JOURNAL
OF THE
SCIENCE OF FOOD AND AGRICULTURE
(INCLUDING ABSTRACTS)

Published by the Society of Chemical Industry

Volume 16 | No. 1 | January, 1965

SOCIETY OF CHEMICAL INDUSTRY

FOUNDED IN 1881 INCORPORATED BY ROYAL CHARTER 1907

Offices of the Society: 14 Belgrave Square, S.W.1
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Annual Subscription to the *Journal of the Science of Food and Agriculture*
£15 post free, single copies £1 17s. 6d. post free

Journal of the Science of Food and Agriculture

Vol. 16 London 1965

INTERACTIONS OF HORMONAL SUBSTANCES IN THE GROWTH AND DEVELOPMENT OF PLANTS*

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Introduction

THE greatest challenge to the biologist of the 1960's is without doubt the elucidation of the factors governing the development of the fertilised egg into the multicellular organism characteristic of its species. Every biologist now accepts that each living cell of an organism contains accurately coded within the genetic material of the nucleus essentially the same basic information as the cells of other organisms of the same species. But every biologist knows that despite the similarity of genetic information in each cell, the cells of different parts and organs of a living creature may be quite different both in form and function. How then is this differentiation of form and function brought about?

The biologist who works with plants is struck by another feature of this problem. He can take a group of living cells from either root or shoot, transplant them to a nutrient medium as Steward and his collaborators have done[1] with phloem cells from carrots, and in suitable cultural conditions they will divide, and give rise to new groups of cells some of which will eventually develop into plantlets which grow, produce flowers and set seed like any normal plant. Although the carrot root is a highly differentiated structure, the phloem cells from this organ are still totipotent and quite capable of giving rise to all the cells that make up a whole carrot plant. The genetic information of the nucleus clearly remains complete and the simplest explanation of cell growth and differentiation is that different parts of genetic information are used during different stages of the growth process.

On this concept there arises the problem of discovering the factors that determine which parts of the whole genetic information should be used at any one stage of growth.

For three decades or more, experimental evidence has accumulated showing how plant growth can be modified by minute quantities of chemical substances which we call hormones. This paper outlines some of the patterns of growth and development in plants which are now known to be under the control and direction of these endogenously produced chemical regulators. Consideration is given first to the nature of plant hormones, then to the kinds of responses they produce in plant cells and in whole plants, and lastly to ways hormones might act as regulators of the biochemical processes of the cell which lead to growth and differentiation.

There are three main groups of natural chemical regulators (or plant hormones)—the auxins, the kinins and the gibberellins—although it may well be that there are more groups of regulators still to be discovered.

The auxins and the gibberellins have been extracted from many kinds of plants and their chemical structure is established. Kinins have been isolated from plants, but so far no natural kinin has been obtained pure or chemically characterised with certainty. This information should soon be available, as several groups of workers claim that they are nearing success with this enterprise. It is known, however, that a number of adenine derivatives are effective

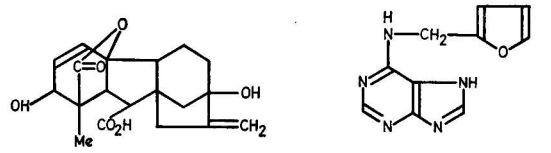
* Read before the Pesticides Group, 17 February, 1964

substitutes for the so-far impure kinin preparations and there is some justification for believing that the natural kinins are purines.*

The most commonly occurring form of natural auxin is indol-3-ylacetic acid (IAA)[2, 3] and auxins, both natural (IAA) and synthetic (e.g., 2,4-dichlorophenoxyacetic acid), are characterised by their stimulation of the rate of growth of stem cells and their ability to induce curvature responses in aerial parts.

Gibberellins and kinins

Gibberellins (Fig. 1) may be considered as modified diterpenes or isoprenoid derivatives,[4] and are characterised by promoting greater total growth of shoot cells (not simply rate of growth). This effect is most marked in plants in which the dwarfing characteristic is controlled genetically.[5]



Gibberellin A_3 (GA) (naturally occurring gibberellin)

Kinetin (K), 6-furfurylaminopurine (synthetic kinin)

FIG. 1

Although it is probable that there is only *one* form of the natural auxin that is active in plants, namely indol-3-ylacetic acid, and all types of plants show growth responses to this compound, at least nine different gibberellins of slightly differing chemical structure have been isolated in pure form from different plant materials, and different plants respond specifically to different gibberellins.[6]

The synthetic kinins (Fig. 1) are characterised by their stimulation of cell division in tissue cultures of root and shoot[6a] and for their ability to stimulate leaf expansion[7] and to release lateral buds from the growth inhibition imposed by a terminal bud.[8] Kinetin was the first synthetic kinin to be discovered,[9] and it shows some activity in all the species tested so far, but it is not known yet whether all plants respond to the same *natural* kinin.[10] The auxins, kinins and gibberellins have been shown to have growth-regulating activity in all groups of green plants, including algae, liverworts, mosses and ferns.[11]

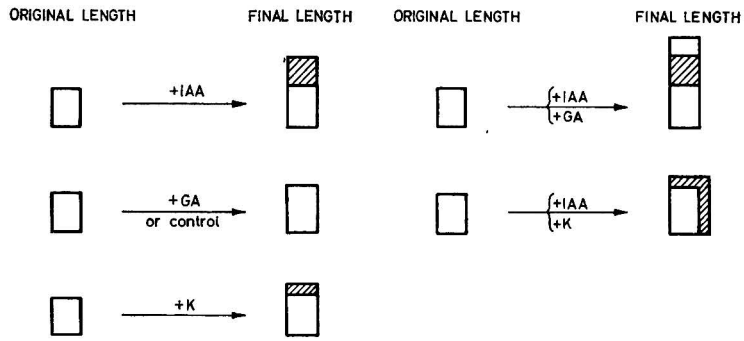


FIG. 2.—*Effect of auxin, gibberellin and kinetin on growth of stem segments of green pea plants*

Presence in tissues

These substances are all present in relatively large amounts in seeds, where they form part of the storage reserves available for the young plant during germination, and it is believed that auxins and gibberellins are synthesised in the aerial parts of plants.

