44	17·89	2·35	15·26
Cow C	21·68	3·05	17·55	20·74	2·66	17·29	17·42	2·43	14·78
Cow D	22·33	2·81	18·41	21·55	2·51	18·17	18·10	2·32	15·48
				Part II					
Hay	11·26	3·06	9·11	10·27	2·07	8·94	8·27	2·02	7·23
Concentrates	6·68	2·97	5·46	6·07	1·68	5·43	5·36	2·02	4·68
Faeces									
Cow A	22·94	2·91	18·77	21·65	2·54	18·21	18·84	2·22	16·23
Cow B	22·31	2·88	18·30	21·07	2·79	17·40	18·35	2·28	15·74
Cow C	21·47	2·58	18·01	20·89	2·76	17·29	18·07	2·35	15·42
Cow D	22·08	2·80	18·22	20·85	2·65	17·40	18·24	2·30	15·62

* Corrected lignin = crude lignin minus (6·25 times the nitrogen content of the crude lignin)

The digestibility of lignin

Digestibility coefficients for crude and corrected lignin, as determined by each method, were calculated from the weights of lignin consumed and excreted, and are given in Table II. The analytical methods giving the higher lignin values also gave higher digestibility coefficients for lignin. There was no appreciable difference between the digestibilities of crude and corrected lignin determined by method A (owing to the related variation in the nitrogen contents of the food and faecal lignins noted in Table I), but higher digestibilities were obtained for corrected than for crude lignin by methods B and C. The values in Table II show that lignin as determined by method C (of Armitage *et al.*[20]), and particularly when not corrected for its nitrogen content, showed a negligible mean digestibility, and only a small variation from cow to cow.

Table II

Digestibility coefficients for crude lignin and corrected lignin, in a hay–concentrate diet, by using three methods of lignin determination

	Method A		Method B		Method C	
	Crude lignin	Corrected lignin	Crude lignin	Corrected lignin	Crude lignin	Corrected lignin
			Part I			
Cow A	8·7	7·7	5·7	10·3	2·1	6·7
B	6·9	6·6	1·6	8·9	− 2·1	2·4
C	10·6	10·5	4·8	8·2	3·2	7·9
D	10·0	8·5	3·8	6·1	1·2	5·4
			Part II			
Cow A	7·4	6·8	4·0	7·5	− 0·7	− 0·2
B	9·0	7·9	5·7	10·5	1·6	3·4
C	13·1	10·3	7·2	12·2	4·7	6·9
D	8·5	8·0	5·0	9·3	0·5	2·4
Mean	9·3	8·3	4·7	9·1	1·3	4·4
Standard error	0·69	0·51	0·59	0·68	0·75	0·99

Discussion

The determination of lignin

The three methods for the isolation of lignin that were compared in this study all depend on the destruction of non-lignin organic matter with 72% (w/w) sulphuric acid after various preliminary treatments to remove fatty material, protein, pentosans etc. A number of workers have commented on the difficulty of carrying out the final filtrations, which tend to be slow, but no difficulty was experienced when using Gooch crucibles packed with asbestos, provided that the sulphuric acid had been maintained at the full specified strength.

The empirical nature of the lignin determinations is clearly shown by the marked differences in apparent lignin contents in Table I. The main difference between the methods is in the treatment given to hydrolyse protein. The simple hydrolysis with 5% (w/w) sulphuric acid in method A is supplemented by pre-digestion with pepsin in method B. Although this enzyme stage appreciably decreased the nitrogen content of food lignin, reduction in the nitrogen content of faecal lignin was small and variable. The nitrogen content of lignin determined by method A is of the same order as that found, by a similar method, for lignin isolated from hay and sheep faeces by Thomas & Armstrong.[2] Also, the nitrogen content of the faecal lignin determined by method B was similar to that observed by Thomas & Armstrong with method B, but, contrary to their results, the food lignin contained less nitrogen than the corresponding faecal lignin—as Forbes & Garrigus[16] and Ely *et al.*[17] have found.

In method C the protein is hydrolysed by digestion with trypsin under alkaline conditions after preliminary treatment with 5% (w/w) hydrochloric acid. This combination of treatments reduced the nitrogen content of the lignin to less than that obtained by methods A or B, and there was a greater reduction of the nitrogen content of the food than of the faecal lignin. The nitrogen values are of the same magnitude as those reported by Armitage *et al.*[20] when method C was applied to clover.

The validity of correcting crude lignin for its nitrogen content on the assumption that the nitrogen is due entirely to contamination with protein or protein-degradation products has been questioned by Bondi & Meyer,[21] who consider that nitrogen is an integral part of the lignin molecule, but subsequent work by Thomas & Armstrong[2] and de Man & de Heus,[22] on the liberation of amino-acids from crude lignin and their similarity to the amino-acids obtained from the same food or faeces as the lignins, confirms the general belief that the nitrogenous material in crude lignin is largely, if not entirely, of protein origin.

Method C consistently gave much lower lignin values than methods A and B, and this difference was only partly due to a lower nitrogen content of the crude lignin. Method C had previously been found by Armitage *et al.*,[20] who developed it, to give lower crude and corrected lignin values than method A, and has recently been found by Moon & Abou-Raya[3] to give lower values than method B.

The digestibility of lignin

To obtain a true value for the digestibility of any food fraction, its composition should be identical when it is determined in the food and faeces. In view of the variation in the amount and composition of the lignin fractions isolated by the different methods, and of the tendency (as shown by MacDougall & DeLong,[23] Ellis *et al.*[12] and Thomas & Armstrong[2]) for the apparent lignin content of the analytical samples to increase slightly with the temperature of drying during preparation, it is not surprising to find that the published coefficients for the digestibility of lignin cover a wide range. In fact, Sowden & DeLong,[24] who investigated by solubility and spectrographic tests the purity of lignins prepared from herbage and faeces by methods based on the use of 72% sulphuric acid, have expressed the view that no true digestibility coefficient for lignin will be obtained when lignin is isolated by these methods.

Nevertheless, a method for the determination of lignin which, although to some extent empirical, is reproducible under specified conditions to yield a lignin of virtually no apparent digestibility, is a procedure of great value in permitting the application of the ratio technique to the dairy cow. Of the three methods for lignin that have been compared in this study, that of Armitage *et al.*[20] (for which lignin digestibility values with cows have not previously been

reported), when the results are expressed as crude lignin, gave the closest, and a very satisfactory, approximation to such a method. The mean digestibility coefficient for the eight trials was $1{\cdot}3 \pm 0{\cdot}75$, with only a small variation from cow to cow (S.D. $\pm$ 2·1).

The reliability of the lignin-ratio technique, when using this method for hay–concentrate diets under our conditions, was subsequently confirmed by a further series of ten conventional digestibility trials with milking cows fed on various hay–concentrate rations. The mean digestibility coefficient for crude lignin was $-0{\cdot}72 \pm 0{\cdot}66$ (S.D. $\pm$ 2·1).

In considering the extent to which the faecal recovery of lignin may vary from 100% and still be acceptable for the ratio technique, it should be remembered that the digestibility coefficient of a given nutrient is calculated by this technique as:

$$100 - \left(100 \times \frac{\%\ \text{lignin in feed}}{\%\ \text{lignin in faeces}} \times \frac{\%\ \text{nutrient in faeces}}{\%\ \text{nutrient in feed}}\right)$$

and that, therefore, the extent to which the lignin recovery deviates from 100% influences, in the same proportion, not the digestibility coefficient of the nutrient, but ' 100 minus the coefficient '. Hence, as the true coefficient for the nutrient increases above 50, the proportional error in this coefficient arising from incorrect lignin recovery progressively diminishes. For example, a lignin recovery of only 95% (taking an extreme instance for the above method) would reduce a true digestibility coefficient of 75 to 73·7, an error of 1·7%; or a lignin recovery of 102% would increase a true digestibility coefficient of 65 to 65·7, an error of 1·1%.

Admittedly, coefficients much below 50 would carry an increasing percentage error, but it is with digestibility coefficients mainly within the range of 55 to 80, for dry matter, nitrogen-free extract, fibre, crude protein etc., that the ratio technique would most frequently be used. The lignin recoveries obtained by the method of Armitage *et al.* in the present investigations should therefore be acceptable as the basis of a ratio technique.

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CHEMICAL ASPECTS OF THE BROWNISH-YELLOW DISCOLORATION OF SALT COD

By PÁLL ÓLAFSSON

The brownish-yellow discoloration of salt cod has been studied chemically. The discoloured fish tissue is found to contain considerably more total calcium, and more calcium in excess of sulphate, than the ordinary tissue. The concentration of sulphate falls in the deteriorating fish and in the salt in the stacks. Calcium is found to have a pronounced catalytic effect on the process of discoloration. Sterilization of the salt suppresses the cause of discoloration. Micro-organisms are considered to play an important role.

Introduction

In the dry salting method salt cod is produced by splitting the fish, removing two-thirds of the backbone, washing the fish and salting in stacks with an excess of salt. When the fish have been held in the salt for 3 or 4 days they are resalted in new stacks, more salt being added. After an additional 10 days or so the fish are resalted again. The curing is completed in about 3 weeks. This method is most commonly used in Iceland. In recent years the salt-cod industry in Iceland has suffered heavy losses by the deterioration of the product caused by brownish-yellow discoloration. The discoloration generally occurs in 10–20 days after salting in stacks, the surface of the fish flesh first becoming cream and yellow and turning brownish-yellow to brown in 3–6 weeks. Such a deterioration is well known in other countries such as France,[1] Canada[2] and Great Britain,[3] and has been attributed to the use of unsuitable fishery salts as it is caused only by the use of certain salts.

Boury[1] mentions different types of yellowing of salt cod. One is characterized by an orange-yellow discoloration of the surface of the flesh, either all over the surface or only in certain parts; this is attributed to excessive salting. Another type is attributed to insufficient salting and the discoloration is said to be caused by chromogenic bacteria. He found that salting of cod with a fishery salt that was almost pure sodium chloride resulted in a translucent brownish-yellow cure, whereas the presence of calcium and magnesium salts, particularly the calcium compounds, gave the cures a whiter and more opaque appearance. Similar results were obtained at the Torry Research Station.[4] These results were taken by the writer as indicating that the discoloration might be due to shortage of calcium salts in the fishery salts used. The salt cod was therefore analysed for content of calcium and sulphate.

Dyer[2] states that the deterioration has previously been assumed to be caused by bacteria forming ' a yellow pigment on the fish either before salting or early in the salting process. The later heavy salting killed off the bacteria and the yellow pigment remained '. This theory was abandoned. He found considerable amounts of iron in the stained layers of fish and much less in the unstained centre portions and the white surface layers. From this and the results of some experiments of staining fish with iron during salting he considered it probable that the yellow stains were due to small amounts of iron in the fishery salts.

Legendre[5] studied the yellowing of three types of dried salt cod of different degrees of curing. In one case the fish were covered with small cream-coloured specks before the experiments began. It is thought probable that the yellowing of this lot may have been of the same type as the deterioration studied in the present paper. It is well known in Iceland that on drying salt cod it may become yellow if stacked slightly warm.

In the Report[3] of the Food Investigation Board for 1951 it is suggested that the browning may be of the Maillard type. The Report further states ' The constituent(s) in the solar salts which prevents or retards browning is not organic in nature, but the addition of Ca, Mg, K and SO_4 ions to pure salts in amounts equivalent to that in solar salts, although reducing the amount of browning, did not completely prevent it, even in North Sea fish.'

Recently it has been found[7] that the addition of a few tenths of one part of copper per million of fishery salts not causing discoloration produces discoloration on salting fish. The content of copper in fishery salts causing discoloration was found to be of the same order.

Analytical methods

Analysis of salt-cod tissue.—For analysis of salt cod, skin and bone-free pieces were cut from the flesh of the fish and disintegrated in a meat-chopper. Each sample was taken from one fish only. Moisture was determined by the toluene distillation method. For calcium and sulphate determinations, 10–20 g. of the fish flesh was ashed in a porcelain crucible at low heat to greyish-white ash in an electric furnace. . The ash was extracted as follows: The whole content of the crucible was washed into a beaker with 200 ml. of dilute hydrochloric acid (1 : 3) and the solution boiled for a few minutes. The solution was then filtered into a 500-ml. volumetric flask and the filter washed with distilled water until the flask was filled to the mark. Calcium was determined on an aliquot by precipitation with oxalate in an ammoniacal solution; the precipitate was washed and dissolved in 125 ml. of dilute sulphuric acid (5 : 95) and the oxalate titrated with 0·01N-potassium permanganate.[6] The sulphate was determined in the usual way as barium sulphate, the precipitate being washed until the filtrate was chloride-free, and weighed after ignition.

Analysis of fishery salts.—The salt was dissolved in dilute hydrochloric acid (1 : 3), the solution boiled for about 10 minutes and filtered into a volumetric flask. Calcium and sulphate were determined as described above.

Results of analysis of salt-cod tissue

In Tables I and II the results of analysis of salt-cod tissue are given. The samples were taken from a commercial salt-curing station. The fish had been salted quite fresh, on board

Table I

Content of moisture, calcium and sulphate in skin and bone-free samples of salt-cod tissue

Sample	Condition	Moisture, %	Ca, %	SO_4, %	Ca in excess of SO_4, %
9	Ordinary, not deteriorated	51·0	0·17	0·26	0·06
10	,, ,, ,,	51·0	0·21	0·21	0·11
11A	,, ,, ,,	51·5	0·15	0·15	0·09
17	,, ,, ,,	49·5	0·20	0·19	0·12
18	,, ,, ,,	56·0	0·09	0·15	0·04
19	,, ,, ,,	53·5	0·13	0·19	0·05
20	,, ,, ,,	51·5	0·16	0·16	0·09
21	,, ,, ,,	51·0	0·19	0·18	0·11
	Average	51·9	0·16	0·18	0·08

Table II

Content of moisture, calcium and sulphate in skin and bone-free samples of discoloured salt-cod tissue

Sample	Condition	Moisture, %	Ca, %	SO_4, %	Ca in excess of SO_4, %
1	Brownish-yellow	52·0	0·20	0·15	0·14
7	Bright yellow	51·5	0·17	0·17	0·10
8	,, ,,	51·0	0·23	0·17	0·16
11B	Yellow cuts from sample 11	51·5	0·41	0·19	0·33
12	Yellow	50·0	0·20	0·15	0·14
13	Bright yellow	51·5	0·37	0·24	0·27
14	Brownish-yellow	49·0	0·28	0·22	0·19
15	,, ,,	50·0	0·31	0·23	0·21
16	,, ,,	52·0	0·35	0·31	0·22
	Average	50·9	0·28	0·20	0·20

trawlers, landed up to about 3 weeks old and resalted in the curing station. Samples 11A and 11B were cuts from the same fish, 11A from unspoiled parts and 11B from the spoiled parts. The results in Tables I and II are considered below (see 'Discussion').

Salting experiments

Before salting experiments were started 100–200 kg. of the fishery salts was thoroughly mixed. Salting experiments were done in the following way: fresh line-caught cod of fairly

uniform size (wt. about 1½ kg.) were washed, split and again thoroughly washed in tap-water and then salted in the usual way with an excess of salt in small stacks. The pickle formed was allowed to drain. When the fish had been a few days in the salt they were taken up and resalted.

The temperature of storage was 8–10°. The contents of calcium and sulphate in the fishery salts used are shown in Table III. Salts B and C were known to cause discoloration in salt cod; salts A and D have a rather low content of calcium.

Table III

Content of calcium and sulphate in fishery salts used in salting experiments

Salt	Ca, %	SO_4, %	Ca/SO_4
A	0·05	0·10	0·50
B	0·17	0·37	0·46
C	0·23	0·62	0·37
D	0·06	0·14	0·43

The sodium chloride used was of pharmaceutical grade and the other salts were of analytical grade. Some typical salting experiments are listed in Table IV.