* Since this review was written, a natural kinin has been isolated in crystalline form from sweet corn and identified as a purine, as predicted, probably 6-(3-methyl-4-hydroxybut-2-enyl) aminopurine (Letham, D. S., *et al.*, *Proc. chem. Soc., Lond.*, 1964, p. 230)

The highest content of auxin is found in the most rapidly expanding tissues, in young stems and unfolding leaves, but falls to levels too low to be determined in old and senescent parts of the plant. It is synthesised in green leaves in the light (particularly by the youngest leaves at the apex), and is transported from the leaves to the stems and the parts below. There it is known to be inactivated in a number of metabolic reactions. The resulting distribution is a falling gradient in auxin concentration from the young to the mature tissues.[12, 13] Tissue cultures of plants will normally not grow, unless supplied with auxin, so it is clear that not all cells can meet their own auxin requirements. Auxin concentrations in green plants have been variously estimated between 10 and 0·1 p.p.m. for young tissues.

Gibberellins also are known to be present in both young and old tissues, and like auxin are in greatest concentration in apical buds and in young expanding leaves.[14] The work of Lockhart[15] suggests that gibberellin, like auxin, is synthesised primarily in the buds. It is also known that the nature and content of gibberellins change in the plant as it ages, and as it progresses from the vegetative to the flowering condition.[16] It is reported that tissue cultures contain endogenous gibberellins,[17] and, since additional gibberellin is not necessary for their continual growth, it is possible that all cells can make some gibberellins.

Few measurements have been made of kinin contents in different parts of plants, but experiments suggest that old tissues are low and probably deficient in kinins.[18] Since tissue cultures require the addition of a kinin for continued growth, it is unlikely that all cells have the capacity to synthesise a sufficiency of kinins.

Physiological effects

Auxins or gibberellins, when applied to one part of a shoot, will induce curvature or elongation responses in other young aerial parts, showing that both these types of hormones are readily transported in the plant. Auxins are moved most readily from the physiological apex to the physiological base in segments of young tissues,[19] i.e., their movement is basipetally polar, whereas gibberellins apparently move with equal ease up or down a piece of stem tissue.[20]

The synthetic kinins do not normally cause curvatures or stimulate elongation of stems when applied to whole plants, and their movement appears to be slight when they are applied to mature tissues,[21, 22] although the natural kinins may well be more mobile. However, when synthetic kinins are applied to segments of stem tissue their movement has been shown to be, like that of auxins, basipetally polar, and this basipetal movement of auxins and kinins is mutually stimulatory (Osborne & Black,[23] and data in press).

Although these different hormones cause readily measurable responses in growth and differentiation in young parts of plants they have little effect on the growth of mature tissue. This does not mean, however, that mature tissues are no longer responsive to these regulators. In recent years it has been discovered that although all visible growth in size has ceased, the longevity and functional life of certain cells, such as leaf cells, can still, depending upon the species, be controlled by auxins, kinins or gibberellins. These hormones, in fact, regulate the biochemistry of every cell in the plant from the time of its production at cell division, until its ultimate senescence and death.

Cell enlargement

For a study of how these hormones together regulate expansion growth of cells, segments of rapidly elongating stems of young green pea plants are rewarding material. A section 1 cm. long cut from just below the apical bud and put into a solution of auxin (IAA, 10 mg./l.) will extend 85% in 24 h. If it is put into a solution of gibberellin 10 mg./l. it will elongate no more than a control in distilled water—perhaps 20%. However, if auxin and gibberellin are supplied together, total extension growth is as much as 115% and is greater than that in auxin alone (Fig. 2). From this, and many other experiments of this kind (Brian & Hemming[5, 5a]), it is clear that both substances are necessary for maximum growth in cell length. It also suggests that both gibberellin and auxin are present in suboptimal amounts in the segments, and that an insufficiency of these hormones limits their growth in length. If kinetin 0·3 mg./l. is supplied to green pea segments, extension growth is hardly distinguishable from that in controls, but if the kinetin is supplied with the auxin (IAA 10 mg./l.) growth in length is *less* than that in

auxin alone. This is a result that requires careful interpretation, however, for it is found that both the fresh weight and the growth in volume of the segments is not less, but is actually slightly enhanced. Kinetin, in fact, causes an increase in the thickness of the section and less growth in length.[24] The presence of kinetin results in similar reductions in length in gibberellin-treated sections, and it seems that kinins may be important in determining the *orientation of the growth* induced by auxins and gibberellins.

It is known, furthermore, that the elongation growth response of cells to applied hormones is different at different ages and at different stages of development. For instance, the cells of the coleoptiles of newly germinated oat seedlings show the greatest elongation response to gibberellin, cells of older coleoptiles are more responsive to kinetin, and in the last stages of elongation growth of the coleoptile, the cells show the greatest growth response to auxin.[25]

One can picture the normal growth in size of stem cells, therefore, as being under the multiple control of all these kind of hormones; each one playing the major determining rôle in turn, but all of them being essential for full growth and differentiation of the cell, and none of them being fully effective without the others.

Cell division

The discovery of the group of hormones known as kinins was the result of a search for the active material present in coconut milk, without which the cells in plant tissue cultures will not multiply. The active factor is not yet isolated, but kinetin, which is a synthetic product formed by degradation of DNA, was found by Miller *et al.*[9] to be an effective substitute for the active factor of coconut milk. Kinins are, by definition, substances which will, in the presence of auxins, maintain continued proliferations of a plant tissue culture.

Over the past 8 years investigation into the hormonal regulation of growth and organ formation in tissue cultures has enormously widened our understanding of the hormonal control of differentiation. For example, Skoog & Miller[6a] cultured small pieces of pith tissue, cut from the middle of tobacco stems, under aseptic nutrient conditions in the presence of auxin or kinetin alone, and in increasing concentrations of kinetin combined with the auxin. They found the remarkable responses shown in Fig. 3. In auxin alone the cells will expand but then cease to grow (they do not divide), in kinetin alone there is no growth at all. With low kinetin concentrations plus the auxin, the tissue will enlarge, divide and produce numerous roots; at higher kinetin concentrations plus auxin a mass of undifferentiated loosely packed callus will develop but *no* roots; if the kinetin concentration is increased further, a callus tissue develops with numerous leafy bud-like outgrowths. The highest kinetin concentration together with auxin, causes the production of an undifferentiated callus, with small closely-packed cells. Thus by suitable modifications of the auxin/kinin balance, similar original tobacco pith cells can be induced to divide and differentiate to produce distinct and morphologically different organs.

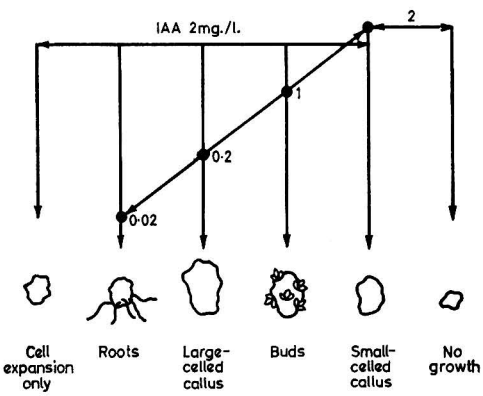


FIG. 3.—*Morphogenetic effects of auxin and kinetin in tobacco pith*
(after Skoog & Miller[6a])
numerals indicate amounts of kinetin in mg./l.

The growth response to auxin and kinin is dependent not only on the concentrations supplied, however, but also on the physiological condition of the recipient tissues. For example Adamson[26] showed that if freshly cut artichoke tuber disks were washed in water at 24° for 24 h., then auxin and kinetin together acted subsequently to enhance cell expansion, but if the disks were washed for 24 h. at 4° before being supplied with auxin and kinetin, the response was not that of cell expansion, but of greatly enhanced cell division. The nature of the physiological changes that take place during the 24-h. washing period will be discussed later, but it is clear that some kind of conditioning occurs in these cells which determines whether the cells will subsequently expand or divide.

Although stimulation in the growth of some tissue cultures has been reported following the addition of gibberellins, they generally have little effect on these systems, and this may be because callus cells can make enough gibberellin of their own. Gibberellins do, however, have marked effects on cell division in intact plants. For instance, if gibberellins are applied to certain rosette, long-day plants growing under non-photoinductive short-day conditions, remarkable results occur. The normal rosette condition is changed. There is a stimulation of meristematic activity in the bud, the internodes elongate and a tall stem is produced which will eventually flower in short days.[27] The effect on meristematic activity is rapid (Fig. 4). Within 24 h. of the application of gibberellin to the bud of a *Hyoscyamus* plant, a three-fold stimulation of cell division is observed in the sub-apical meristem and about 70% of these divisions are in a plane perpendicular to the stem axis, i.e., in the direction to give increased stem length.[28] Gibberellin is clearly a regulator of mitotic activity in these cells. This view is strengthened by the results of Lang[14] and Harada & Nitsch[16] who showed that the endogenous content of gibberellins is increased and changes during the bolting and flowering in long-day plants under normal photoinductive conditions, and more recently Radley[29] showed that the content of gibberellin in spinach plants temporarily increased tenfold after 1–3 long-day photoinductive periods.

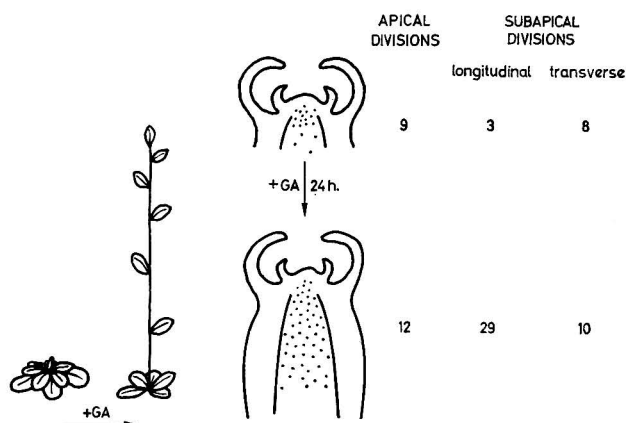


FIG. 4.—*Effect of gibberellin on cell division in meristem of rosette plant* (after Sachs & Lang[28])

There are two other good examples of gibberellin control of cell division and growth in intact tissues and both of these are associated with the breaking of dormancy. Firstly, dormant potato tubers can be induced to sprout by the application of gibberellin directly to the buds. Furthermore, a spray of gibberellin applied to a whole potato plant before the tubers are lifted will actually cause the tubers to sprout beneath the soil.[30] That gibberellin is a natural regulator of bud growth and development in the potato tuber is supported by the results of Smith & Rappaport[31] who have since shown that the content of endogenous gibberellins in the tuber increases several-fold during the normal breaking of bud dormancy. Gibberellin has also

been shown to stimulate the growth and cell division that occurs during the germination of many kinds of seeds. In barley grains, for example, the stimulation of germination causes a corresponding increase in enzyme production by the grain, particularly of proteases and amylases.[32]

Before considering the mechanism of the hormonal control of growth processes in the intact plant there is a further stage in plant development which must be discussed and that is the final phase of plant growth, namely senescence.

An important fact about senescence in leaves is that it is apparently associated with a deficiency of one or other of the plant hormones. In a herbaceous plant, *Xanthium* (Richmond & Lang[33]) or *Nicotiana* (Mothes *et al.*[34]), senescence of the blade can be temporarily retarded by applying a kinin. Leaves of many deciduous trees such as *Prunus* can be preserved by supplying them with additional auxin, or in some cases with gibberellin, but not by additional kinin.[35] In all these cases the leaf remains, as far as can be detected, in a fully functional condition.

Hormonal regulation of growth processes

To attempt a solution to the rôle of hormones in the control of these very varied growth and developmental processes, one must consider the effects of auxins, gibberellins and kinins from a biochemical standpoint.

In the following, some evidence is collected which suggests that the auxins, kinins and gibberellins may all have a common primary action in the regulation of cell processes, and that this primary action is the modification of the control exerted by the nucleus, on the synthesis of nucleic acid and protein (and hence of enzymes) within the cell.

Rôle in cell expansion

It is accepted without question that growth is a metabolic phenomenon associated with a utilisation of cellular energy. Many years of investigations have been spent in the search for the primary metabolic process which is stimulated during auxin-induced cell enlargement, and until recently there have been a large and miscellaneous collection of clues, none of which gives a clear indication of the site of auxin action. Although it would be unwise to dismiss any of these clues, for any theory of hormone action must accommodate the known facts, it seems likely that very many of the effects shown by auxin, such as increased respiratory activity or increases in pectin methylation, for instance, are secondary in nature and result from other more fundamental changes in the biochemical control mechanisms of the cell.

In 1959, Biswas & Sen[36] showed that rapidly expanding *Avena* coleoptiles growing in the presence of indol-3-ylacetic acid showed a 50% increase of incorporation of ^{32}P into nucleic acids within 2 h. and concluded that the auxin induced a rapid increase in nucleic acid synthesis. The stimulation of incorporation of labelled intermediate into RNA has since been repeated and confirmed by other workers. It is also known that under certain conditions, auxins induce a net increase in total protein in, for instance, expanding cells of beetroot or potato tuber tissue,[37] but in many other expanding tissues a net loss of protein occurs. The proper question to ask is not whether this is net gain or loss of protein but whether there is a change in the rate of synthesis of protein. Do auxins in fact stimulate the synthesis of protein ?