Table IV

Salting experiments

Experiment	Salt	Treatment	Remarks on salt fish
1	A		No discoloration in 6 weeks
2	A + 1·25% $CaCl_2$		Discoloured in 7–10 days
3	A + 2·25% $MgSO_4,7H_2O$		No discoloration in 6 weeks
4	A + 1·25% $CaCl_2$ + 2·25% $MgSO_4,7H_2O$		Discoloured in 7–10 days
5	B		,, ,, 14 days
6	B + 0·5% $CaCl_2$		,, ,, 7 days
7	C		,, ,, 12 days
8	C	Sterilized by heating in electric oven for 3 h., at 130°	No discoloration in 6 weeks
9	A + 1·25% $CaCl_2$ + 2·25% $MgSO_4,7H_2O$	Sterilized by heating for 4 h. at 150–180°	,, ,, ,, ,, ,,
10	B	,,	,, ,, ,, ,, ,,
11	D		,, ,, ,, ,, ,,
12	D + 1% $CaCl_2$		,, ,, ,, ,, ,,
13	D + 2·5 p.p.m. of $CuSO_4$		Partially discoloured in 2 weeks
14	D + 1% $CaCl_2$ + 2·5 p.p.m. of $CuSO_4$		Discoloured in 7 days
15	D + 1% $CaCl_2$ + 2·5 p.p.m. of $CuSO_4$	Salt sterilized; fish washed in 0·5% $NaNO_2$	No discoloration in 6 weeks
16	NaCl		Fish rather translucent, slightly yellowish, but not brownish-yellow
17	NaCl + 1% $CaCl_2$		No discoloration in 6 weeks
18	NaCl + 1% $CaCl_2$ + 0·75 p.p.m. of $CuSO_4$		Discoloured in 2 weeks
19	NaCl + 1% $CaCl_2$ + 0·75 p.p.m. of $CuSO_4$	Fish washed in 0·5% $NaNO_2$	No discoloration in 5 weeks; slightly discoloured in 6 weeks
20	NaCl + 0·75 p.p.m. of $CuSO_4$		No discoloration in 5 weeks; slightly discoloured in 6 weeks

Changes in the ratio Ca/SO_4 in the salt in the stacks.—In two of the stacks where discoloration was apparent the contents of calcium and sulphate in the salt were determined. The results of the analysis are given in Table V. There was a greater excess of salt in experiment 7 than in experiment 5.

Table V

Content of calcium and sulphate in the salts of stacks of deteriorating fish

Salt	Experiment	Original salt			Time from salting, days	Condition of fish	Salt in stack		
		Ca, %	SO_4, %	Ca/SO_4			Ca, %	SO_4, %	Ca/SO_4
B	5	0·17	0·37	0·46	43	Brownish-yellow	0·11	0·08	1·38
C	7	0·23	0·62	0·37	17	Yellow to brownish-yellow	0·23	0·50	0·46
					36	Brownish-yellow	0·23	0·42	0·55
C	8	Sterilized		0·37	36	Ordinary	0·27	0·65	0·42

Discussion

Tables I and II show that the content of calcium in discoloured salt-cod tissue is considerably higher than in the ordinary tissue. This is particularly clear from a comparison of samples 11A and 11B. It will also be noted that the content of calcium in excess of sulphate is higher in the deteriorated fish tissue than in the undeteriorated. The content of calcium in excess of sulphate is of the order of 0·2% in the brownish-yellow tissue, but less than 0.1% in the ordinary tissue. These results would indicate a disappearance of sulphate in the deteriorating fish, as generally there is very little or no calcium in excess of sulphate in the fishery salts. Thus of ten samples of fishery salts recently investigated by the writer four had a content of calcium in excess of sulphate from 0·01–0·06%. The content of sodium chloride in salt-cod tissue is about 18%.

The disappearance of sulphate is apparent from the results of analysis of the salt in the stacks of deteriorating fish (Table V). Thus the ratio Ca/SO_4 in the salt B in experiment 5 changed from the original value of 0·46 to 1·38 in 43 days. At that time the fish had become brownish-yellow to brown. The ratio Ca/SO_4 in salt C in experiment 7 increased from 0·37 to 0·46 in 17 days and to 0·55 in 36 days. That stack had a greater excess of salt than the other stack. The ratio Ca/SO_4 in experiment 8 with the sterilized salt was 0·42 after 36 days.

The salting experiments 1–6 show that calcium has a pronounced catalytic effect on the process of discoloration. Neither magnesium nor sulphate was found to have any influence on that effect. Experiments 8–10 show that the causes of discoloration in salts A, B and C are suppressed by sterilization.

The results discussed here suggest that the cause of discoloration is microbiological. It is thought that the micro-organisms reduce the sulphate in calcium sulphate, so as to convert the calcium into a soluble form.

The salting experiments did show that salt fish is discoloured by the addition of traces of copper to the salts. The appearance of the discoloration is accelerated by calcium. It is, however, not possible from the work reported here to decide whether this type of discoloration is of the same nature as the discoloration caused by the fishery salts investigated. The discoloration noted in experiments 13–14 was of a particularly bright-yellow (golden) tint and it changed after a time to orange. The discoloration observed in experiment 18 was more uniformly distributed over the whole surface of the fish and of a more orange colour than the brownish-yellow discoloration caused by the fishery salts.

From these results it is deduced that calcium-requiring halophilic micro-organisms probably play a prominent role in the brownish-yellow discoloration of salt cod. They are considered to be transferred mainly by the salt.

Summary

1. The content of calcium in brownish-yellow salt-cod tissue is considerably higher than in the ordinary tissue.

2. The content of calcium in excess of sulphate is also higher in the discoloured tissue.

3. The content of sulphate in the salt in the stacks of deteriorating fish is reduced and the ratio Ca/SO_4 is increased.

4. The addition of calcium to the salts causing discoloration accelerates its appearance.

5. Sterilization of the salt suppresses the causes of discoloration.

6. Calcium-requiring halophilic micro-organisms are considered to play a prominent role in the process of discoloration. They are believed to be transferred mainly by the salt.

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THEOGALLIN, A POLYPHENOL OCCURRING IN TEA

By R. A. CARTWRIGHT and E. A. H. ROBERTS

A polyphenol, now named theogallin, detected by paper chromatography in tea-leaf, has been shown not to be identical with *m*-digallic acid as originally thought. A method of preparation is described but the final product still contains appreciable impurities. Its reactions suggest it to be a depside of gallic acid with some highly hydroxylated organic acid.

Introduction

As a result of a study of the polyphenols in tea-leaf by paper chromatography Roberts & Wood[1, 2] recorded the presence of one polyphenol that differed in many respects from the flavan-3-ols isolated by Bradfield & co-workers.[3–5] A tentative identification of this polyphenol as *m*-digallic acid is now withdrawn as a result of comparison with authentic samples described below. The substance does not seem to have been previously recognized and the name theogallin is provisionally proposed for it.

Methods

Methods employed for the paper chromatography of green-leaf extracts and of crude theogallin extracts are similar to those described earlier.[1, 2] In most experiments a reconstituted juice has been used, obtained by grinding dried green tea-leaf with two to three volumes of water. The dried green tea-leaf used for these experiments and in the preparation of crude theogallin had been obtained from the Tocklai Experimental Station, Assam. The phenol solvent used has been slightly modified and now has the composition 80% phenol, 2% acetic acid and 18% water. It has also proved advantageous to run chromatograms with butanol–acetic acid and then with water, rather than in the reverse order as previously done.[2] The most convenient proportions of the solvent were butanol–acetic acid–water (4 : 1 : 2·2), which corresponds with the mixture[1] of butanol (80%) and acetic acid (20%) to which water had been added in amounts just short of that required for saturation.

The position of the theogallin spot on the dried paper chromatogram is revealed by its fluorescence in ultra-violet light. This fluorescence is intensified by a preliminary exposure of the paper to ammonia vapour. In addition to sprays previously described, including 0·2% ferric alum and 1% potassium cyanide, the following spray or dip reagents have been employed :

Mixed ferric chloride and potassium ferricyanide.—This was prepared in the way described by Kirby, Knowles & White.[6]

Bis-diazotized benzidine.—This was as described by Kirby, Knowles & White.[7] A useful alternative to this, which is very easily made up and gives substantially the same results, is an aqueous solution of 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate (NNCD reagent) at about pH 2.

Vanillin reagent.—Vanillin (0·5 g.) and toluene-*p*-sulphonic acid (0·5 g.) were dissolved in butanol (5 c.c.) and the mixture diluted to 50 c.c. with xylene.[8]

Ethylenediamine reagent.—A 2M-solution of ethylenediamine dihydrochloride in water was mixed with ethylenediamine to a pH of just above 10·0, and the mixture diluted with an equal volume of water. The use of this reagent was suggested by its employment in the estimation of adrenaline by Weil-Malherbe & Bone.[9]

Results

Spot reactions on paper chromatograms.—Under ultra-violet light the theogallin spot appears a dark blue-violet, intensifying on exposure to ammonia vapour. Similar colours are observed with gallic acid and the galloyl esters of the flavan-3-ols. (—)-*epi*Gallocatechin shows up as a dark patch under ultra-violet light, turning to blue on exposure to ammonia. The blue–violet colour therefore appears to be characteristic of galloyl groups.

With the ethylenediamine reagent the six flavan-3-ols found in green tea-leaf give yellow colours which intensify under ultra-violet light. Neither gallic acid nor theogallin gives an appreciable colour reaction in visible light, but under ultra-violet light both give an intense blue fluorescence. The two galloyl esters, (—)-*epi*catechin gallate and (—)-*epi*gallocatechin gallate, also show this fluorescence as a blue centre to the yellow spots obtained after spraying with the reagent.

Theogallin resembles gallic acid and the flavan-3-ols in giving positive reactions with both ammoniacal silver nitrate and the ferric chloride–potassium ferricyanide reagents. Ferric alum gives the blue–black colour typical of pyrogallol derivatives, but the colour reaction is often not fully developed unless the paper is subsequently exposed to ammonia vapour. The gallic acid spot behaves in a similar way. As the blue colour reaction with ferric salts is optimal at pH 7–8, it is possible that the acidic nature of the gallic acid prevents full development of the colour, and that neutralization of the free acid permits it. This behaviour of theogallin, therefore, is an indication that it may be acidic.

Potassium cyanide gives a pink colour with the theogallin spot, the colour fading rapidly. The colour reaction is similar to that given by gallic acid, but much less intense.

The colour reactions of both gallic acid and theogallin with bis-diazotized benzidine and the NNCD reagent are very much weaker than the reactions of the flavan-3-ols. In the latter cases the strong reactions are presumably due to the phloroglucinol groups. This suggests that theogallin differs from the flavan-3-ols in not containing a phloroglucinol group, a conclusion that is confirmed by the complete absence of any reaction between theogallin and the vanillin reagent.

Indicator sprays show theogallin to have an acidic reaction.

These reactions show that theogallin, although a strongly reducing polyphenol with three hydroxyl groups adjacent to each other, is essentially different in structure from the flavan-3-ols. Its reactions suggest that it contains a galloyl group, and its acidic nature suggests that theogallin is the galloyl derivative of some acid. It is unlikely that the galloyl group is linked to another molecule by one of its phenolic hydroxyl groups, as the reaction with ferric alum indicates the presence of three adjacent hydroxyl groups. This conclusion would not be valid if two gallic acid molecules were linked, as in *m*-digallic acid.

Chromatographic behaviour of theogallin.—Fig. 1 shows the positions taken up on a paper chromatogram, which is run first with butanol–acetic acid and then with water, by the six

flavan-3-ols, theogallin, gallic acid, chlorogenic acid, caffeic acid and *m*-digallic acid. Except with gallic acid, and caffeic and *m*-digallic acids, which have not previously been investigated, the positions on the paper chromatogram are not appreciably affected by changing the order of the solvents. The variation with gallic acid will be discussed later elsewhere.

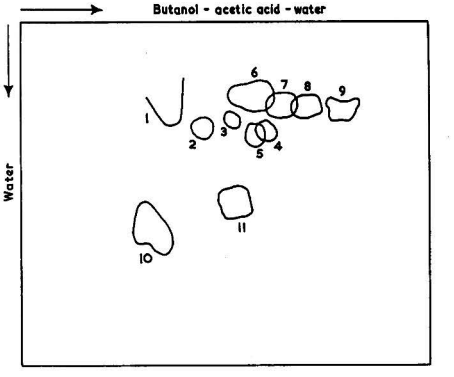

FIG. 1.—*Paper chromatogram for mixed polyphenols. The chromatogram was run first from left to right with butanol–acetic acid–water, and then downwards with water*

(1) (−)-*epi*Gallocatechin
(2) (+)-Gallocatechin
(3) (−)-*epi*Catechin
(4) (+)-Catechin
(5) Gallic acid
(6) (−)-*epi*Gallocatechin gallate
(7) *m*-Digallic acid
(8) (−)-*epi*Catechin gallate
(9) Caffeic acid
(10) Theogallin
(11) Chlorogenic acid

The position occupied by *m*-digallic acid establishes the non-identity of this substance with theogallin. The R_F values in phenol also show these substances to be distinct from each other, values for theogallin and *m*-digallic acid being approximately 0·22 and 0·12 respectively.

The R_F of theogallin in 80% phenol is increased by about 0·10 if 2% acetic acid is added to the solvent. The R_F values of gallic and chlorogenic acids are increased to a similar extent, but those of the flavan-3-ols remain unaffected. This shows a further difference of theogallin from the flavan-3-ols, which is also consistent with an acidic nature for theogallin.

In the chromatogram illustrated in Fig. 1 it will be observed that the position of theogallin relative to gallic acid is very similar to that of chlorogenic acid relative to caffeic acid. This suggests a possibility that theogallin may be a depside of gallic acid and quinic acid. At present this suggestion remains an untested hypothesis, and there are other equally likely possibilities. The evidence discussed above, however, is consistent with the identification of theogallin as a depside of gallic acid with some other highly hydroxylated acid such as quinic acid. The identification of theogallin as a glycoside of gallic acid would also account for its position on the chromatogram relative to gallic acid, but it appears doubtful whether a glycoside would have such definite acidic properties if the link with the gallic acid were through the carboxyl group. This remains to be established.

Preparation of theogallin from dried green tea-leaf.—To determine the structure of theogallin a reasonably pure preparation is needed. Some progress has been made in freeing it from associated impurities, but the final preparation is still unsuitable for studies of its structure.

Dried green leaf (150 g.) is roughly powdered and percolated with acetone until further extraction takes out little further colour. This removes some pigments, lipids and considerable amounts of flavan-3-ols, but leaves the theogallin. Partial removal of the lipids facilitates further working-up of the material, and the extraction of some of the flavan-3-ols reduces the bulk of the chloroform precipitate in the next stage.

The residual tissue is then percolated with methanol, which extracts the theogallin, together with the remaining flavan-3-ols, pigments and lipids. Amino-acids are also extracted. The methanol extract is then concentrated *in vacuo* until a trial portion gives a satisfactory precipitate with chloroform (if concentrated too far an intractable gum results). The concentrate is poured, with stirring, into three volumes of chloroform, and the precipitate washed successively with chloroform and light petroleum. After drying, the precipitate is redissolved in methanol and the procedure repeated. The final precipitate is dried *in vacuo* over paraffin wax (yield 32 g.).

The crude product is then dissolved in water (250 c.c.) and extracted continuously with ethyl acetate for six hours to remove associated flavan-3-ols. The residual aqueous solution is then precipitated by an excess of a saturated aqueous solution of lead acetate. This precipitates the polyphenols and leaves the bulk of the amino-acids and other components in solution. The precipitate, after washing, is suspended in water and made acid (pH 2) with glacial acetic acid. Insoluble matter is rejected, and 5N-ammonia added slowly, with stirring, to the filtrate. Material precipitated between pH 2 and 5 contains little theogallin and is rejected. Most of the theogallin comes down between pH 5·0 and 6·5, and this fraction is collected separately and washed free from ammonium acetate. The precipitate is then suspended in methanol and decomposed by hydrogen sulphide. After removal of lead sulphide the extract is finally evaporated to dryness *in vacuo*, yielding 1·2 g. of crude theogallin.

Impurities in this product include coloured polyphenolic oxidation products, chlorogenic acid, flavonol glycosides and theanine,[10] together with other amino-acids. Further purification was effected by the Craig procedure, by using eight separating funnels and water and butanol as the two solvents. The amino-acids remain in the first two tubes and the flavonols are found in the two last tubes, which also contain a considerable proportion of the chlorogenic acid. Evaporation of the contents of the four middle tubes yielded a substantially purer preparation of theogallin (yield 350 mg.).

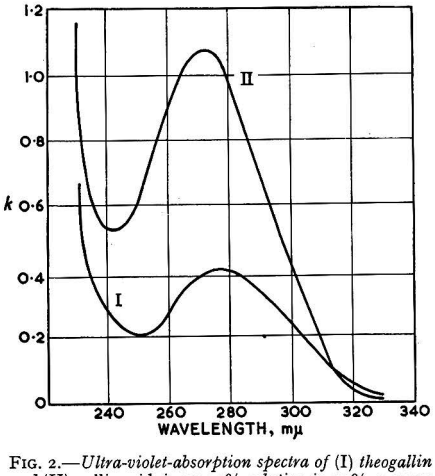

FIG. 2.—*Ultra-violet-absorption spectra of* (I) *theogallin and* (II) *gallic acid, in* 0·002% *solution in* 50% *aqueous ethanol*

Paper chromatograms showed this final product to be free from amino-acids, but traces of flavan-3-ols, chlorogenic acid and what are believed to be leuco-anthocyanins[11] were detected. It is intended to carry out further purification of the product in a Craig countercurrent apparatus, which is now available.

The ultra-violet-absorption spectrum of the final product (Fig. 2) shows a well-developed peak at 275 mμ, with k (extinction coefficient) equal to 0·42 for a 0·002% solution in 50% aqueous ethanol. The spectrum is similar to but weaker than that recorded for gallic acid, for which k was found to be 1·07 under the same conditions. Assuming that a galloyl group is the only one making any significant contribution to the absorption in the ultra-violet, this would indicate that the molecular weight of theogallin is about 430. This is approximate, as the presence of impurities will be likely to modify this value.