In 1963 Nooden & Thimann[38] cut disks from artichoke tuber tissue, washed them overnight in water or in a solution of an auxin (indol-3-ylacetic acid) and then transferred them to a solution of ^{14}C-leucine for 5 h. At the end of this period they separated the protein into soluble or supernatant protein and insoluble protein. They found a 17% increase in the ^{14}C-leucine that was incorporated into the insoluble protein fraction, but a 50% increase in the incorporation of ^{14}C-leucine into the supernatant of soluble protein fraction. They next asked the very important question : is this auxin-induced stimulation in protein synthesis associated with auxin-induced stimulation of growth ?

The answer is given in their experiment with *Avena* coleoptile sections (Table I) in which they used the antibiotic chloramphenicol as an inhibitor of protein synthesis. It is clear that the results must be interpreted as an inhibition of auxin-induced growth which parallels the inhibition of auxin-induced protein synthesis.

Table I

Inhibition of auxin-induced growth and protein synthesis by chloramphenicol
(after Nooden & Thimann[38])

Concentration of chloramphenicol	% Inhibition of Extension growth	% Inhibition of Incorporation of ^{14}C leucine into protein (c.p.m. in protein)
	40%	5×10^3
4×10^{-4}M	3	6
8×10^{-4}M	5	14
2×10^{-3}M	22	33
6×10^{-3}M	38	59

In other experiments they used two different inhibitors of protein synthesis, namely the antibiotics puromycin and actinomycin D, the former of which prevents the formation of peptide bonds in the ribosomes by specifically blocking the template sites of the transfer-RNA–amino-acid complex, while antinomycin D has the property of binding to the guanine base in the DNA of the nucleus and thereby preventing the synthesis of DNA-dependent RNA. It is believed that DNA-dependent RNA synthesis includes the entire RNA synthesis of the cell. Nooden & Thimann showed that both these specific inhibitors of protein and RNA synthesis also inhibit cell expansion.

More recently still, Key & Shannon[39] have extended their work on the effects of auxins on nucleic acid and protein synthesis in excised segments of soya-bean stems. They have shown that the incorporation of ^{14}C-ADP into RNA is increased twofold in the presence of growth stimulatory concentrations of indol-3-ylacetic acid or 2,4-dichlorophenoxyacetic acid. They have shown, furthermore, that the presence of 10 μg./ml. of actinomycin D will reduce incorporation of ^{14}C-ADP into RNA by approximately 90%, while under these conditions, expansion growth of the segments is inhibited by 80%.

One can conclude from such experiments that the addition of auxin to plant cells potentially capable of further expansion not only causes a stimulation of growth, but also a stimulation of synthesis of RNA and protein. If either of these synthetic pathways is blocked by suitable inhibitors, then growth is also inhibited by roughly the same order of magnitude.

What happens in cells that have completed their expansion growth and which are so highly differentiated that they will no longer undergo cell expansion? What is then the effect of auxins or other hormones on the synthesis of protein and nucleic acid?

Rôle in senescence

Auxins, kinins and gibberellins are all effective in delaying senescence in leaf cells of different plant species. Senescence in leaves is a physiological phenomenon associated with a declining ability of the cells to synthesise RNA and protein. This results in a progressive decline in the levels of nucleic acid and protein. Chlorophyll is degraded, leading to yellowing of the blade, a visible symptom of leaf senescence.[18]

A single application of an auxin, in this case the synthetic auxin 2,4-dichlorophenoxyacetic acid, to the blade of a *Prunus* leaf in the autumn, when protein synthesis has already started to decline, will arrest senescence for periods up to 3 weeks. Investigations of the ability of these leaf cells to make protein by supplying leaf disks with radioactive leucine and subsequently isolating and counting the protein, have shown that protein synthesis has been maintained at its original level (in some cases it is enhanced) by the auxin, while in the controls protein synthesis has continued to decline (Fig. 5).[39a]

These experiments clearly show that auxins can regulate protein synthesis in mature and highly differentiated cells, and that their activity in this respect is not confined to expanding or dividing cells.

Not all species require the same hormone to retard senescence, however. The survival of leaves of *Xanthium*, for example, is little affected by the addition of auxin, but the application of the kinin, kinetin, effectively retards yellowing. Once again it can be shown that kinetin not only prevents the decline in RNA and protein synthesis in the senescent leaf, but within

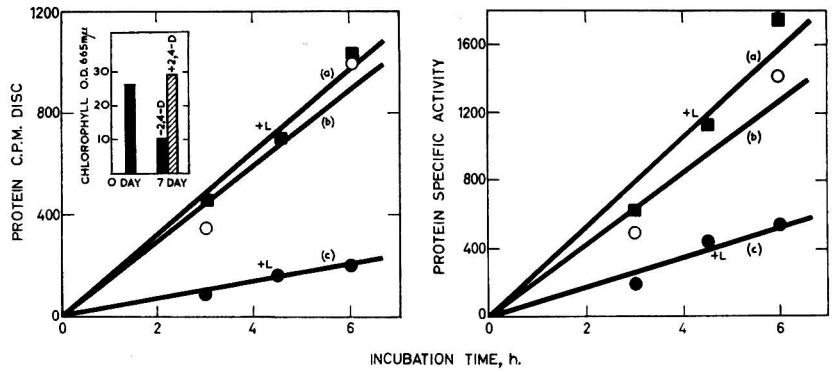


FIG. 5.—*Incorporation of ^{14}C-leucine (L) into protein in disks of cherry leaf cut from 2,4-D-treated and untreated areas of blade*

(after Osborne & Hallaway[39a])

■ 2,4-D ○ control

curve *a* 7 days with 2,4-D
c 7 „ without 2,4-D
b 0 day control

a few hours measurably increases the incorporation of labelled precursors into these two fractions.[18] Experiments are now reported* designed to give information on the site of action of kinetin in these processes. If puromycin is supplied to the disks so that the incorporation of amino-acids into the RNA template in the ribosome is partly blocked (about 50%), then kinetin is no longer effective in either stimulating protein synthesis or retarding senescence. One may conclude therefore that kinetin does not regulate protein synthesis directly at the stage of peptide-bond formation. However, if actinomycin D is supplied to the leaf disks, so that DNA-dependent RNA synthesis is partly blocked, then kinetin still exerts a stimulatory effect on protein synthesis which is roughly proportional to the amount of DNA-dependent RNA synthesis still occurring in the tissues. On evidence of this kind it is proposed that the action of kinetin in preventing senescence could be directed through an effect on DNA-controlled RNA synthesis, and it is not unreasonable to suggest that the regulatory action of kinetin may take place at the stage of messenger-RNA synthesis. Nor would it be contrary to present evidence to suggest that senescence, in an organ like a leaf, is the result of a progressive turning off of the genetic information in the nucleus. If fewer and fewer of the genes remain functional as templates for DNA-dependent RNA synthesis, the cells would eventually stop making protein and pass into a state of dormancy. But the cells of dormant tissues such as winter buds and seeds can be made metabolically active again by many kinds of treatments, including the addition of hormones. However, a senescent leaf cannot be successfully rejuvenated, protein and RNA synthesis cannot be restored to their original levels and the genetic formation seems permanently repressed. During dormancy the genes are apparently only temporarily repressed and if suitably activated within a reasonable period they become de-repressed again and the cells resume both active nucleic acid and protein synthesis and active growth.† If this interpretation of senescence should prove to be correct, and it is found that the genes do become functionally inactivated or repressed in an irreversible manner, then auxin in *Prunus* and kinetin in *Xanthium* must play a rôle in keeping genes de-repressed and preventing the genetic material from being irreversibly turned off.

Rôle in flower induction

What kinds of changes occur when a vegetative plant is transformed to a reproductive plant, and the meristematic buds begin to differentiate flower primordia instead of leaf primordia? Gifford & Tepper[40] have shown that this stage of development coincides in *Chenopodium* with sharp increases in RNA and later in protein content, in the cells of the meristem. This would

* See Osborne, D. J., 'Hormonal Regulation of Leaf Senescence', *Xth Int. Bot. Conf.* (Edinburgh), 1964.
† See Tuan, D. Y. H., & Bonner, J., *Plant Physiol.*, 1964, **39**, 768.

be expected if the transformation from the vegetative to flowering condition were associated with a whole new part of the genome becoming operative, and many previously repressed genes being turned on. The following experiments add support to the premise that flower induction involves changes at the gene level.

It is known that the leaves are the receptor organs for the flowering stimulus as well as being the site of synthesis of the flowering hormone, and that once the leaves (or just one young leaf) receive the appropriate number of hours of darkness, the chemical stimulus is transmitted to the bud which initiates the new kind of differentiation in the meristem. The transport of the stimulus from leaf to bud may take 16 to 40 h. depending on species and environmental conditions.

Xanthium plants growing in non-photoinductive conditions can be induced to flower if given a single long night exceeding 8½ h. If, during this period, RNA synthesis in the bud is interrupted by supplying it with 5-fluoro-uracil (5FU), an antimetabolite which is incorporated into RNA instead of uracil to give fraudulent RNA, then the plant does not flower (Fig. 6). If 5FU is given before or after the dark period the plant flowers. It will also flower if a ' good ' precursor for uracil (in this case orotic acid) is added together with the 5FU during the inductive dark period so that at least some non-fraudulent RNA is made.[41] One can conclude then, that RNA synthesis in the bud during the dark period is necessary for flower induction in *Xanthium*. In another plant, *Pharbitis nil*, Zeevart[42] has shown that flowering will occur only if actual DNA replication takes place at the time the flowering stimulation from the leaf reaches the bud. In these experiments 5-fluorodeoxyuridine (5FDU) was used as an antimetabolite of thymidine, and when this was applied to the bud some hours before the stimulus arrived or some hours after its arrival, flowering proceeded normally (Fig. 7). If it was applied at the time of arrival of the flowering stimulus, then flowering was prevented. Further, if thymidine was added with 5FDU so that some normal DNA was synthesised, then once again some flowering was obtained.

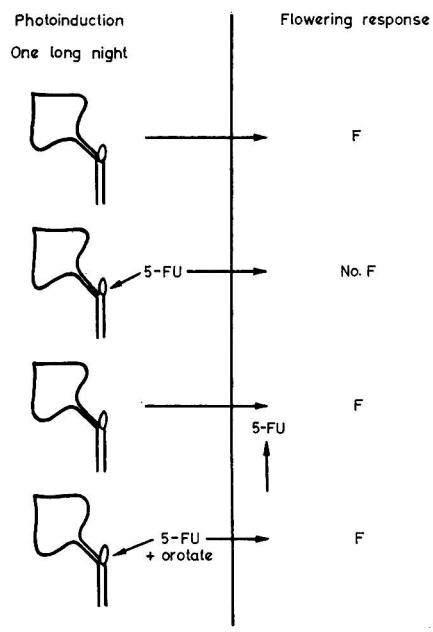


FIG. 6.—*Effect of 5-fluoro-uracil on flowering induction in* Xanthium
(after Bonner & Zeevart[41])

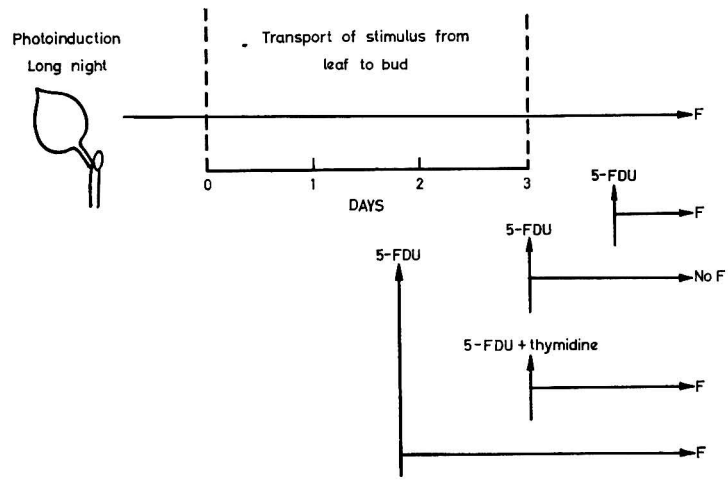


FIG. 7.—*Effect of 5-fluorodeoxyuridine (FDU) on reception of flowering stimulus in buds of* Pharbitis (after Zeevart[42])

So it seems that RNA synthesis in the bud at the time of photoinduction is required for flowering in *Xanthium*. In *Pharbitis* it is DNA replication in the bud that is required at the time when the floral stimulus formed in the leaves arrives in the bud. Active nucleic acid synthesis is therefore a prerequisite for these two species to be transformed from the vegetative to the flowering condition.

Rôle in cell differentiation

At this point the experiments with artichoke tuber tissue can be reconsidered.[43] It was noted above that if the disks were washed in water at 24° for 24 h., then subsequent additions of auxin and kinetin stimulated cell expansion, but if they were washed in water at 4°, then kinetin and auxin stimulated cell division (Fig. 8). What is the nature of the regulatory mechanism that determines the growth response to auxin and kinetin ?