Potentiometric titration shows that theogallin (40 mg.) requires 0·94 c.c. of 0·1N-sodium hydroxide for neutralization, corresponding with a molecular weight of 425, if it is a monobasic acid. If non-acidic impurities are present, the true value is likely to be somewhat lower than this.

Discussion

The results in this paper are all in accordance with the view that theogallin is a depside of gallic acid, the nature of the other acid linked to the carboxyl of the gallic acid being unknown at present. What is probably an upper limit for the molecular weight is the value of 425 obtained by titration. This value receives some support from the absorption in the ultra-violet, as compared with that of gallic acid.

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THEANINE, AN AMINO-ACID *N*-ETHYL AMIDE PRESENT IN TEA

By R. A. CARTWRIGHT, E. A. H. ROBERTS and D. J. WOOD

Theanine, isolated from Japanese green tea by Sakato, has been shown to be identical with a previously uncharacterized amino-acid reported in both fermented and unfermented tea-leaf.

Introduction

A study of the amino-acids in tea-leaf[1] revealed the presence of one substance, reacting with ninhydrin, that was present in greater concentration than any of the identifiable amino-acids. It was known that this substance, which received the code-number 10G, yielded glutamic acid on hydrolysis. Further work on its characterization was in progress when Sakato[2] reported the isolation of theanine, an *N*-ethyl amide of glutamic acid, from Japanese green tea. It at once appeared probable that theanine and 10G were identical; the present paper reports the establishment of this identity and some further analytical data that confirm Sakato's establishment of the structure of theanine.

Experimental

Microanalytical tests with 10G concentrates.—Dilute aqueous solutions of 10G were prepared according to the following procedure. Stems (10 g.) were separated from freshly plucked tea-shoots, and extracted for one hour with 100 c.c. of boiling water. The infusion was extracted once with chloroform and twice with ethyl acetate to remove caffeine and the bulk of the flavan-3-ols, and then concentrated *in vacuo* to about 2 c.c. This concentrate was then applied, in 5-μl. spots along the starting line of two sheets of Whatman No. 1 paper (22 in. × 18 in.). Thirty-seven spots were accommodated along each starting line. The papers were then irrigated with phenol, which separates 10G from all amino-acids occurring in high concentration in tea-leaf. Marker spots, sprayed with ninhydrin, revealed the position of the 10G, and strips containing the 10G were cut out from the chromatograms and eluted with water. The resulting aqueous extract was almost free from amino-acids other than 10G and was used for the following microanalytical determinations.

One such extract, which contained 170 μg. of nitrogen per c.c. (micro-Kjeldahl), was found to contain 282 μg. of carboxyl per c.c., as determined by the modified Willstätter & Waldschmidt-Leitz procedure described by Pregl.[3] These figures correspond with 12·15 and 6·26 μmoles per c.c., which indicates the presence of two atoms of nitrogen for every carboxyl group.

Hydrolysis of this solution by N-hydrochloric acid for one hour at 100° yields glutamic acid as the only product detectable on a paper chromatogram by ninhydrin. It follows that in 10G a nitrogenous substance is probably linked to one of the carboxyl groups of glutamic acid. This is of course fully consistent with the identification of 10G with *N*-ethyl-γ-glutamine, $EtNH{\cdot}CO{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$.

In another extract of 10G, containing 31·2 μg. of nitrogen per c.c., the ammonia-nitrogen determined by the standard Conway technique[4] was found to be nil. The solution (2 c.c.) was then refluxed with 0·2 c.c. of 10N-hydrochloric acid for one hour, after which the ammonia-nitrogen was found to be 15·7 μg. per c.c. of the original solution. This indicates that one of the two nitrogen atoms present for each carboxyl group has an amide-like character, which is again consistent with the identification of 10G as *N*-ethyl-γ-glutamine.

Non-identity of 10G with peptides.—Partial hydrolysis of 10G by 0·1N-hydrochloric acid gave a product which, when examined by paper chromatography, was found to contain glutamic acid and 10G as the only substances reacting with ninhydrin. Had 10G been a peptide, more complex than a dipeptide, other products arising from partial hydrolysis might have been formed.

Interaction with nitrous acid destroys the ability of 10G to form coloured reaction products with ninhydrin. However, if 10G were a peptide, acid hydrolysis of the product formed by the action of nitrous acid should result in the production of a substance again reacting with ninhydrin. Results of such a test were negative, supporting the view that 10G is not a peptide.

Paper chromatography of 10G and theanine.—The foregoing results suggested strongly that 10G was a simple amide of glutamic acid. There can be no doubt from R_F values quoted in previous work[1] that 10G is not to be identified with γ-glutamine, and the stability of 10G at pH 4 and its resistance to hydrolysis by saturated K_2CO_3 solution confirm this point. Before Sakato announced his isolation of theanine the possibility was considered that 10G might prove identical with α-glutamine [*iso*glutamine, $HO_2C{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO{\cdot}NH_2$]. Dr. A. C. Chibnall very kindly carried out some tests on α-glutamine, from which it followed that, on the basis of R_F values, 10G could not be identified with α-glutamine. Dr. Chibnall's R_F values for α-glutamine and our values for 10G and γ-glutamine (which agreed with those found by Dr. Chibnall) are as follows:

Solvent	R_F values		
	α-Glutamine	γ-Glutamine	10G
Butanol–acetic acid–water	0·12	0·11	0·50
Phenol–water	0·39	0·58	0·85

It may be pointed out that the relatively high R_F values for 10G in both solvents would be consistent with the identification of 10G as the *N*-ethyl derivative of γ-glutamine. Opportunity for direct comparison arose, however, as Dr. Sakato kindly presented us with a large sample of theanine. Its R_F values were identical with those of 10G in both butanol–acetic acid–water and phenol–water, and addition of theanine to a solution containing 10G intensified the 10G spot on the chromatograms. The evidence from paper chromatography is therefore fully in accord with the identification of 10G as theanine.

Isolation of theanine from tea.—Although the evidence for the identity of 10G and theanine so far quoted is very strong, further confirmation was sought by isolation.

It has already been shown[5] that theanine is extracted from dried green tea-leaf by methanol, and is precipitated from this extract by chloroform, together with theogallin and other polyphenols. The theanine remains in aqueous solution during the subsequent ethyl acetate extraction, and only a little is removed when the theogallin is precipitated by lead acetate. The solution left after lead acetate precipitation in the preparation of theogallin is therefore a source of theanine.

In one preparation this solution was freed from lead by treatment with hydrogen sulphide, and the filtrate evaporated almost to dryness *in vacuo*. A gum resulted which was dissolved in the minimum quantity of water and then precipitated with excess of acetone. A thick yellowish gum separated out and adhered to the sides of the vessel, and a finely dispersed white solid remained in suspension. This suspension was separated from the gum by decantation and then centrifuged. The white solid thus deposited consisted of crude theanine (10G) and after one recrystallization from aqueous ethanol no longer contained polyphenols, sugars or other amino-acids in amounts sufficient to be detected on paper chromatograms.

The product so obtained was indistinguishable from Sakato's specimen [Found: ash, 1·73; C, 48·40; H, 8·05; N, 16·04 (all ash-free). Calc. for $C_7H_{14}O_3N_2$: C, 48·26; H, 8·10; N, 16·08%]. M.p. 216–217°, identical with that found for Sakato's specimen and for a mixed m.p. of the two specimens. Sakato[2] records the m.p. of recrystallized theanine as 217–218°.

The method of preparation described above is somewhat unsatisfactory and yields are small, so that alternative procedures are being developed. In the most successful of these an infusion, obtained by extracting made tea (200 g.) with boiling water (5 l.) for five minutes, was treated with excess of saturated lead acetate solution (110 c.c.). The filtrate was freed from lead by H_2S and then passed through a column of Zeo-Karb 215. The procedure from this point on was the same as that described by Westall[6] in the isolation of glutamine from beet. The theanine is displaced from the resin by 0·17N-ammonia immediately after alanine and just before valine, but with considerable overlapping. Fractions rich in theanine, totalling about 150 c.c., were mixed and passed through another Zeo-Karb 215 column (100-mesh) and eluted with 0·17N-ammonia, as before. Fractions rich in theanine were again collected, but these still contained small amounts of glutamine, alanine, valine, proline and leucine. By recrystallization from aqueous acetone theanine was obtained, which on paper chromatograms showed no traces of these contaminants. The yield was only 200 mg., which is rather less than a tenth of the amount of theanine believed to be present in the tea. This low yield may be attributed in part to incomplete extraction in the infusion, and to adsorption by the lead acetate precipitate.

Discussion

The results may be taken as establishing the identity of the major component of the amino-acid fraction present in green tea-leaf or made tea as theanine, shown by Sakato[2] to be *N*-ethyl-γ-glutamine. From available figures for amino-acid nitrogen in teas, which will be reported later, and the preponderance of theanine in this fraction, it may be estimated that theanine accounts for from 1–2% of the total dry matter of tea. Theanine may therefore be expected to contribute quite appreciably to the liquoring characters of a tea, and the importance of establishing its nature beyond reasonable doubt will be readily apparent. The extent of this contribution to liquoring characters will receive further attention.

Acknowledgments

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THE SUGARS OF MANUFACTURED TEA

By R. A. CARTWRIGHT and E. A. H. ROBERTS

An aqueous extract of made tea, after removal of polyphenolic matter and passage through ion-exchange resins, was examined for sugars by paper chromatography. Glucose, fructose, sucrose, arabinose and ribose were detected. Less positive identifications were made of maltose, raffinose and rhamnose. *meso*Inositol was also found.

Introduction

In their investigations into the chemical composition of tea, Bradfield & Penney[1] fractionated the water-soluble extract into polyphenolic matter, caffeine, inorganic ash and ' non-phenolic gum '. The last accounted for about 10% of the dry matter of the tea. This gum was stated to contain carbohydrate material, but was not further investigated as it was believed to contribute little or nothing to the liquoring characters of the tea.

From what is known about the chemical composition of tea it would be expected that the chief components of this gum would be the amino-acids, carbohydrates and pectins. It has already been shown[2] that the gum contains appreciable amounts of the recently discovered *N*-ethyl amide of glutamic acid, theanine,[3] together with appreciable amounts of other amino-acids, the most abundant of which were aspartic and glutamic acids, alanine and valine.

In view of the availability of large quantities of this fraction containing gum, resulting as a by-product from the isolation of polyphenolic fractions (to be reported later), it was decided to examine the material by paper chromatography in order to determine what sugars were likely to be present. Previous work[4] has already shown that reducing sugars, expressed in terms of glucose, are to be found in appreciable amounts (1·4%) in fermented tea, and the osazone of glucose has been prepared from an extract of unfermented tea-leaf.

Methods

One-way paper chromatography proved inadequate to resolve the mixture of sugars present in the gum, so that a two-way method had to be developed. For most solvents used in the paper chromatography of sugars, the R_F values are dependent only upon the molar fraction of water in the mixture, the only exception being solvents which contain phenols as the organic component.[5] A suitable two-way system must therefore include one of these latter. Chromatographic solvents in general use in this Laboratory include phenol–acetic acid–water (80 : 2 : 18) and butanol–acetic acid–water (4 : 1 : 2·2), and this combination has been found to resolve quite complicated mixtures of sugars satisfactorily.

After two-way chromatography with these two solvents, the papers are dried and sprayed with *p*-anisidine hydrochloride (a 3% solution in water-saturated butanol). As is well known, this reagent gives characteristically coloured spots for many sugars. These colours are much more intense under ultra-violet light, and traces of sugars may be detected in this way that would be missed if the chromatograms were examined only in visible light. Chromatograms were also treated according to the silver method of Trevelyan, Procter & Harrison.[6] This method does not distinguish between pentoses and hexoses as *p*-anisidine does, but offers a convenient method of detecting *meso*inositol, which does not react with *p*-anisidine. Polyphenols, if present, are also detected.

Experimental

A suitable concentrate of sugars for paper chromatography was prepared in the following way. A strong infusion of made tea was obtained by extracting the tea (15 g.) with boiling water (375 c.c.) for five minutes and filtering the extract through a cotton-wool plug. The filtrate (250 c.c.) was extracted continuously with ethyl acetate for six hours, which removed caffeine and some of the polyphenolic matter.[1] The residual aqueous layer was then precipitated with excess of saturated lead acetate solution (20 c.c.). The filtrate was freed from lead by treatment with hydrogen sulphide and concentrated to 150 c.c. This filtrate contains both the gum and inorganic ash. The ash interferes considerably with paper chromatography ; the filtrate was therefore passed successively through columns of De-Acidite and Zeo-Karb 215

in order to remove cations, organic acids and the amino-acids. The solution, after passing through the two columns, was then evaporated *in vacuo* to a thin syrup and examined on two-way paper chromatograms.

The type of result obtained is illustrated in Fig. 1. Strong spots are obtained corresponding with glucose and fructose, with a rather weaker one for sucrose. Some confirmation of these identifications is provided by the intensifications observed when any of these three sugars is added to the mixture applied to the origin of the chromatogram. The almost universal distribution of these sugars in the plant kingdom makes it most probable that these identifications can be accepted without the usual reservations that must be made when relying upon paper chromatography alone.

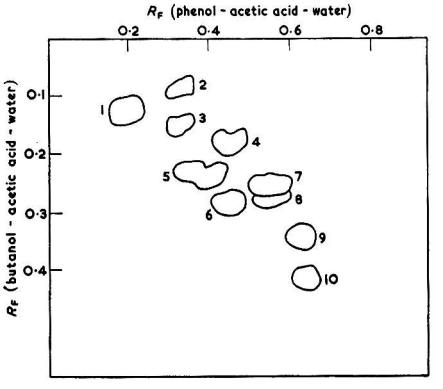

FIG. 1.—*Paper chromatogram of sugar concentrate from made tea plus added xylose. The chromatogram was run first from left to right with phenol–acetic acid–water, and then downwards with butanol–acetic acid–water*

(1) *meso*Inositol
(2) Raffinose
(3) Maltose
(4) Sucrose
(5) Glucose
(6) Xylose
(7) Fructose
(8) Arabinose
(9) Ribose
(10) Rhamnose

The identifications of arabinose and ribose, which are based on similar considerations, must be assigned a high level of probability. A very faint spot below ribose corresponds with the position taken up by rhamnose. As tea contains rutin,[7] which is a rhamnoglucoside, the presence of rhamnose in tea is not unlikely. There is less certainty about the tentative identifications of maltose and raffinose. These comparatively weak spots are intensified by addition of maltose and raffinose to the mixture, and these sugars are likely components of plant tissues; but it must be remembered that there are a large number of possible di- and tri-saccharides that will not be very much separated from each other by the solvent systems employed, and there is a reasonably high probability that other di- and tri-saccharides would occupy the same position on the chromatograms as maltose and raffinose.

Xylose cannot be present in significant amounts, as addition of this sugar to the mixture results in the production of a spot not found on chromatograms of the mixtures investigated.

With the alkaline silver nitrate reagent of Trevelyan, Procter & Harrison, an additional very strong spot is observed on the chromatograms, which is intensified on addition of *meso*-inositol to the mixture. The presence of *meso*inositol in tea has previously been reported,[8–10] and this observation may be taken as confirming the earlier reports.

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The authors wish to express their thanks to Dr. L. H. Lampitt for his interest in this work, and to the Indian Tea Association (London) for permission to publish this paper.

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THE PLANT-GROWTH-PROMOTING PROPERTIES OF GIBBERELLIC ACID, A METABOLIC PRODUCT OF THE FUNGUS *GIBBERELLA FUJIKUROI*

By P. W. BRIAN, G. W. ELSON, H. G. HEMMING and MARGARET RADLEY

Gibberellic acid is a metabolic product of the fungus *Gibberella fujikuroi*, similar in physiological properties to the gibberellins described by Japanese investigators. Supplied in a nutrient solution to wheat plants growing in water culture, it causes increased growth of the shoots, as a result of increased length of stem internodes and leaves. The leaves are narrower and paler than those of untreated plants. Under similar conditions pea seedlings develop much elongated stem internodes, but the leaves are little changed in size. These morphological responses are similar to those seen in etiolation.

The total dry weight of both peas and wheat so treated is increased; if shoots alone are considered the increase in dry weight is even greater; the dry weight of roots is reduced, though this does not occur if the gibberellic acid is applied to the shoots in a lanolin paste. The increased dry weight is mainly attributable to increased carbon assimilation. Treated plants contain more soluble carbohydrate than controls, but this accounts only for a small part of the increased carbon assimilation. The increase in glucose content is particularly striking.

Gibberellic acid is rapidly biologically degraded in soil.