Further experiments showed that if gibberellin was added together with the auxin and kinetin *after* the washing period at 24° the growth response was similar to that with auxin and kinin alone. Gibberellin did not affect cell expansion (Fig. 9). The situation with freshly cut disks is, however, very different. As already seen, the cells expand very little for the first 24 h. in water and they grow very little more in the presence of auxin and kinin together or if they are supplied with gibberellin alone. But, if fresh disks are supplied with auxin, kinin and gibberellin all added together, rapid cell expansion ensues at once (Fig. 9). Apparently gibberellin has some special rôle in predisposing the cells to expansion under the influence of

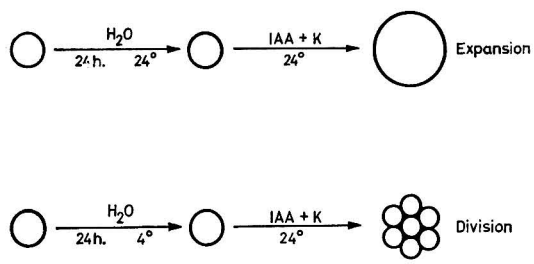


FIG. 8.—*Effect of pretreatment temperature on growth of artichoke tuber disks in auxin and kinetin* (after Adamson[26])

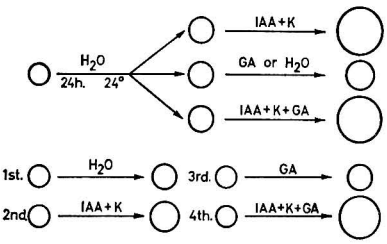


FIG. 9.—*Effect of gibberellin on cell expansion in disks of artichoke tuber*
(1st–4th tests and for 24 h. at 24°)
(after Setterfield[43])

auxin and kinin. It seems that the addition of gibberellin results in cellular changes similar to those occurring when the disks are washed for 24 h. at 24°, for both gibberellin or a washing treatment permit rapid cell expansion in auxin and kinin.

Setterfield and his colleagues then carried out an experiment to determine if the changes that occurred during the washing period were prevented if the formation of new and functional RNA was blocked. They found that if the antimetabolite 5FU was added during the 24-h. washing period at 24° auxin and kinetin together no longer induced rapid cell expansion, but if applied after the 24-h. washing period, 5FU did not prevent the rapid expansion induced by auxin and kinetin (Fig. 10). Setterfield concludes that if the cell is not able to make functional RNA during the washing period, then it does not become programmed to expand rapidly under the influence of auxin and kinetin—in fact it behaves as if it had had no washing period at all. In other words the cells have not received the new information which permits the processes of rapid expansion.

One may reasonably ask the questions, is there evidence that new information is actually produced and is there evidence that the process is regulated by hormones? If there is production of new information, one would expect new kinds of RNA, new kinds of protein and new enzymes to be produced during the washing period. It is now known that this does, in fact, take place. Freshly cut artichoke tuber disks have negligible invertase activity, but after they have been washed in water for a day they exhibit considerable invertase activity. A number of tissues washed in this way, for example potato tuber slices, or red beet slices,[37] show a net increase in total protein, and it is clear that this washing period is associated with the production of new protein. Edelman & Hall[44] have recently shown very elegantly, that the production of invertase by artichoke tuber disks is dependent on the production of new RNA. If DNA-dependent RNA formation is blocked by actinomycin D, invertase formation does not occur although protein synthesis is not then immediately stopped for we know that plant messenger RNA remains functional for some hours. If protein synthesis is blocked by puromycin, again, invertase activity does not develop. Edelman & Hall then investigated two plant hormones in this connexion and found that auxin greatly reduced the amount of invertase formed, but gibberellin greatly enhanced it (Fig. 11). It is reasonable to suggest that, in the

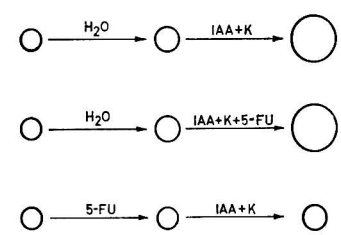


FIG. 10.—*Effect of 5-fluoro-uracil (FU) on cell expansion in disks of artichoke tuber*
(after Setterfield[43])

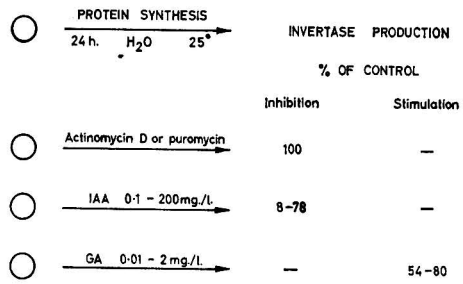


FIG. 11.—*Development of invertase activity in artichoke tuber disks*
(after Edelman & Hall[44])

case of these artichoke tuber disks, auxin causes the genes for making invertase to remain repressed, while gibberellin causes them to be de-repressed.

There is another enzyme system in plants whose formation is inducible by a plant hormone, and this is α-amylase. Paleg[32] showed that germination and the development of enzyme activity (including protease activity and amylase activity) were all stimulated when barley seed was germinated in the presence of gibberellin. One of the enzymes, α-amylase, was not present in the ungerminated seed and was clearly produced only during germination. If the embryo were removed from the seed, however, only traces of α-amylase could be detected in the aleurone, but if gibberellin were added to the excised aleurones, large amounts of α-amylase were formed. It appeared that gibberellin could substitute for the embryo in inducing the aleurone to synthesise α-amylase. Most interesting of all, however, is the fact that if chloramphenicol or puromycin are added to the aleurone at the same time as gibberellin or if actinomycin D is added some while before the gibberellin, no α-amylase is formed (Fig. 12). So it is clear that here again DNA-dependent RNA synthesis and protein synthesis are prerequisites for α-amylase formation.[45]

This result is now even more convincing following further experiments soon to be published by Varner & Chandra,[46] who have chemically isolated α-amylase from excised barley aleurone treated with gibberellin in the presence of a number of radioactive amino-acids. They have shown that the ^{14}C-label is incorporated into the purified α-amylase, and that this incorporation is blocked by puromycin and actinomycin D. Gibberellin is therefore responsible in barley aleurone for the induction of α-amylase activity. One is thus led to conclude that gibberellin functions in this system by acting as a de-repressor of the genes that control the synthesis of the protein, α-amylase.

From a consideration of these experimental facts it seems that we should now look for the primary site of hormone action in plants at the gene level.[47] A wealth of evidence is accumulating that suggests that animal hormones and insect hormones also function at this level.

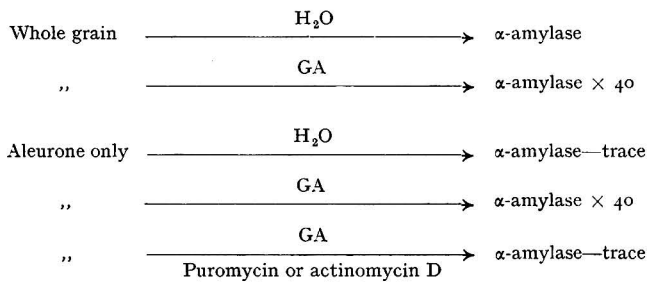


FIG. 12.—*Development of α-amylase by germinating barley*
(combined data)

Conclusion

Hormones could be the effector substances that control repression and de-repression of different parts of the genome so that different parts are repressed or de-repressed at different times. It is known that the external environment, the light intensity and the daylength can affect the levels of hormones within plant cells, and we can begin to see how these different contents of auxins, kinins and gibberellins could be operative in regulating a progressively changing pattern of enzyme systems resulting in the complex growth, differentiation and maturation of the whole organism.

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PRODUCTION OF AMYLASE ON VERMICULITE BY *ASPERGILLUS ORYZAE*

By J. MEYRATH

The production of amylase on the conventional substrate wheat bran has a serious drawback in so far as the enzyme extracts are thoroughly contaminated with solubilised material from wheat bran which make a purification of the extract rather difficult. Successful attempts have been made to use synthetic media absorbed on vermiculite for amlyase production by *Aspergillus oryzae*. Since under these conditions the rate of amylase production is very much higher than in stationary or deep cultures on the same substrate or similar substrates, the described method is of interest from the industrial point of view.

Introduction

There is a certain trend nowadays to use the deep culture method for the production of enzymes by moulds. Nevertheless the greatest proportion of microbial amylase and protease is apparently still produced by solid cultures using especially one particular substrate, wheat bran (Underkofler, personal communication). This medium is well known to give high yields of amylase and/or protease. It also affords the advantages of cultivation techniques common to this type of substrate, i.e., few contamination problems, because infections remain localised; requirement of less-skilled labour; use of low-pressure fans for aeration.[1] One of the disadvantages of bran cultures is that the enzyme extracts are very difficult to purify in view of the great number of undefined compounds which have been solubilised by the mould.

The method described below consists of using synthetic substrates (thus allowing easy purification of the amylase) in the presence of an inorganic absorbing material (vermiculite), which renders the whole substrate solid, and, furthermore, has a strong stimulating action on the rate of amylase production. Application has been made for a patent.[2]

Experimental

Production of inoculum

A strain of *Aspergillus oryzae* which has been used in previous work for enzyme formation,[3] served as amylase producer in the present investigations. The inoculum consisted of a standard amount of conidia which can reliably be produced in large numbers on substrate E of which the composition is indicated below. The conidial suspension was prepared[4] by adding sterile distilled water to the sporulated cultures, shaking them vigorously, filtering the suspension through sterile absorbent cotton wool in order to free the suspension from pieces of mycelium, and finally washing the suspension three times by centrifuging and resuspending the conidia in sterile distilled water. Counting of the conidia was carried out by a haemocytometer; the size of inoculum was adjusted to 10^6 conidia per 25 ml. of substrate.

Preparation of substrates

For the vermiculite cultures a substrate with a low concentration of phosphate can be used. The pH is maintained approximately constant by including an organic anion[5, 6] (in this case acetate) which serves as a carbon source, thus counteracting the increase in acidity due to assimilation of ammonium-nitrogen and production of non-utilisable organic acids. In previous tests it was found that a high concentration of the nitrogen source was essential for the strain of *Aspergillus oryzae* used here, as it has been shown previously to be the case for *A. niger*[5] and for *A. oryzae*.[7] The substrate used in the present experiments (substrate D) contained per l. of deionised water: starch, 40 g.; citric acid monohydrate, 5 g.; acetic acid, 3·6 g.; $FeCl_3,6H_2O$, 15 mg.; $MgSO_4,7H_2O$, 0·3 g.; $(NH_4)_2SO_4$, 8 g.; KH_2PO_4, 2 g.; NaOH was included to obtain pH values of 5, 5·5, 6·1 and 6·8 for the various experiments shown below.

The substrates were distributed in 10-ml. amounts in 50-ml. conical flasks, thus forming a very shallow layer (approximately 8 mm.), or they were added in 25-ml. portions to 5 g. of a large-grain vermiculite in 150-ml. conical flasks, thus forming a layer of approximately 2 cm. The substrate was completely absorbed in the pores of the vermiculite.

Substrate E, which was strongly buffered, contained per l. of deionised water : starch, 40 g. ; $(NH_4)_2SO_4$, 8 g. ; $MgSO_4,7H_2O$, 0·3 g. ; $FeCl_3,6H_2O$, 3 mg. ; KH_2PO_4, 34 g. ; Na_2HPO_4, 35·5 g. The pH of this substrate is 6·8 ; it was distributed in 25-ml. amounts in 150-ml. conical flasks, thus forming a layer of approximately 1 cm. and was also added in 25- or 10-ml. amounts to 5 g. of vermiculite (see under substrate D).

Sterilisation of the substrate was effected by autoclaving for 15 min. at 15 p.s.i. The liquid substrates (for stationary cultures) were autoclaved in the culture flasks, and inoculated with 0·5 ml. of a suspension of conidia per 10 ml. of substrate, whereas the media for the vermiculite cultures were sterilised in bulk, inoculated in the same proportions as above and then mixed in the indicated amounts with vermiculite.

All cultures were incubated at 25°.

Assay of amylase activity

The amylase activity was determined on a 1 : 100 diluted solution of the culture filtrate obtained by pressing the content of the flasks on to a piece of nylon fabric in a Büchner funnel. The procedure of the assay chosen is very similar to the spectrophotometric method described earlier[3] (see also Fuwa[8]), except that starch and not amylase is used as substrate. In principle, one determines the rate of breakdown of starch indicated by the decrease in intensity of the purplish dextrin–iodine colour. The assay procedure consists in transferring 0·7-ml. amounts of starch into test tubes placed in a water bath at 20°, adding to each portion 0·5 ml. of a suitably diluted enzyme solution, and stopping the reaction at convenient time intervals in the various tubes by adding 10 ml. of iodine solution B.[3] The intensity of the colour is recorded and subtracted from that of a blank in which the enzyme solution had been replaced by water. In the present experiments an EEL absorptiometer was used with neutral intensity filter. The amylase units were calculated from the following formula :

$$(A) = (100 - x)/t$$

where (A) stands for amylase units per ml.,
100 is the initial intensity of the starch–iodine colour,
x is the intensity of the dextrin–iodine colour after time t, in minutes.