Introduction

A soil-borne fungus, *Gibberella fujikuroi* (Saw.) Wr. (conidial stage *Fusarium moniliforme* Sheld.), is the cause of a disease of rice seedlings, not uncommon in Japan and other countries of the Far East, and recently found in Italy. A characteristic early symptom of the disease is elongation of the shoot, so that, in an infected crop, diseased plants are much taller than healthy ones, giving an impression that the crop has germinated unevenly. In the later stages of the disease fungal invasion causes considerable necrosis of basal tissues of the plant and the seedlings become weakly or die.

Kurosawa[1] showed in 1926 that the symptoms of overgrowth could be induced in rice seedlings by applying cell-free filtrates from liquid cultures of *G. fujikuroi* to the roots. This observation was confirmed and extended by other Japanese workers[2–7] shortly afterwards. The main features of the action established by this work were: (*a*) that though overgrowth of shoots was produced by culture filtrates, growth of roots was unaffected; (*b*) that not only rice plants, but other plants, including dicotyledons as well as monocotyledons, responded by increased shoot growth; (*c*) that the active material in culture filtrates could be adsorbed on charcoal and eluted by acetone or ether; (*d*) that the increased height of plants was sometimes associated with an increase in dry weight, though more frequently dry weight was unaffected.

An active material was isolated from culture filtrates in pure form by Yabuta & Hayashi,[8] which was named gibberellin A.[9] Another active material, gibberellin B, was also obtained, but gibberellin A was the main substance isolated. Its biological properties were described in a number of papers which will be referred to elsewhere in this paper. Gibberellin A produced overgrowth of seedlings of a great variety of plants if introduced into culture solutions in which they were growing at concentrations[10] as low as 1·4 μg./ml.

The properties of gibberellin appear to have been generally overlooked by plant physiologists. It was suggested to us by Dr. W. A. Sexton, of I.C.I. Ltd., Dyestuffs Division, that further study of this substance was desirable. We have as yet been unable to isolate gibberellin A from the cultures of *G. fujikuroi* that we have examined, but have isolated instead a substance with similar biological properties but different in chemical and physical properties in several important respects; this new substance has been named gibberellic acid.[11] The biological properties of gibberellic acid seem to us to be of unusual interest and we have considered it desirable to publish a general account of our results at an early stage of our investigation.

Experimental methods

Plant culture.—Unless otherwise stated all plants were grown in water culture by the method described by Stokes.[12] All experiments were carried out in quadruplicate; 15–20 wheat

seedlings were grown in each culture vessel and 4 pea seedlings in each vessel. Wheat (variety Victor) was sown directly in the culture vessels; a week later, by which time the first leaf had usually emerged, the nutrient was removed and replaced by a fresh nutrient, containing gibberellic acid where appropriate. Peas (variety Meteor) were germinated in moist sand, and a week after sowing the seedlings were transferred to the water-culture vessels, the solutions including gibberellic acid if required. In most experiments Long Ashton nutrient solution[13] was used; in one experiment (G_1 in Tables III and IV) a Pfeffer nutrient[12] was employed. The Pfeffer solution at standard strength was similar to the 1/5th-strength Long Ashton solution. Unless otherwise stated gibberellic acid was used at 5 μg./ml. The culture vessels containing the experimental plants were kept in a shallow water bath, maintained at 25° in a glasshouse.

Dry weights.—Plant material was dried rapidly at 70° in a current of dry air.

Ash constituents.—Samples (200 mg.) were ashed[14] and the ash was dissolved in dilute nitric acid. Phosphorus was estimated by a vanadomolybdate method.[15] Potassium was estimated by flame photometry.

Total nitrogen was determined by micro-Kjeldahl digestion.[14, 16, 17]

Total carbon was estimated either by a potassium dichromate reduction method[18] (Tables V, VI and experiments G_1, G_2 and G_3 in Table IV) or by a gravimetric wet combustion[19] (all other experiments).

Total soluble carbohydrate.—Plant material was dried as for dry-weight determinations and finely ground in a beater mill; 50 mg. of this material was refluxed in 80% ethyl alcohol and the extract passed through a sintered-glass filter. The alcohol was removed under reduced pressure and the resulting aqueous extract shaken with chloroform. The chloroform extract was washed twice with water and the water washings were retained. The aqueous extract (chloroform-extracted) and the water washings were then bulked and made up to a convenient volume. Carbohydrates were estimated by a modification[20] of Dreywood's anthrone method.

Carbohydrates in expressed sap.—Plant material was dropped in boiling water immediately after harvesting and then drained. The sap was expressed in a pneumatic press.[21] Total carbohydrates were determined by the anthrone reagent. Chromatograms of expressed sap were run on Whatman's No. 1 paper, with a *n*-butanol–acetic acid–water (4 : 1 : 5) solvent system. The sugars could be located by spraying with aniline hydrogen oxalate or anisidine. For quantitative estimation of separated sugars, a series of spots of expressed sap were run in parallel. Both vertical edges of the sheet were sprayed with one of the reagents mentioned above, and strips corresponding to the indicated sugars cut out at right angles to the line of flow. Sugars were eluted from these strips with water and estimated by the anthrone method.

Results

Effect of gibberellic acid on bacteria and fungi

We have been unable to detect any effect, either inhibitory or stimulatory, on the growth of a number of bacteria and fungi. The bacteria, tested on tryptone yeast agar, included strains of *Bacillus subtilis, Bacterium aroideae, Bact. carotovorum, Bact. coli, Bact. phytophthorum, Bact. tracheiphilum, Pseudomonas marginalis, Ps. solanacearum, Salmonella typhi, Staphylococcus aureus* and *Xanthomonas campestris*. The fungi, tested in a germination test in a glucose–ammonium tartrate–salts medium, included strains of *Absidia glauca, Aspergillus niger, Botrytis allii, Fusarium coeruleum, F. graminearum, Mucor erectus, Myrothecium verrucaria, Penicillium digitatum, P. expansum, P. gladioli, Stemphylium* sp., *Thamnidium elegans* and *Trichoderma viride*.

Morphological response of higher plants to gibberellic acid

Wheat.—Wheat seedlings grown in nutrient solutions containing gibberellic acid show certain characteristic features (Fig. 1), the most striking of which is increase in height. Increases in height of the order of 50% are commonly obtained after 2–3 weeks' growth in a cool glasshouse. In one experiment the mean heights of plants in various concentrations of gibberellic acid were: untreated plants, 29 cm.; 0·01 μg./ml. of gibberellic acid, 31 cm.; 0·1 μg./ml., 35 cm.; 1·0 μg./ml., 43 cm.; 10 μg./ml., 50 cm. Other examples will be found in Table III.

The increase in height is due to increase in length of both stem and leaves. The width of

the leaf-blades decreases. Some typical measurements are shown in Table I. In this experiment the first leaf had almost completed growth before treatment was commenced, and the second leaf was already beginning to extend. The dimensions of the first leaf were little affected by gibberellic acid treatment, the second leaf noticeably increased in length and decreased in width, and the

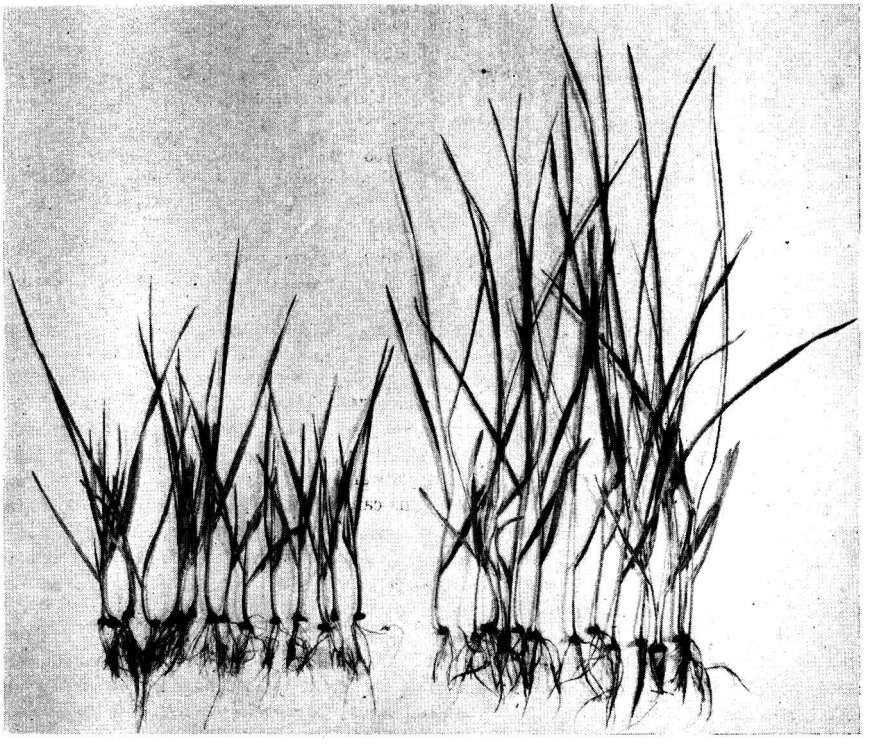

FIG. 1.—*Effect of gibberellic acid on growth of plants*
Wheat, var. Victor: gibberellic acid applied in culture solution, control plants on left, treated on right

third leaf was still more markedly affected. The fourth leaf had not finished growth when the measurements were made. It thus appears that gibberellic acid causes extension of only actively growing tissues and organs.

The following features of other responses to gibberellic acid may be noted: (*a*) Treated plants show some chlorosis; this feature is much more noticeable in plants grown in weak nutrient solutions; by raising the nutrient status the chlorosis can be offset to a considerable extent; thus, in the experiment illustrated in Table I, the chlorosis was most marked in those plants grown in 1/5 standard-strength nutrient without renewal, and least noticeable in those grown in standard-strength nutrient renewed weekly. (*b*) Leaf-roll develops progressively, especially in culture solutions of low nutrient status, so that plants appear spindly and seem to lack turgor. (*c*) The first leaf frequently becomes reflexed, apparently an epinastic response. If treatment of the plant is commenced at an early stage, for example by applying gibberellic acid to the seed, a noticeable response is a great extension of the mesocotyl, which extends well beyond the protecting coleoptile sheath, so that the seedlings develop a marked tendency to ' lodging '.

Increase in the gibberellic acid concentration above 10 μg./ml. leads to no increased response. Very high concentrations, of the order of 250–1000 μg./ml., are inhibitory. At 1000 μg./ml. the

plants extend little if at all more rapidly than untreated plants, and chlorosis, leaf-rolling and reduction in width of leaf-blades are much exaggerated.

Table I

Effect of gibberellic acid (5 μg./ml.) supplied in Long Ashton nutrient solution at standard (S) and 1/5 standard (S/5) concentrations on dimensions (mm.) of leaf-blades and the interval between bases of leaf-blades of wheat seedlings (L_1, L_2 etc. = first leaf, second leaf etc.)

Treatment	Nutrient level	Distance between bases of leaf-blades			Length of leaf-blades				Maximum width of leaf-blades			
		L_1–L_2	L_2–L_3	L_3–L_4	L_1	L_2	L_3	L_4	L_1	L_2	L_3	L_4
Untreated	S	45	22	16	110	214	293	253	4·3	4·7	4·9	5·5
	S/5	51	14		111	212	239		4·3	4·0	4·0	
	S, renewed weekly	40	19	17	103	200	279	262	4·2	4·6	5·3	6·3
	S/5, renewed weekly	44	14	10	98	195	251	117	4·3	4·1	4·1	4·1
Gibberellic acid	S	56	56	36	115	225	393	320	4·4	4·2	4·0	4·0
	S/5	58	59	22	104	223	333		4·3	3·7	3·2	
	S, renewed weekly	56	66	23	115	265	436	317	4·5	4·4	4·3	4·7
	S/5, renewed weekly	59	65		108	246	387		4·1	3·7	3·0	

Peas.—The effect of gibberellic acid on peas is very striking; increases in height of 500% have been observed. This is due to a great extension of internodes (Table II). The thickness of stems is reduced. Leaves are little affected in size, though they are usually a little larger than those of comparable untreated plants; petioles are greatly lengthened. Treated plants are yellower than control plants but, as in wheat, this can be to a considerable extent offset by supply of extra nutrients.

Table II

Effect of gibberellic acid (5 μg./ml.) supplied in Long Ashton nutrient solution on dimensions (mm.) of internodes, stipules and leaflets of pea seedlings (L_1, L_2 etc. = first leaf, second leaf etc.)

	Untreated controls			Gibberellic acid		
	L_1	L_2	L_3	L_1	L_2	L_3
Mean length of stipules	15·9	20·4	18·5	16·8	21·8	18·5
,, diameter of stipules	9·9	12·1	11·0	10·8	11·5	11·6
,, length of leaflets	20·8	21·0	17·7	25·0	23·7	15·6
,, diameter of leaflets	13·5	16·4	14·2	14·0	15·8	10·8
,, length of petioles	12·4	14·3	15·5	19·6	29·9	34·6
	L_1–L_2		L_2–L_3	L_1–L_2		L_2–L_3
Mean length of internodes	23·8		22·4	75·7		99·0

As with wheat, the maximum effect is produced by a concentration in the range 1–10 μg./ml.; further increase in the concentration to 100 μg./ml. does not increase the response (see Table XI). If the concentration is increased still further to 1000 μg./ml., treated plants are no taller than controls and the leaves are much reduced in size.

Other plants.—The following plants also respond to gibberellic acid in the same general way: oats, various grasses, clover, cucumber, tomato, linseed and dwarf bean (*Phaseolus*). The effect on dwarf bean is remarkable. In dwarf beans apical dominance is lost at an early stage of development, and side-shoots develop to the same extent as the main axis, resulting in a bushy habit. After treatment with gibberellic acid, lateral branching is inhibited and the main axis extends to produce very long internodes, which assume a twining habit as in climbing beans.

Methods of application

In most of the work described in this paper gibberellic acid has been applied to the plants through the roots. Similar responses are obtained if it is applied locally to leaves or stems in

lanolin, if sprayed on the foliage, or even if applied to the seed as a dry seed-dressing before sowing.

Effect of gibberellic acid on the chemical composition of wheat seedlings

Fresh and dry weight.—The percentage increases or decreases in fresh and dry weight of wheat seedlings, resulting from addition of gibberellic acid (5 μg./ml.) to nutrient solutions, obtained in four experiments, are presented in Table III.

Table III

Mean percentage increase or decrease in height, fresh weight (FW) and dry weight (DW) of wheat seedlings, resulting from gibberellic acid treatment (5 μg./ml.)

Expt. No.	Nutrient concn.		2 weeks' growth Height	FW	DW	3 weeks' growth Height	FW	DW
G_1	Pfeffer	Shoot	+54	+ 9	+22	+86	+ 9	+24
		Root		+ 1	− 8		−24	− 5
		Total		+ 7	+11		− 3	+12
G_1	Pfeffer, × 5 concn.	Shoot	+62	+21	+15	+108	+37	+33
		Root		+ 2	+ 8		−13	0
		Total		+ 6	+13		+23	+25
G_2	Long Ashton, 1/5 concn.	Shoot	+49	+17	+ 8	+74	+21	+26
		Root		−12	−10		−12	−17
		Total		+ 8	+ 3		+ 7	+14
G_2	Long Ashton, standard concn.	Shoot	+50	+27	+15	+80	+11	+14
		Root		−24	− 8		−25	−14
		Total		+12	+ 8		− 3	+ 6
G_3	Long Ashton, 1/5 concn.	Shoot	+58	+19	+24	+93	+17	+14
		Root		−29	+ 6		−35	−34
		Total		− 3	+10		− 4	− 3
G_3	Long Ashton, standard concn.	Shoot	+54	0	+ 5	+69	+ 4	− 1
		Root		−18	− 6		−29	−16
		Total		− 6	+ 2		− 5	− 5
G_4	Long Ashton,* standard concn.	Shoot	+46	+ 6	+21	+63	0	+19
		Root		−21	− 6		−25	−12
		Total		− 1	+15		− 8	+ 9

* Renewed every 2 days

In three experiments (G_1, G_2 and G_4) gibberellic acid consistently increased the dry weight of shoots, and in nearly all cases also the fresh weight. On the other hand, fresh and dry weights of roots were consistently decreased. The dry weight of the whole plant was consistently increased, the fresh weights somewhat less regularly. From these three experiments as a whole there can be no doubt that the increased growth of shoots, which is the most obvious symptom of gibberellic acid treatment, is associated with a substantial increase in dry-matter accumulation in the plant as a whole. The increases observed in shoots result from two processes: (*a*) an increase in over-all assimilation and (*b*) a redistribution of material within the plant, a greater proportion of the total dry matter being found in the shoots than in normal plants.

The results obtained in experiment G_3 exhibit some points of difference. Here the increase in dry weight of shoots and loss in roots is again shown well, but there was little or no effect on total dry weight. The increase in weight of shoots in this case is accounted for entirely by redistribution of the dry matter between root and shoot. This experiment was carried out during a period of particularly low light intensity, and it is believed that the consequent limitation of the rate of photosynthesis explains the absence of increases in total dry matter.

The detailed dry- and fresh-weight results for one experiment (G_2) are given in Tables V and VI. A point worth notice is that increase in nutrient concentration increases dry weight of roots and shoots, but in no way obscures the effects of gibberellic acid. In experiment G_4, where the nutrient solution was changed every two days, so that no nutrient would be limiting, increases in dry weight of shoot and whole plant were very marked as a result of gibberellic acid treatment, and losses in dry weight of roots were equally well marked.

A further analysis of the dry-weight increases in three of the experiments is summarized in Table IV Tables V and VI give results for one experiment (G_2) in detail.

Table IV

Mean percentage increase or decrease in ash, carbon (C), nitrogen (N), phosphorus (P) and potassium (K) contents of wheat seedlings, resulting from gibberellic acid treatment (5 μg./ml.)

Expt. No.	Nutrient concn.		2 weeks' growth Ash	C	N	P	K	3 weeks' growth Ash	C	N	P	K
G_1	Pfeffer	Shoot	+ 3	+33	−17	+ 3	+10	+ 2	+36	−13	0	+20
		Root	−13	− 4	−25	−20	−40	−22	−15	−20	−20	
		Total	0	+ 8	−18	0	+ 5	− 2	+19	−15	− 3	
G_1	Pfeffer, × 5 concn.	Shoot	+ 7	+ 6	+ 9	−12	− 6	+24	+40	+16	+ 2	+35
		Root	+16	+10	0	+26	−24	−10	0	−13	−23	−42
		Total	+ 8	+ 7	+ 7	+ 3	− 7	+17	+29	+12	− 6	+28
G_2	Long Ashton, 1/5 concn.	Shoot	+ 3	+ 6	+ 2	+10	+10	+ 5	+30	+10	+ 3	+20
		Root	−11	− 5	−11	−20	−41	−11	−17	−15	−17	
		Total	0	+ 3	0	+ 6	+ 4	+ 2	+16	+ 5	0	
G_2	Long Ashton, standard concn.	Shoot	+13	+17	+17	+14	− 6	+13	+13	− 1	+ 8	+35
		Root	−11	− 5	−12	0	−24	−18	−13	−15	−30	−42
		Total	+ 7	+10	+ 3	+10	− 7	+ 4	+ 6	− 4	− 8	+28
G_4	Long Ashton,* standard concn.	Shoot	+24	+26	+11	0	+ 8	+11	+24	+ 5	+13	+11
		Root	− 9	− 3	−10	−18	−28	−22	− 1	− 8	−23	−30
		Total	+15	+18	+ 8	−10	+ 3	− 3	+18	+ 3	−15	+ 5

* Renewed every 2 days

Table V

Effect of gibberellic acid (GA), at 5 μg./ml. in 1/5 standard-strength Long Ashton nutrient solution, on mean fresh weight (FW), dry weight (DW) and ash, carbon (C), nitrogen (N), phosphorus (P) and potassium (K) contents of wheat seedlings, all expressed as mg./plant

Period of treatment, weeks		Shoot Control	GA	Diff.	Root Control	GA	Diff.	Whole plant Control	GA	Diff.
2	FW	249	291	+42	111	98	−13	360	389	+29
	DW	24	26	+2	10	9	−1	34	35	+1
	Ash	4·0	4·1	+0·1	0·9	0·8	−0·1	4·9	4·9	0
	C	9·5	10·1	+0·6	4·3	4·1	−0·2	13·8	14·2	+0·4
	N	1·24	1·27	+0·03	0·27	0·24	−0·03	1·51	1·51	0
	P	0·28	0·31	+0·03	0·05	0·04	−0·01	0·33	0·35	+0·02
	K	1·25	1·37	+0·12	0·14	0·08	−0·06	1·39	1·45	+0·06
3	FW	293	355	+62	218	191	−27	511	546	+35
	DW	31	39	+8	12	10	−2	43	49	+6
	Ash	3·7	3·9	+0·2	0·9	0·8	−0·1	4·6	4·7	+0·1
	C	12·5	16·3	+3·8	5·2	4·3	−0·9	17·7	20·6	+2·9
	N	1·26	1·38	+0·12	0·27	0·23	−0·04	1·53	1·61	+0·08
	P	0·33	0·34	+0·01	0·06	0·05	−0·01	0·39	0·39	0
	K	1·21	1·45	+0·24	n.d.	0·08	n.d.	n.d.	1·53	n.d.

n.d. = no potassium determinations were made

Ash, nitrogen, phosphorus and potassium.—In general, the contents of ash, nitrogen, phosphorus and potassium increased in shoots and decreased in roots as a result of gibberellic acid treatment. Increases in the plant as a whole were frequent, especially in the more concentrated nutrients. If the concentration of these constituents on a dry-weight basis is calculated it is found that gibberellic acid treatment reduces their concentration in shoots and in the whole plants, as well as in roots. This is probably accounted for by a marked increase in carbon content (see below). No consistent differences in the uptake of nitrogen, phosphorus and potassium have been detected.

Carbon.—Gibberellic acid caused large increases in the carbon content of shoots, and, despite a reduction in the carbon content of roots, there is also an increase in the carbon assimilated by the plant as a whole. When the carbon concentration is worked out on a dry-weight basis it

is found that there is a consistent increase in the concentration of carbon per unit of dry weight in shoots, in whole plants and also in roots. It thus seems certain that one of the main effects of gibberellic acid is a considerable increase in the amount of carbon fixed by photosynthesis, since

Table VI

Effect of gibberellic acid (GA), at 5 μg./ml. in standard-strength Long Ashton nutrient solution, on mean fresh weight (FW), dry weight (DW) and ash, carbon (C), nitrogen (N), phosphorus (P) and potassium (K) contents of wheat seedlings, all expressed as mg./plant

Period of treatment, weeks		Shoot			Root			Whole plant		
		Control	GA	Diff.	Control	GA	Diff.	Control	GA	Diff.
2	FW	259	330	+71	110	84	−26	369	414	+45
	DW	27	31	+4	12	11	−1	39	42	+3
	Ash	5·5	6·2	+0·7	1·9	1·7	−0·2	7·4	7·9	+0·5
	C	9·0	10·5	+1·5	4·2	4·0	−0·2	13·2	14·5	+1·3
	N	1·50	1·60	+0·10	0·41	0·36	−0·05	1·91	1·96	+0·05
	P	0·35	0·40	+0·05	0·16	0·16	0	0·51	0·56	+0·05
	K	3·12	2·94	−0·18	0·37	0·28	−0·09	3·49	3·22	−0·27
3	FW	381	424	+43	214	153	−61	595	577	−18
	DW	36	41	+5	14	12	−2	50	53	+3
	Ash	7·1	8·0	+0·9	2·8	2·3	−0·5	9·9	10·3	+0·4
	C	12·7	14·3	+1·6	4·6	4·0	−0·6	17·3	18·3	+1·0
	N	2·02	1·99	+0·03	0·48	0·41	−0·07	2·50	2·40	−0·10
	P	0·39	0·42	+0·03	0·27	0·19	−0·08	0·66	0·61	−0·05
	K	3·34	4·51	+1·17	0·34	0·19	−0·15	3·68	4·70	+1·02

uptake of gibberellic acid could account only for an increased carbon content in each plant of the order of 4 μg., and probably accounts for much less. Inspection of the results in Tables III and IV shows that one-third to one-half of the dry-weight increase in shoots or whole plants is accounted for by increase in carbon ; if the carbon is considered to appear in the plant as carbohydrate, virtually the whole dry-weight increase can be accounted for ; the absolute increases of ash constituents are in any case of a much smaller order.

We have as yet little detailed information on the form in which this extra carbon accumulates in the plant, but there is some evidence that an increase in monosaccharides and disaccharides is of importance. After a preliminary experiment had indicated that gibberellic acid increased the carbohydrates soluble in 80% ethanol, we investigated the sugars in expressed sap. Wheat seedlings were grown for 14 days in Long Ashton nutrient, with and without 5 μg./ml. of gibberellic acid. Sap was expressed in the manner described above from 17 plants in each treatment. Untreated plants yielded 2·5 ml. of sap, treated plants 2·3 ml. The concentrations of soluble carbohydrate, and of glucose, fructose and sucrose after separation on a paper chromatogram, are given in Table VII. Total soluble carbohydrate was almost doubled as a result of gibberellic acid treatment. The results for separate sugars must be regarded with some caution,

Table VII

Effect of gibberellic acid treatment on concentration of carbohydrates (mg./ml.) in expressed sap from wheat seedlings

Treatment	Total carbohydrate (glucose equiv.)	After elution from chromatogram		
		Sucrose	Glucose	Fructose
None	3·03	1·06	0·73	0·44
Gibberellic acid	5·90	1·46	2·24	0·87

since there were losses on the chromatogram (see Table VII), but it is clear that although sucrose and fructose concentrations were greatly increased as a result of gibberellic acid treatment, the main increase was in the glucose concentration. In the sap of untreated plants the main sugar is sucrose ; in the treated plants it is glucose. These experiments were carried out during winter without supplementary lighting and no starch could at any time be detected in leaves.

Effect of gibberellic acid on the chemical composition of pea seedlings

Fresh and dry weight.—Addition of 5 μg./ml. of gibberellic acid to culture solutions in which pea seedlings are growing causes marked increases in dry and fresh weight; the results of a typical experiment are presented in Table VIII. As in wheat the effect is complex, weights of shoots being greatly increased and of roots decreased.

Table VIII

Effect of gibberellic acid (GA), at 5 μg./ml. in standard-strength Long Ashton nutrient solution, on mean fresh weight (FW), dry weight (DW) and ash, carbon (C), nitrogen (N), phosphorus (P), potassium (K) and carbohydrate (soluble in 80% ethanol) contents of 15-day pea seedlings, all expressed as mg./plant

	Shoot			Root			Whole plant		
	Control	GA	Diff.	Control	GA	Diff.	Control	GA	Diff.
FW	1099	1697	+598	922	734	−188	2021	2431	+410
DW	86	112	+26	34	25	−9	120	137	+17
Ash	11·3	14·1	+2·8	8·3	5·6	−2·7	19·6	19·6	0
C	34·3	45·2	+10·9	11·3	8·1	−3·2	45·6	53·3	+7·7
N	7·7	9·1	+1·4	2·0	1·3	−0·7	9·7	10·4	+0·7
P	0·96	1·24	+0·28	0·86	0·47	−0·39	1·82	1·71	−0·11
K	3·37	4·07	+0·70	3·12	1·88	−1·24	6·49	5·95	−0·54
Soluble carbohydrate	5·2	7·2	+2·0	0·9	0·6	−0·3	6·1	7·8	+1·7

Ash, nitrogen, phosphorus and potassium.—The contents of ash, nitrogen, phosphorus and potassium also increase in shoots and decrease in roots, as was found with wheat. The actual concentrations per unit of dry weight fell in both shoots and roots, as in wheat, as a result of a relatively great increase in carbon.

Carbon.—As in wheat, gibberellic acid treatment markedly increases carbon assimilation; in the experiment illustrated in Table VIII there was an over-all increase of 17%, considering the plant as a whole. Root and shoot were differentially affected, the shoots showing a 32% increase and the roots a 29% decrease in carbon content. The increase in carbon accounts for 38% of the total dry-weight increase of the plant.

Soluble carbohydrate is increased as a result of gibberellic acid treatment (Table VIII), but this accounts for only a small part of the increased carbon content. No starch was found in these plants. It seems likely, therefore, that the main forms in which the additional carbon appears will be protein or cell-wall material. Since the increase in total nitrogen is small, cell-wall material is probably of greater importance.

The carbohydrates in expressed sap have been examined (Table IX) with results similar to those found with wheat. Treated plants yielded approximately twice the volume of sap yielded by control plants. The sap from plants treated with gibberellic acid contained a higher concentration of soluble carbohydrates. As in wheat, the increase is largely due to an increase in the glucose concentration. The greater carbohydrate content of sap from treated plants was seen both at 9 a.m., when the concentration in control plants was relatively low, and at 4 p.m., when it was high.

Table IX

Effect of gibberellic acid (GA) on concentration of carbohydrates in expressed sap of pea seedlings (FW and DW in mg./plant; sap volume in ml./plant; all carbohydrates in mg./ml. of sap)

Age	Time of harvest	Treatment	FW	DW	Sap volume	Total soluble carbohydrate	After elution from chromatograms		
							Sucrose	Glucose	Fructose
7 days	9.0 a.m.	Control	408		0·21	8·4	1·9	3·1	3·2
		GA	658		0·43	13·8	1·7	8·3	3·0
	4.0 p.m.	Control	448		0·18	10·7	4·2	3·5	3·3
		GA	719		0·45	15·0	3·7	8·3	3·2
16 days	9.0 a.m.	Control	768	56	0·48	6·8	0·8	2·3	2·3
		GA	1378	71	0·95	7·6	1·3	3·4	2·0
	4.0 p.m.	Control	627	48	0·40	11·9	4·9	3·0	2·1
		GA	1254	73	0·75	14·8	3·1	3·4	1·9

Biological breakdown of gibberellic acid in soil

In this study the soil used was a John Innes Compost No. 2, known to contain a vigorous bacterial and fungal microflora. Four 20-kg. batches of this soil were prepared as follows: untreated, untreated + gibberellic acid (100 mg./kg. of soil), heat-treated (autoclaved 30 min. at 15 lb./sq. in.), heat-treated + gibberellic acid (100 mg./kg.). Each of these four lots of soil was stored at 25° in closed sterile containers. Four tall 400-ml. beakers were filled with each soil immediately after its preparation, and in each beaker 8 pea seeds, var. Meteor, were sown. This process was repeated with samples of each batch of soil taken at intervals during a total period of 38 days. The beakers sown with pea seeds were placed in a greenhouse and the heights of the seedlings produced were measured at frequent intervals. Mean-growth curves were drawn from these results for each treatment at each time of sampling. The time when pea seedlings in untreated soil had reached a mean height of 100 mm. was determined from these curves for each time of sampling, and the mean heights of seedlings in treated soils at these times were similarly derived. The effects on growth caused by the various treatments are presented in Table X.

Table X

Persistence of gibberellic acid (GA) in heat-sterilized and unsterile soil as measured by the effect on height of pea seedlings

Days storage	Mean height of seedlings, mm.			
	Unsterile soil	Sterile soil	Unsterile soil + GA	Sterile soil + GA
0	100	110	340	328
1	100	110	300	358
2	100	120	360	376
3	100	116	362	362
6	100	102	230	300
8	100	118	218	380
13	100	102	152	348
20	100	112	126	360
24	100	118	126	360
31	100	112	110	364
38	100	110	124	374

Heat-treatment of the soil caused a small but constant increase in the height of plants. Addition of gibberellic acid to untreated soil at first caused a great increase (about 240 mm. over controls) in the height of seedlings, but this effect fell off with time, the increase being only of the order of 100 mm. after 8 days and negligible after 31 days. In heat-treated soil the original increment in height was similar to that in untreated soil and this initial increment was maintained throughout the experiment.

This affords strong evidence for supposing that gibberellic acid is broken down in soil by the metabolic activity of the soil microflora. The breakdown of gibberellic acid is in fact more rapid than might be supposed by inspection of Table X, since this takes no account of the dose/response relations of gibberellic acid to pea seedlings. Table XI shows the effect of gibberellic acid included in heat-treated soil at various concentrations, the effect being measured in the same way as in Table X. Although it is scarcely possible to derive an accurate dose/response curve from these results, it is fairly clear that a maximum effect is produced by gibberellic acid included in this soil at about 6 mg./kg., and that the median growth-promoting dose is about 1 mg./kg. Interpreting Table X in the light of this dose/response relationship, it appears that in the untreated soil nearly all the gibberellic acid added to the soil (100 mg./kg.) had been inactivated in 6 days, in spite of an initial lag of 3 days.

Discussion

The most obvious effect of gibberellic acid on plants is to cause a marked increase in height of the shoot by increase in length of the stem internodes; it affects only the length of internodes still extending at the time of treatment. The question will naturally be asked, is this due to extension of cells, to cell multiplication or to both? We have not sufficient data to give a final

answer to this question; with pea seedlings there is undoubtedly a great increase in length of cells of the stem internodes, and this is of an order sufficient to account for the increased length of the internodes. Yabuta & Hayashi,[10] while noting the increased cell extension produced by gibberellin, concluded that some cell multiplication must also take place, but presented no quantitative evidence in favour of their view. This is a point which needs further investigation.

Table XI

Effect of gibberellic acid included at several concentrations in sterile soil on mean height of pea seedlings, measured at time when controls had reached mean height of 100 *cm.*

Concn. of gibberellic acid, mg./kg.	Mean height of pea seedlings, mm.
100	310
50	320
25	300
12·5	312
6·25	320
3·125	290
1·6	264
0·8	200
0·4	128

The morphological response of wheat seedlings, consisting of (*a*) great increase in length of mesocotyl, (*b*) increase in length of stem internodes, (*c*) increased length of leaves and (*d*) diminished width of leaves, is strikingly like the morphological response to reduced light intensity, namely the morphological component of etiolation. In pea seedlings the similarity is less striking, in that, although increased length of internode is characteristic of both gibberellic acid treatment and etiolation, the reduced area of leaf laminae characteristic of etiolation is not in our experience produced by gibberellic acid treatment.

In many ways the most interesting result of gibberellic acid treatment is increased dry weight of plants. The results presented in this paper indicate that under suitable conditions substantial increases in dry weight of whole plants are produced. If the shoot alone is considered, the increases in dry weight are even greater, because, in addition to the over-all increase in dry-matter accumulation, gibberellic acid treatments of the kind reported here appear also to result in a higher proportion of the total dry matter being directed to the shoot and a smaller proportion to the root. Although this redistribution between shoot and root has been characteristic of all experiments reported here, we do not consider that it should be supposed to be a necessary consequence of gibberellic acid treatment, but rather of the specific method of application (i.e. to the roots) used in the experiments quoted. On two occasions we have applied gibberellic acid in a lanolin paste to the shoots of pea seedlings and this resulted in considerable dry-weight increases of both shoots and roots. It may be that when gibberellic acid is applied to the roots too high a local concentration is built up.

The means by which this gross increase in dry weight is built up needs further investigation. Our results show, as might indeed be expected, that it is mainly due to increased carbon assimilation. This might be achieved either by an increase in the photosynthesizing surface or by an increase in the net assimilation rate. Such results as we have (see Tables I and II) indicate that gibberellic acid treatment does produce a small increase in leaf area; in pea seedlings the green internodes are of importance in photosynthesis and the area of these is greatly increased. It seems possible, therefore, that the increased carbon assimilation is achieved without change of the net assimilation rate, though that possibility cannot be finally ruled out. Yabuta & Hayashi[10] consider that leaf area in many plants is reduced by gibberellin treatment.

Japanese investigators[10, 22, 23] have on occasion noted small increases in dry weight of gibberellin-treated plants but, in most of their experiments, dry weight was unaffected or even reduced. They did note a frequent reduction of dry weight of roots where the gibberellin was supplied in nutrient solution to the roots.

The physiological activity of gibberellic acid and gibberellin, particularly in so far as they promote cell extension, is reminiscent of that of the auxins. Sumiki[24] has reported that gibberellin A does not stimulate callus formation, does not cause epinasty of tomato petioles, and is

inactive in the standard *Avena* and Went pea tests for auxins. Similarly, we have found that gibberellic acid at most produces a very slight extension in the wheat-coleoptile-section test devised by Luckwill.[25] On the other hand, no auxins supplied in a nutrient solution to the roots of intact plants cause increased growth as does gibberellic acid or gibberellin. The physiological relationship between the auxins and gibberellic acid and gibberellin requires further study.

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STUDIES ON EGG SHELLS. V.*—Some Physical and Chemical Characteristics of the Egg Shells of Five Different Types of Pheasant

By C. TYLER and F. H. GEAKE

A study has been made of a number of physical and chemical characteristics associated with five different types of pheasant-egg shells. These include shell thickness, porosity coefficients, pore counts, membrane-, pore- and matrix-protein, calcium, carbonate, magnesium, phosphorus and citric acid. With most of these characteristics there were considerable variations between types, and, in particular, the wild type falls at one end or other of the range when the values are set out in order of magnitude. Certain relationships such as shell thickness : weight per unit area, shell weight : calcium content and calcium content : carbonate content were found, but some of these differed significantly from those found for hen-egg shells.

In an earlier paper (Part III of this series), Tyler & Geake[1] have described certain physical and chemical properties of hen-egg shells. From this work it was clear that a group of hens

* Part IV: *J. Sci. Fd Agric.*, 1954, **5**, 335

(Rhode Island Red × Light Sussex) showed pronounced differences between individuals in relation to a number of the characteristics studied.

A supply of about 100 pheasant eggs was made available and the shells of these eggs have now been examined in a manner similar to that used for the hen-egg shells. The findings are reported below.

Experimental

Material

The pheasant eggs were obtained from five types. The Blackneck (*Phasianus colchicus colchicus*), Chinese ringneck (*Phasianus colchicus torquatus*), Mongolian (*Phasianus colchicus mongolicus*) and the Melanistic mutant (*Phasianus colchicus tenebrosus*) have been kept free from crosses at the I.C.I. Game Research Station for five years and no striking variation has appeared in this time. These can therefore be regarded almost as sub-species. The wild type might be a cross of almost any kind and the birds producing the eggs for this experiment had been trapped and penned just before the beginning of laying.

Eggs, as laid, were taken from the different pens. Comparisons have been made between the five different types, and not between individuals within a type.

All the birds were fed alike, but it must be remembered that although the Blackneck, Chinese, Mongolian and Melanistic mutant types had been reared in captivity, and hence had been on the diet for some time, the wild type had received this diet for only a short time.

About 20 eggs of each type were originally available, but, owing to accidents or shortage of material, the full set of measurements was not made on every egg. The number of eggs from which each mean was calculated is shown in the Tables.

Methods

The methods for physical measurements and chemical analysis were the same as for hen-egg shells with the following exceptions: (*a*) Calcium was determined by the method of Biedermann & Schwarzenbach.[2] (*b*) The treatment of the true shell with 10% sodium hydroxide solution for the removal of pore protein was shown to be as successful over a 24-hour period as it was over the original 12-hour period. The longer time was therefore used since this was more convenient. (*c*) Pheasant-egg shells are thinner than hen-egg shells and it was therefore necessary when preparing the shell for pore counting to immerse it for only 10 seconds in nitric acid, instead of the 25 seconds used for hen-egg shells. This period gave maximum and reproducible values for pore counts.

Statistical treatment of the results

Analysis of variance was used on most of the results. The wild type was taken as a standard, because it is probably a mixture of some or all of the other types. The other four types were then compared with it. The various Tables do not show the full analysis of variance, but only the value and significance of the F test, with the means of each type and the significance of their respective differences from the wild type.

Results

There are far fewer results available on pheasant eggs than on hen eggs and it has therefore been decided to present information on fresh weight and shape index as well as the other information.

Fresh weight and shape index (Table I)

It is clear that the Chinese type gave the heaviest eggs and the wild birds the lightest, but Blackneck and Mongolian also laid significantly heavier eggs than the wild type.

Shape index is here taken as breadth divided by length. Only the Blackneck differed significantly from the wild type, being broader in relation to its length than the rest.

Shell thickness (Tables II and III)

In Part III it was suggested that, since there was such a highly significant regression of

Table I

Weight of fresh eggs, g., and shape index

Type	Variance ratio, $F = 11{\cdot}6$ ***		Variance ratio, $F = 6{\cdot}56$ ***
	No.	Wt., g.	Shape index
Blackneck	19	33·6 ***	0·804 ***
Chinese	19	35·3 ***	0·772 N.S.
Mongolian	19	34·8 ***	0·773 N.S.
Melanistic mutant	16	31·7 N.S.	0·783 N.S.
Wild	22	30·6	0·766
General mean		33·2	0·779

In this and succeeding Tables, *, ** and *** indicate significance at $P < 0{\cdot}05$, $< 0{\cdot}01$ and $< 0{\cdot}001$ respectively, and N.S. indicates non-significant values.

shell thickness on weight per unit area of true shell, then for hen eggs the latter value could be used as a quick and reliable means of calculating shell thickness, by means of the equation:

$$Y = 4{\cdot}83X - 15{\cdot}8$$

where X is weight of true shell in mg./sq. cm. and Y is the thickness in μ. It was therefore decided to consider the corresponding relationship with the pheasant-egg shells.

Table II shows that, with each type, there was a highly significant relationship, and that for all birds taken together the result was:

$$Y = 4{\cdot}39X + 0{\cdot}3$$

There is thus no doubt that, as with hen-egg shells, it is possible to use weight per unit area to calculate thickness with a very great degree of accuracy. It must be remembered, however, that this will give only the average thickness over the whole shell, and will not indicate variations over the different parts of the shell.

Table II

Relationship between weight, X, of true shell (mg./sq. cm.) and shell thickness, Y (microns)

Type	No.	Regression equation
Blackneck	19	$Y = 4{\cdot}26X + 10{\cdot}2$ ***
Chinese	18	$Y = 4{\cdot}25X + 5{\cdot}6$ ***
Mongolian	19	$Y = 4{\cdot}65X - 14{\cdot}5$ ***
Melanistic mutant	16	$Y = 4{\cdot}22X + 13{\cdot}0$ ***
Wild	22	$Y = 4{\cdot}56X - 10{\cdot}2$ ***
All types	94	$Y = 4{\cdot}39X + 0{\cdot}3$ ***

There is another point to be noted in connexion with these equations. The 'hen' and 'pheasant' equations give regression coefficients that are significantly different from each other ($P < 0{\cdot}05$). Now these equations can be used to calculate a series of apparent densities of the shell, and the average value for the hen is 2·19 and that for the pheasant 2·28, with very little variation on either side of these values. It is well known that the true shell contains only about 1–2% of protein and that the rest is almost all calcium carbonate, present as calcite. Thus, since calcite has a density of about 2·7 and the density of protein is about 1·0, it follows that the apparent density of the shell is much lower than would be expected. Further, one would not expect to find very great differences between species.

There may be a number of factors involved but the major one seems to be related to the structure of the shell. The inner layer of the shell consists of mammillae, which are knob-like projections, and the rest is solid shell. If the minute error caused by the curvature of the shell is ignored, the faces of the micrometer screw gauge will fit to the shell as shown in Fig. 1; hence the measurement made is one of maximum thickness. This maximum thickness multiplied by the unit surface area will thus give the apparent volume of that piece of shell and the relation to weight will give its apparent density. The difference between apparent

density as measured by us and true density as calculated, and the difference between apparent density of hen- and pheasant-egg shells, are therefore, in part at least, caused by the failure to measure true thickness, and hence to calculate true volume. This failure is related to the size of the projecting mamillae in relation to the rest of the shell. For example if there were no mamillae, but only solid shell, then there would be no discrepancy in the apparent volume measurement, i.e. it would be the same as the true volume, but if the shell consisted entirely of mamillae, regarded ideally as hemispheres, then the apparent to true volume ratio would be 1·5, i.e. 50% error. It may therefore be suggested that, the nearer the apparent density is to the true density, the smaller are the mamillae in relation to the shell as a whole.

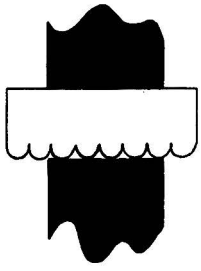

FIG. 1.—*Diagrammatic representation of a piece of shell between the jaws of a micrometer screw gauge*

The shell-thickness values and weights per unit area are given in Table III, but only the weights per unit area have been analysed, and from this it is clear that the Chinese and Mongolian types give shells significantly thicker than the wild type; the Blackneck and Melanistic mutant types are not significantly different from the wild in this respect.

Table III

Shell thickness and weights per unit area of true shell

Type	No.	Shell thickness, μ	$F = 2{\cdot}51$ * Shell wt., mg./sq. cm.
Blackneck	19	246	55·6 N.S.
Chinese	19	250	57·0 *
Mongolian	19	254	57·8 **
Melanistic mutant	16	247	55·3 N.S.
Wild	22	232	53·1
General mean		245	55·7

Pore counts and porosity coefficients (Table IV)

Table IV shows that there were considerable differences between pore counts for the different types, and that the wild type gave a significantly higher count than either Blackneck, Chinese or Mongolian. The pheasant-egg shells gave a general mean of 180 pores per sq. cm., compared with 154 for hen-egg shells.

Similarly, the porosity coefficients showed that the wild type gave the greatest value, but it was only significantly greater than the Blackneck and the Mongolian. The general mean for pheasants was 2·04, compared with 1·86 for hens.

Table IV

Pore count as number of pores per sq. cm. and porosity coefficients based on rate of loss of weight in mg./sq. cm./day at 15° over calcium chloride

Type	$F = 4{\cdot}42$ ** No.	Pore count	$F = 3{\cdot}23$ * No.	Porosity coefficient
Blackneck	17	180 *	19	1·81 **
Chinese	18	173 **	19	2·14 N.S.
Mongolian	19	164 ***	18	1·88 *
Melanistic mutant	16	184 N.S.	15	2·19 N.S.
Wild	21	198	21	2·20
General mean		180		2·04

From these two sets of figures the porosity coefficient per pore was calculated separately for each egg, but when the results were submitted to the analysis of variance it was found that there were no significant differences. It would thus appear that, generally speaking, the increase in porosity coefficient was chiefly caused by increasing numbers of pores, although other factors, such as shape of pores, must clearly have some influence. The general mean

porosity coefficient per pore for pheasants was 11·4 μg. and that for hens was 12·2 μg., but the values for each species covered roughly the same range.

Membrane protein (Tables V and VI)

As with hen eggs, the results (Table V) have been expressed as weight of membrane in mg./sq. cm. The Blackneck and Melanistic mutant show significantly larger values than the wild type, but the other two, Chinese and Mongolian, are not significantly different from it.

Table V

Weight of membrane (plus cuticle), mg./sq. cm.

Type	$F = 6{\cdot}90$ *** No.	Mean
Blackneck	19	3·00 ***
Mongolian	19	2·59 N.S.
Chinese	19	2·32 N.S.
Melanistic mutant	16	2·88 **
Wild	22	2·32
General mean		2·61

The general mean is 2·61 mg./sq. cm., which is considerably less than the 3·99 mg./sq. cm. given for hens in Part III. By using the same method of calculation as used then, the thickness is 0·033 mm. As far as the authors are aware there are no other published data for pheasant-egg-shell membranes, but Asmundson, Baker & Emlen[3] state that membrane thickness is roughly related to size of egg. In this respect there is no doubt that the smaller pheasant egg has a thinner membrane than the hen eggs studied, but for the five types of pheasant there is no indication of any such relationship, as will be seen in Table VI. The heaviest and lightest eggs have the thinnest membranes. Further, there appears to be no relation between thickness of shell and thickness of membrane amongst these five pheasant types.

Table VI

Type	Egg wt., g.	Shell thickness, μ	Membrane thickness, μ
Blackneck	33·6	246	38
Chinese	35·3	250	29
Mongolian	34·8	254	32
Melanistic mutant	31·7	247	36
Wild	30·6	232	29

True shell protein; pore protein and matrix protein (Table VII)

With the exception of the Melanistic mutant, the other types were all significantly lower in true shell protein than the wild type and the difference was considerable. However, the mean value for all birds was not greatly different from the general mean for hens, the two values being 1·59 and 1·53% respectively.

The results for pore protein (Table VII) indicate that once more there are type differences. Blackneck, Melanistic mutant, and wild types were not significantly different, but Chinese and Mongolian were significantly lower than the wild. Compared with hens, which gave a general mean of 0·77%, the pheasant general mean was 0·81%. The pore protein per pore was calculated for individual eggs, but it was so variable that there were no significant differences between types. However, the mean value for the pheasant-egg shells was much lower than for hens, these being 2·48 μg. and 3·85 μg. respectively.

When the matrix protein in hen-egg shells was studied, significant differences were found between individual birds, but it is evident that there were no significant differences between

Table VII

True shell protein, pore protein and matrix protein, %

Type	No.	$F = 6{\cdot}73$ *** Shell	$F = 6{\cdot}30$ *** Pore	$F = 1{\cdot}05$ N.S. Matrix
Blackneck	19	1·57 *	0·84 N.S.	0·73 N.S.
Chinese	19	1·53 **	0·74 **	0·79 N.S.
Mongolian	19	1·42 ***	0·64 ***	0·78 N.S.
Melanistic mutant	16	1·72 N.S.	0·92 N.S.	0·80 N.S.
Wild	22	1·72	0·94	0·78
General mean		1·59	0·81	0·78

any of the pheasant types. Apart from the Blackneck, results were in very close agreement; the general mean was 0·78% compared with a value of 0·76% for hens.

Calcium and carbonate (Table VIII)

The results for hen-egg shells discussed in Part III showed that there were no significant differences between birds for the calcium in the nitrogen-free shell, and many analyses carried out since confirm this. This also holds for carbonate.

The values for calcium and carbonate for the pheasant eggs showed that significant differences exist between types, but these were so minute that they fell within the limits of the error of the analytical methods and therefore they can have little meaning. The results are not shown, but the general mean for calcium is 38·6% compared with 38·7% for hens; the respective carbonate values are 42·8 and 42·9%. This shows how even slight variations in analytical technique may give a quite erroneous picture when superimposed upon a factor that is almost constant.

For hen-egg shells it was found that there was a highly significant regression of g. of carbonate (Y) on g. of calcium (X) in the shell. One set of 73 shells and another of 49 shells gave, respectively, the equations:

$$Y = 1{\cdot}118X - 0{\cdot}002$$
$$Y = 1{\cdot}117X - 0{\cdot}015$$

Despite the errors in analysis, which were admittedly present, but very small, the pheasant-egg shells gave the equation:

$$Y = 1{\cdot}115X - 0{\cdot}007$$

Apparently then there is an almost identical relationship between the absolute amounts of calcium and carbonate in the shell of both hen and pheasant eggs, which is to be expected with such a constancy of calcium and carbonate percentages.

For hen eggs it was also shown that an equation relating weight of calcium in g. (X) to weight of shell plus membrane in g. (Y) had a very high degree of significance. A similar equation, also highly significant, has been obtained for pheasants. These two equations are given in Table VIII.

Table VIII

Values of shell calcium, g., calculated from the equations:

$X = 0{\cdot}374Y - 0{\cdot}072$ (Hen)
$X = 0{\cdot}368Y - 0{\cdot}014$ (Pheasant)

Shell wt., g. (Y)	Calcium in shell, g. (X)	
	Hen	Pheasant
2	0·676	0·722
4	1·424	1·458
6	2·172	2·194

In Part III it was suggested that the equation could profitably be used for determining the amount of calcium in an egg shell without the labour of analysis, and that such a procedure was well suited to be used in balance experiments. Comparison of the two equations for this purpose is therefore of interest. The equations have been used to calculate the values in Table VIII, but it is important to remember that in practice they should be used only over the actual range of data they cover. Clearly the error is greatest at the lower shell-weight values and can be very large. This is caused by the large difference in magnitude of the independent term, which itself is chiefly a measure of the relative amount of membrane present, and a lower regression coefficient means more protein in the true shell. Thus, if this method of evaluating shell calcium is to be used it will be necessary to obtain the correct equation at least for each species. Alternatively, it may be worth while to remove the membrane and get the weight of true shell, then simply multiply by a constant factor, since the protein in the true shell varies far less than the membrane. It has already been shown that the percentage of calcium in the nitrogen-free shell is almost constant for both hens and pheasants at a level of about 38·7%, and unpublished results show a similar level for other species.

Allowing for the percentage of protein in the true shell this would give the percentage calcium in the true shell as about 38·0%, and the use of this factor would almost certainly be sufficiently accurate to ensure that the error was little more than ± 1% for the eggs of any species. Thus the weight of true shell in g. multiplied by a factor of 0·38 will give an accurate measure of the g. of calcium in the shell. This is probably a more satisfactory method and one of wider application than the suggestion put forward in Part III, that is the use of an equation that referred to the shell with membrane still present. The process of removing the membrane and weighing the true shell is one that takes very little time compared with a calcium determination.

Magnesium, phosphorus and citric acid (Table IX)

From Table IX it can be seen that there is no significant difference between the Melanistic mutant and the wild type for percentage magnesium in the nitrogen-free shell, but the Blackneck, Chinese and Mongolian show significantly lower values than the wild type. This result differs from that for individual hens, where there were no significant differences; it is possible, however, for individuals of one breed to show no differences, but for types within a species to do so. The mean value for the pheasant eggs was 0·43% and for hen eggs 0·59%; there thus appears to be much less magnesium in the pheasant-egg shell than in the hen-egg shell.

With phosphorus, there are again significant differences between types, Blackneck and Mongolian being lower than the wild, but Chinese and Melanistic mutant not differing significantly. The respective general means for hen and pheasant are 0·13 and 0·20%.

Table IX

Percentages of magnesium, phosphorus and citric acid in the nitrogen-free shell

Type	$F = 13{\cdot}2$ ***		$F = 13{\cdot}5$ ***		$F = 13{\cdot}9$ ***	
	No.	Mg	No.	P	No.	Citric acid
Blackneck	19	0·37 ***	19	0·15 ***	19	0·13 N.S.
Chinese	19	0·43 **	19	0·21 N.S.	18	0·18 ***
Mongolian	19	0·41 ***	19	0·20 *	19	0·14 N.S.
Melanistic mutant	16	0·46 N.S.	16	0·22 N.S.	16	0·16 ***
Wild	22	0·48	22	0·22	22	0·13
General mean		0·43		0·20		0·15

The citric acid values show that the birds are in two groups: Blackneck, Mongolian and wild show no significant differences, but Chinese and Melanistic mutant are significantly greater in citric acid content. The general mean for pheasant eggs was 0·15% and for the hen 0·11%. In Part III it was found that there was a significant relationship between the weight of citric acid and the weight of true shell protein. By taking all the pheasant eggs together a relationship was again found.

If X is mg. of true shell protein and Y is mg. of citric acid, the equations are:

$$Y = 0{\cdot}09X - 1{\cdot}56 \text{ ***} \quad \text{(Hen)}$$
$$Y = 0{\cdot}45X + 1{\cdot}98 \text{ *} \quad \text{(Pheasant)}$$

The two relationships are quite different in type and the one for pheasants is significant only at $P < 0{\cdot}05$. Further, when pheasant types were considered separately no significant regression coefficients were found. No further comment will therefore be made about this until more results have been collected.

Discussion

From the results it is clear that there are pronounced differences between the shell characteristics of these different types of pheasant. Moreover, in relation to almost every factor studied, the wild type occupied one end or other of the range. Thus, it gave the smallest eggs, the narrowest eggs, the thinnest shells, the thinnest membranes, the lowest matrix protein (apart from the Blackneck) and the lowest citric acid in nitrogen-free shell (except for Blackneck), but the greatest porosity, pore count, protein in true shell, pore protein, magnesium in nitrogen-free shell and phosphorus in nitrogen-free shell (except for the Melanistic mutant).

The other types varied in their relative position to each other when the values for each characteristic were arranged in order of magnitude, and sometimes they differed significantly from the wild type and sometimes they did not. There can be little doubt, therefore, that the wild type used in this experiment laid eggs that showed the greatest differences from all the others and in relation to the greatest number of characteristics. It is difficult to advance any explanation at this stage because of the history of these birds. The wild type were trapped just before laying, and there may be dietary factors because the rest of the types had been fed in pens throughout their lives. On the other hand, genetic factors may be operative.

Finally, the question of porosity per pore and pore protein per pore might be mentioned. The former value was similar to that for hens, but with pore protein per pore the hen gave much higher results. This shows that the amount of protein per pore is not directly related to porosity in this case. On the other hand, the longer the pore channel the greater will be its protein content, and the values for pore protein per pore divided by the general mean shell thickness give values much closer together. Thus for pheasants it is 1·01 μg./100-μ length of pore and for hens it is 1·11 μg. This is only a rough calculation which assumes, wrongly, that the pore is a cylinder, but it serves to indicate that the discrepancy in the values for pore protein per pore between hen and pheasant is perhaps not as large as appears at first. Nevertheless, considerable work is required on the shape of pores before this question of porosity and pore protein can be fully cleared up, and, as pointed out in Part III, the possibility that the shell itself is porous cannot be ruled out.

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THE BEHAVIOUR OF METHYL BROMIDE IN THE VACUUM FUMIGATION OF JUTE BAGS *

By H. A. U. MONRO † and J. E. KING ‡

Two methods of vacuum fumigation were compared under practical conditions, by using methyl bromide in 3- or 4-hour treatments of bales of jute bags: (1) sustained-vacuum treatments, in which the low pressure of the system was sustained after introduction of the fumigant; (2) treatments in which atmospheric pressure was restored after 1 or 2 hours of exposure at the initial low pressure. The second method led to greatly increased concentrations of fumigant at or near the centre of the bale and correspondingly improved insecticidal results in this region. In both methods, gas concentrations in the free space of the chamber were lower than in the bales, and insecticidal effect was poor in this part.

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Introduction

The technique of vacuum fumigation is practised commercially in a number of countries, notably the U.S.A., France and Canada, where specially designed metal chambers, capable of withstanding external air pressures, are available for the treatment of material contaminated with insects or other organisms. The actual process followed in conducting a fumigation has varied according to the recommendations of different investigators who have studied the technique. The more important methods employed have recently been reviewed by Brown & Heuser.[1] Also, the special techniques favoured in the three countries mentioned have been described respectively by Lindgren,[2] Lepigre[3] and Monro.[4]

The review by Brown & Heuser provides a description of the various methods as they apply to stored products or 'non-perishable' commodities. It should be pointed out, however, that vacuum-fumigation equipment is often used to treat living plants, fruits and vegetables. When such materials are fumigated great care has to be taken to ensure that the degree of low pressure produced, in combination with the fumigant, has no adverse effect. Thus, with 'perishable' commodities, the method ensuring the most effective penetration of the fumigant may have to be considerably modified to prevent damage to living cells.

In laboratory tests, Brown & Heuser[1, 5] have compared the methods of vacuum fumigation now employed, by using methyl bromide as the test fumigant on boxes of dates and bags of wheat feed. As an index of effectiveness they have proposed a 'penetration factor', expressed as a percentage of the product of the nominal concentration and the time, found at any given point within the material. With the methods commonly employed they observed that the highest factors were obtained with the sustained-vacuum technique. In this method, after the introduction of the fumigant, the lowest possible pressure is held in the chamber for the duration of the exposure period. In addition, these authors found that a new modification of the sustained-vacuum process gave even higher penetration factors. This variation consisted in restoring the pressure in the chamber to that of the atmosphere some time before the end of the treatment.

In the United Kingdom, at present, there are no vacuum-fumigation installations suitable for testing the effectiveness of this new modification under commercial conditions, and therefore it was suggested that the vacuum-fumigation chamber operated in Montreal, Quebec, by the Canadian Department of Agriculture be utilized for a field test.

Some curious terms have been used to describe the procedures connected with vacuum fumigation. Recently Page *et al.*[6] have objected to such terms, among others, as 'released-vacuum' and 'dissipated-vacuum'. They propose other more precise descriptions of the various processes.

The present writers agree with these authors that more precise descriptions, devoid of jargon, are needed to describe the techniques. In this paper the new suggestions are followed as closely as possible. However, in describing the process of restoring atmospheric pressure after a period of sustained vacuum it has been found difficult to avoid completely the term vacuum released', especially in graphs, tables and passages in the text where concise phraseology is needed. It may prove difficult to eliminate this term completely from descriptions of vacuum-fumigation procedures.

Experimental conditions

As a commodity for use in a field trial under commercial conditions the choice fell on hydraulically pressed bales of jute bags. This commodity was particularly suitable for a number of reasons:

(*a*) The material is 'inert', and a given load can be fumigated repeatedly if it is satisfactorily aerated between tests.

(*b*) At the time that the bags are being assembled for pressing in the baling machine, test insects and inlet tubes for extracting gas samples can be inserted in predetermined positions.

(*c*) The jute is highly sorptive and, as sorption of gases is an important modifying factor in fumigant penetration, this material lends itself to a study of such phenomena.

(*d*) The present authors had already made some observations on the material which

indicated that a relatively severe treatment is necessary to ensure complete mortality of contaminating insects.

(*e*) The commodity is regularly fumigated in routine commercial treatments at the Montreal installation, and was thus readily available for the tests.

The commodity as used consisted of pressed bales, bound with wire, containing 500 jute bags formerly used for shipping Brazilian coffee. The average weight of the bales was 570 lb. The area of each bag when empty was 41 in. × 29 in. When pressed the bale had dimensions 41 in. × 29 in. × 37½ in., with a bulk of 25·8 cu. ft. Bales of this type are illustrated in Fig. 1.

Choice of treatment

Methyl bromide was selected as the fumigant for these experiments as it was the one used by Brown & Heuser[1, 5] in their laboratory studies. Moreover, it is commonly used as a fumigant to control insects in used bags.

In order to make a comparison between the effectiveness of various treatments, one dosage and two exposures were selected which would not bring about a complete mortality of the test insects employed. Accordingly, a dosage was chosen of 56 mg./l. (3·5 lb./1000 cu. ft.) of empty chamber space, for exposures of 3 or 4 hours. In this study four different combinations of exposure periods and pressures were employed as follows:

Three-hour exposures.—I. Vacuum sustained throughout.

II. Vacuum sustained 1 hour, then pressure restored to atmospheric (' vacuum released ') for the remaining 2 hours.

Four-hour exposures.—III. Vacuum sustained throughout.

IV. Vacuum sustained 2 hours, then pressure restored to atmospheric (' vacuum released ') for the remaining 2 hours.

Details of treatment

The vacuum-fumigation chamber used for the tests is illustrated in Fig. 1. It has a capacity of approximately 1200 cu. ft. The bales were mounted three at a time on wooden platforms of length 57 in. and width 48 in. Eight platforms were used to charge the chamber, which thus contained 24 bales. Hence the total volume occupied by the bales and the platforms was approximately 640 cu. ft., or a little more than half the total volume of the chamber. No artificial heating was applied to the chamber as the work was carried out in the middle of August. At the time of loading the temperatures in the chamber ranged from 75° to 80° F and those in the bale from 70° to 75° F. Exhausting the air from the chamber caused the temperature in the free space to fall about 10°. If the vacuum was sustained the temperature rose gradually to the original point before the end of 3 hours. When atmospheric pressure was restored the temperature rose about 2° above the original and then gradually fell again to the original reading before the end of the exposure. The temperatures of the bales themselves were not appreciably affected by evacuation or by restoration of atmospheric pressure.

After the chamber was loaded it was evacuated by means of a 45-h.p. pump to a pressure of 25 mm. (Throughout this paper pressures are to be understood as mm. Hg.) The fumigant of standard commercial grade containing 99·5% of methyl bromide by weight was then introduced from a cylinder containing the liquid under pressure. The fumigant flowed as a liquid in a ½-in. pipe coiled through a heated volatilizer and thus entered the chamber as a gas, where it impinged on a 19-in. × 26-in. metal baffle plate, used to ensure that the fumigant was not discharged directly against that portion of the load nearest to the orifice. The introduction of the dosage increased the pressure in the system by 8 mm., whereas the calculated increase for an empty chamber would be 10·9 mm. The difference is ascribed to the effect of partial sorption of the gas by the load.

In the sustained-vacuum treatments the system was left unchanged for the whole exposure period of 3 or 4 hours. However, in common with many commercial vacuum installations, a slight leakage caused an increase of pressure at the rate of 6 mm. per hour. In the treatments when atmospheric pressure was restored 1 or 2 hours after the beginning of the treatment, a large valve at the side of the chamber was opened and air flowed in rapidly, filling the system

completely in 2·5 minutes. At the end of the fumigation period three ' air-washes ' were carried out, each represented by a reduction in pressure in the system to 50 mm. followed by introduction of air to raise the pressure to 635 mm., a point at which it was convenient to start the pump again. In the sustained-vacuum treatment it was necessary to increase the pressure to 635 mm. before beginning the air-washing cycle. When gas samples were not being taken the whole air-washing cycle took 20 minutes.

On completion of air-washing the door of the chamber was opened and the two bales containing the test insects were removed, together with the insect test-capsules in the free

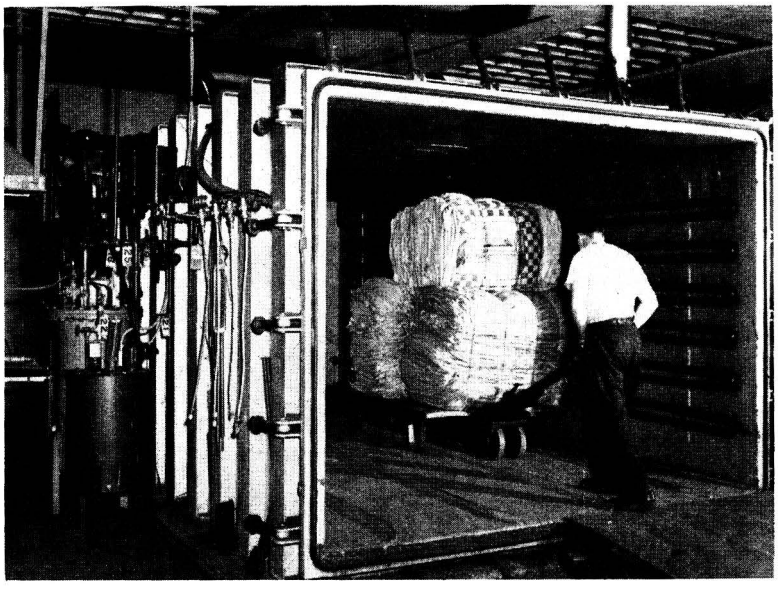

FIG. 1.—*Canadian Government vacuum-fumigation chamber, Montreal: loading hydraulically pressed bales of bags*

space and those lying on top of the bales. The door was closed again and the system partially evacuated to 709 mm. for the balance of the working day. At 5.00 p.m. the usual procedure was followed of further evacuation to 380 mm. to keep the door firmly closed during the night. The following morning four further air-washes were carried out before the greater part of the load was removed for aeration outdoors at summer temperatures.

Of the two bales removed after the actual fumigation, one was unpacked immediately and the capsules and bags containing the test insects were removed. The remaining bale was left untouched in a well-ventilated room overnight and unpacked for removal of insects the following morning. This procedure was designed to test the difference in the mortalities effected by the fumigation itself, as opposed to the fumigation and post-fumigation effects combined. Throughout all the tests the bales containing the insects were made up from fresh non-fumigated bags. However, as the number of bales was limited, the remaining 22 making up the load were used throughout all the tests. Chemical analyses were made of gas samples extracted from one of these bales before each test. The highest recorded residue was 1 mg./l. of methyl bromide in the centre of the bale after aeration between tests.

Chemical analysis

Three gas-sampling tubes of copper with inside diameter of 1·5 mm. were inserted in a bale while it was being assembled for pressing. On loading the chamber these were connected

to similar tubes leading to sampling points outside the chamber. The ends of these tubes were placed so as to extract samples from (*a*) the centre of the bale, (*b*) a point 9 in. from the nearest surface and (*c*) a point 3 in. from the surface. In addition, a fourth tube extracted samples from the free air at a point midway between two bales, which were 12 in. apart. The sampling points within the bales were so arranged as to prevent overlapping of sampling zones during extraction.

Air–gas samples were taken by drawing them into calibrated 200-ml. flasks containing 5 ml. of ethanolamine. Before sampling, each flask was evacuated to a pressure of 2·0 mm. or less and 15 seconds were allowed for the withdrawal of the sample. The flasks were left to stand overnight and the bromide was then estimated by the Volhard method as described by Stenger *et al.*[7]

Use of test insects

Small linen bags containing 25 g. of grain heavily populated with fourth-instar larvae of the granary weevil, *Sitophilus granarius* L., and metal testing-capsules partially filled with rolled oats and containing 7 or 8 fourth-instar larvae of the cadelle, *Tenebroides mauritanicus* L., were placed at various depths in the bales. To avoid creating too much air space around the test containers the bags with grain were placed a distance of ten layers away from the corresponding capsules containing the cadelles. The capsules with the cadelle larvae were laid in groups of four to give a total of 30 larvae at each level.

The grain samples were removed from the bales after fumigation or post-fumigation, and dispatched to the Science Service Laboratory at London, Ontario, where the kernels were *X*-rayed in a 'grain-inspection unit' according to the method of Milner *et al.*[8] Thus an absolute determination was made of the population in each sample, and by this means an accurate mortality figure could be calculated after adults of surviving larvae had emerged from the grain. The mortalities for the weevils are based on samples of 25 g. of infested grain, yielding an average of 253 insects per sample. The cadelle larvae remained in Montreal and were placed with whole-wheat flour in individual glass cells to await mortality counts made one week later.

Results and discussion

Fumigant concentrations during treatment

The results of the chemical analysis of the fumigant concentrations for the two types of treatment of 3 hours (Treatments I and II) are set out in Figs. 2 and 3.

It is clear that the general pattern of fumigant diffusion observed by Brown & Heuser in their laboratory studies has been confirmed in practice. The great increase in concentration found by these workers at the centre of the commodity, when atmospheric pressure had been restored in the chamber after 1 hour of sustained-vacuum treatment, was repeated in the large-scale fumigation. However, there are several differences of detail which are worthy of note. In the sustained-vacuum fumigation, Treatment I [Fig. 2(A)], the concentration in the free space fell below that observed in all three sampling points within the bale. A tendency towards this effect was also seen in the shorter periods of sustained vacuum in the vacuum-released fumigation, Treatment II [Fig. 2(B)]. Also, when atmospheric pressure was restored the concentration in the free space fell very markedly, a phenomenon confirmed by all series of samplings for treatments or air-washes [Fig. 2(B) and Fig. 3(A) and (B)]. Further, at the time of restoration of atmospheric pressure the concentrations at the point 3 in. deep also fell slightly.

The increase in concentration toward the centre of the bales, following restoration of atmospheric pressure, could be explained in two ways. In the first place, desorption of some of the fumigant from surfaces within the commodity could take place as a result of the introduction of large amounts of air into the system. Secondly, fumigant present in the free air space of the chamber or near the surface of the bales could be carried toward the centre of the mass in the stream of incoming air. The belief that this second mechanism must play some part in bringing about the high concentrations at the centre is supported by the evidence of the observed fall in concentration in the free space and near the surface of the bales.

The penetration factors for the diffusion of the fumigant into the bales during the field tests, calculated according to the method of Brown & Heuser, are shown in Table I.

Test-insect mortalities

The results of the study of insect mortality in fumigated bales are set out diagrammatically for *T. mauritanicus* L. in Fig. 4, and for the larvae of *S. granarius* L. in Table II.

In general, the insect mortalities conform to the pattern of gas concentration revealed by chemical analysis. The larvae of *T. mauritanicus* were not so susceptible to the various fumigation techniques as were those of *S. granarius*. The principal discussion will therefore be devoted to the results with the former insect. However, in passing, it should be noted that the mortalities of *S. granarius* also tended to be higher towards the centre of the bales.

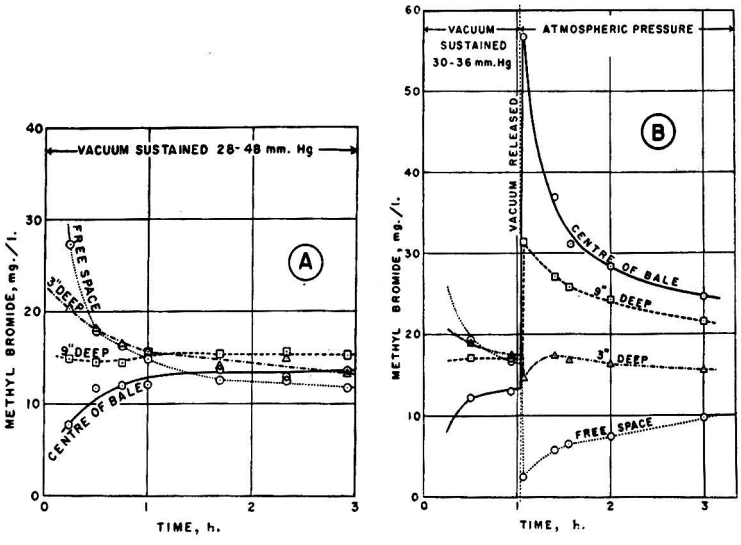

FIG. 2.—*Methyl bromide concentrations in vacuum fumigation of bales of used bags*

(A) Vacuum sustained for 3 h.
(B) Vacuum sustained for 1 h., followed by 2 h. at atmospheric pressure (vacuum released)

A noteworthy effect with *T. mauritanicus* is that in the sustained-vacuum treatment for 3 hours, the mortalities, although incomplete throughout, were higher towards the centre of the bale and low in the free space. In the comparable released-vacuum experiment at 3 hours, the result of restoring atmospheric pressure after 1 hour was to effect complete mortality toward the centre but to leave survivors near and on the surface and in the free space. Prolonging the exposure periods to 4 hours still failed to give complete control inside the bale with a sustained-vacuum treatment, but by restoring atmospheric pressure after 2 hours the mortality was 100% throughout the bale. In both treatments failure was again experienced on the surface and in the free space. Thus the main weakness of all treatments is found not within the bales, but near or on the surface and in the free space.

A similar effect has been reported by El Nahal[9] in the sustained-vacuum fumigation with ethylene oxide of wheat in sacks. This author, using chemical analysis only, found higher concentration–time products of ethylene oxide in the centre of the sack than in the free space during the fumigation period of 4 hours. Also the residual ethylene oxide (p.p.m.) after air-washing was higher in the centre of the sack than at the top (perimeter).

Air-washing technique

In this study, sampling for gas concentrations was continued during the 'air washing' in order to obtain information both on the efficiency of the process and on the prolongation

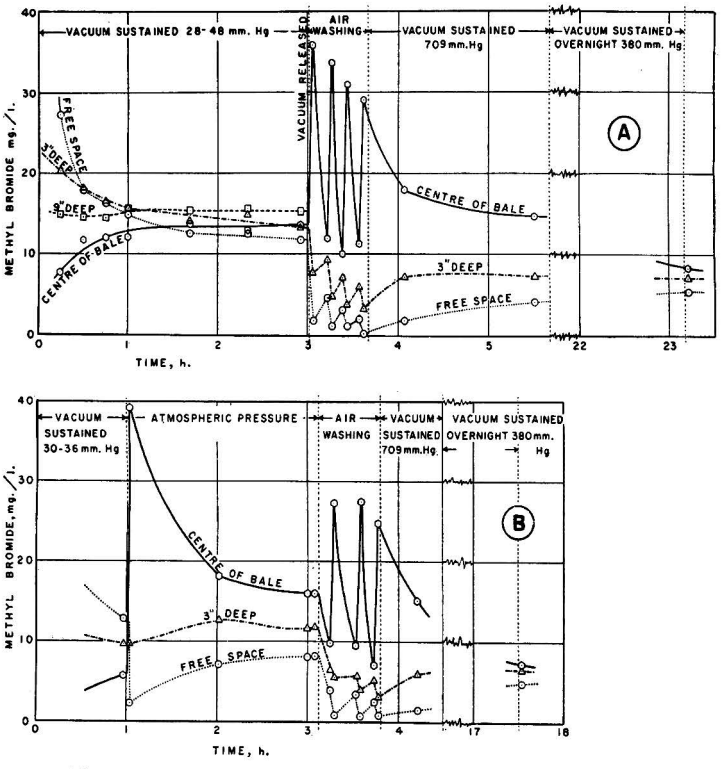

FIG. 3.—*Methyl bromide concentrations in vacuum fumigation of used bags and in subsequent air-washing procedure*

(A) Sustained vacuum
(B) Vacuum-released treatment

of insecticidal effect. These samples were taken after a test from which no insecticidal data were obtained, as the gas sampling prolonged the air-washing process to 45 minutes. The results are shown in Fig. 3. Graph A illustrates the condition after the sustained-vacuum

Table I

Penetration factors and corresponding mortalities of larvae of Tenebroides mauritanicus *L.*

Position	I. Vacuum sustained for 3 h.		II. Vacuum sustained for 1 h.; atm. press. for 2 h.	
	Penetration factor	Mortality, %	Penetration factor	Mortality, %
Free space*	25	23	19	0
3 in. from outside of bale	27	32	28	80
9 in. from outside of bale	26	50	39	100
Centre of bale	21	65	44	100

* Free space factor is calculated on same basis as other positions, although no penetration was, of course, involved.

fumigation and Graph B the effects of air washing after a treatment during which atmospheric pressure was restored. The results confirm the opinion that with highly sorptive commodities, such as jute bags, the conventional air-washing process of alternate evacuations and restorations of atmospheric pressure is not efficient for removing residual vapours from the centre of the commodity. The effect is alternately to remove the gas from the centre and then to

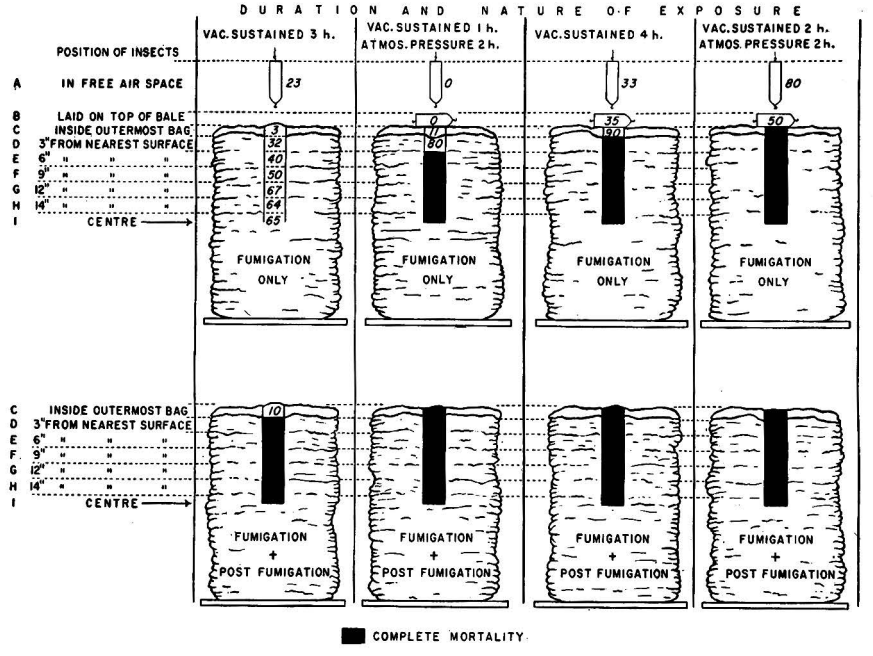

FIG. 4.—*Diagrammatic representation of mortalities of* T. mauritanicus *L. in the vacuum fumigation of used bags; methyl bromide applied at the rate of* 56 *mg./l.* (3·5 *lb./*1000 *cu. ft.*)

'carry' it back. A rough projection of the lines connecting each peak of concentration at the middle of the bales, resulting from the introduction of fresh air, indicates that a period of at least 3 hours of continuous pumping and air washing would be required to remove all but a small fraction of gas from the bales.

On the other hand, a possible advantage of the process is that the final restoration of atmospheric pressure by the sudden inrush of air serves two purposes: (1) to remove dangerous concentrations of fumigant from the free space so that persons may enter the chamber to unload it; (2) to leave residual fumigant in the bale, where it is available to act in the post-fumigation treatment.

From the results illustrated by the graphs in Fig. 3 it is clear that during the air-washing process the average concentrations of fumigant at and near the centre of the bales are higher than those at the surface. It may be assumed, therefore, that near the centre the fumigation treatment actually continues during air washing.

However, extension of the fumigation period from 3 to 4 hours in both types of treatment still failed to bring about complete mortalities in the free space and on top of the bale. On the basis of these tests, therefore, there is ground for suspecting the operation of some factor or factors, at present unaccounted for, which bring about an unsatisfactory fumigation of the insects at the periphery of the load.

Post-fumigation effect

In Fig. 4 and Table II are also shown the results from the observations of the post-fumigation effect within the fumigated bales, as demonstrated by the test insects.

Although in practical work it is desirable to obtain complete mortality throughout a given commodity before it is removed from a fumigation chamber, this is not always possible. Reliance has sometimes to be placed on the action of the residual vapours to complete the

Table II

Percentage mortality of fourth-instar larvae of Sitophilus granarius *L. in vacuum fumigation of used bags; methyl bromide applied at the rate of* 56 *mg./l.* (3·5 *lb./*1000 *cu. ft.*)

Position of insects	Duration and nature of exposure							
	I. Vacuum sustained for 3 h.		II. Vacuum sustained for 1 h.; atm. press. for 2 h.		III. Vacuum sustained for 4 h.		IV. Vacuum sustained for 2 h.; atm. press. for 2 h.	
	Fumigation	Fumigation and post-fumigation	Fumigation	Fumigation and post-fumigation	Fumigation	Fumigation and post-fumigation	Fumigation	Fumigation and post-fumigation
A In free space	100	—	87·5	—	98·8	—	99·6	—
B Laid on top of bale	—	—	89·8	—	99·2	—	100	—
C Inside outermost bag	93·9	94·2	88	99·6	98·5	99·6	100	100
D 3 in. from nearest surface	98·7	100	97·7	100	100	100	100	99·6
E 6 in. from nearest surface	99·4	100	100	100	—	—	—	—
F 9 in. from nearest surface	99·4	100	100	100	100	100	100	100
G 12 in. from nearest surface	100	100	100	100	—	—	—	—
H 14 in. from nearest surface	99·5	100	100	100	—	—	—	—
I Centre	100	100	99·7	100	100	100	100	100

treatment. The importance of this post-fumigation has previously been noted with hydrocyanic acid gas on cotton by Johnson *et al.*[10] and on tobacco by Reed & Livingstone.[11] That this effect may be an important factor in the fumigation of bales of bags is clearly shown from the results of the 3-hour treatments. In the vacuum-released test complete mortality was only achieved as the result of post-fumigation, whereas in the sustained-vacuum treatment the concentrations of fumigant present during aeration were not sufficient to effect complete mortality inside the outermost bag.

General conclusions

The new method of vacuum fumigation developed by Brown & Heuser has been tested on a commercial scale with methyl bromide on pressed jute bags containing two species of insects. In this technique, after the introduction of the fumigant, the low pressure is sustained for a certain period, such as 1 hour, after which atmospheric pressure is restored and the fumigation continued at this pressure for the remainder of the exposure. By restoring atmospheric pressure the fumigant concentrations near the centre of the bales were greatly increased.

The increase in concentration was reflected in the insecticidal results and, in comparison

with the sustained-vacuum technique, the new method gave better mortalities within the bales, both during the actual fumigation and as a result of the combined fumigation and post-fumigation exposures.

This investigation revealed, however, that under the conditions of these tests, with the new method ' penetration factors ' are lower near the surface of the bale than they are deeper in. This observation was confirmed by the results with test insects.

In both the sustained-vacuum and atmospheric-pressure-restored treatments there appears to exist a region of insecticidal weakness on or near the surface of bales of jute bags. This phenomenon and the means of overcoming it are now being investigated.

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