If the value of x is not allowed to drop below 30, there is linearity between the enzyme units thus defined and enzyme concentration.

Results

The enzyme activities obtained with substrate D at various pH values in liquid surface cultures as well as in vermiculite cultures are shown in Table I. It can be seen that, under the conditions chosen, the enzyme activity in vermiculite cultures has practically reached a maximum after 4 days of incubation, with 50–56 units at all pH values. At an initial pH of 5 and 5·5 the enzyme appears to be more stable than at initial pH values of 6·1 and 6·8. The liquid cultures show a very slow increase in enzyme titre, and even after 8 days there is a yield of

Table I

Amylase production (units/ml.) in vermiculite and liquid stationary cultures at various initial pH values in substrate D

Time of cultivation, days	Initial pH			
	5	5·5	6·1	6·8
	Vermiculite			
3	35	30	24	21
4	55	56	50	56
6	59	57	50	42
	Liquid			
3	1·1	1·5	1·7	1·4
4	1·7	4·0	1·5	3·0
6	1·5	11·0	12·5	18·0
8	18	22·5	22	50

only 18–22·5 units attained in the substrates with initial pH of 5·0, 5·5 and 6·1. If the initial pH is 6·8, a more rapid production of amylase can be observed, but the maximum yield of 50 units, however, is reached only after 8 days.

With a high phosphate concentration (substrate E) a more rapid production of amylase in liquid cultures is obtained, as is shown in Table II. After 4 days the yield is already considerable (36 units), and more than ten times higher than the substrate D. The highest yield in substrate E, 48 units, approaches the maximum yield of 59 units under the most favourable conditions in vermiculite cultures with substrate D. If substrate E is used in the presence of vermiculite, a marked stimulation on rate of production and maximum yield can again be observed. With 25 ml. of substrate per 5 g. of vermiculite the results are near enough comparable to those in vermiculite with substrate D, pH 6·8. A smaller amount of substrate, i.e., 10 ml. per 5 g. of vermiculite, does not appear to be as suitable, as the yields are almost 20% lower.

Table II

Amylase production (units/ml.) in vermiculite and liquid stationary cultures in substrate E

Time of cultivation, days	Vermiculite		Liquid
	5 g. + 25 ml.	5 g. + 10 ml.	
3	11	—	4·0
4	55	47	36
6	46	44	48

Discussion

At present it is difficult to give an explanation for the increase in rate of amylase production in vermiculite cultures. There does not appear to be as good a mycelial production as in liquid cultures, according to visual observation. The determination of growth in vermiculite cultures has not been carried out because of technical difficulties. This would appear to be an important point to consider in future experiments in order to obtain some indication of the functions of vermiculite. In past experiments it was frequently observed that good amylase production can be obtained even if growth is relatively poor. This may well be the result of an increased excretion of organic nitrogenous compounds (including enzymes such as amylase). This excretion might be stimulated by a high concentration of the nitrogen source. Poor growth combined with pronounced excretion of organic nitrogen[9] and amylase[10] have recently also been observed with the presently used strain of *A. oryzae* with small inocula in vibrating culture.[11] Poor growth and good amylase production can also be observed in substrates with certain trace element compositions.[12]

Substrate E, with a high phosphate content, is superior to substrate D when used in liquid form. From the present experiments it could not be decided with certainty whether this effect is due to phosphate as the two substrates are not exactly comparable. However, in other experiments (with glucose as carbon source) there was a marked increase of amylase production when the phosphate concentration was increased from M/15 to M/2, which could be shown not to be due to unfavourable pH in the low-phosphate medium. It should be remembered that, although large amounts of phosphates afford a means for increasing the rate of amylase production, they do not result in the formation of solid substrates which have a definite advantage technically because contaminations do not spread over the whole culture.

The presently used mould strain gives on bran cultures approximately four times the yield of amylase on vermiculite cultures in almost the same time of cultivation under the conditions that the bran cultures are obtained from 10 g. of bran plus 20 ml. of water, and the vermiculite cultures from 5 g. of vermiculite and 25 ml. of substrate D. The amount of starch present in 10 g. of bran is approximately 5 g., whereas in 25 ml. of substrate D there is only 1 g. It is known from other experiments carried out under different conditions, that an increase of the concentration of the carbon source from 40 to 80 g./l. results in almost double the yield of amylase. Therefore it is probable that the enzyme yields presently obtained in vermiculite cultures can be considerably increased, and could possibly reach those of bran cultures.

In deep-culture conditions with substrate E, the strain does not give as high yields of amylase as in stationary cultures. If the starch concentration is only 10 g./l., the enzyme titre is higher in deep cultures (20 units/ml.), than in surface cultures (10–12 units/ml.). The rate of enzyme production under the best conditions in deep cultures is smaller than in vermiculite cultures, as the maximum of 20 units in deep cultures, produced at fairly constant rate, is reached after 8 days only, whereas vermiculite cultures can give a yield of 55 units after 4 days.

Bindal & Sreenivasaya[13] used asbestos for amylase production with *A. oryzae*, but they did not carry out any comparisons with other methods of cultivation. In preliminary experiments with the present strain on asbestos the yield of amylase was only about 1/4 of that in vermiculite cultures.

There may be some future in the use of purified bran as a solidifying agent for mould substrates. Wheat bran can be freed from most of the undesirable material by cultivating *A. oryzae* in the usual way for enzyme production, and after extraction of the enzymes the material can be washed further with hot water and is then suitable for cultivating *A. oryzae* in a synthetic medium. The yield of amylase obtained in preliminary tests was approximately 1/2 of that of vermiculite cultures.

A considerable increase in rate of amylase production in both liquid and vermiculite cultures can be expected with our strain if the temperature of incubation is increased, as there is a considerable increase in rate of production with bran cultures if the temperature is increased from 25 to 35°.[3]

There is at present considerable confusion in the literature regarding amylase units because so many different methods have been used for estimating enzyme activity. Work is under progress to make an evaluation of the various units in order to compare the present yields with those of other workers.

Conclusions

The rate of amylase production by *Aspergillus oryzae* under various conditions of pH and substrate composition is strongly increased if the mould is cultivated in the presence of vermiculite in the form of solid substrates. This increase refers to both stationary and submerged cultures in liquid form.

Some stimulation of the rate of amylase production in liquid stationary cultures is also obtained if a large amount of phosphate (beyond that required for buffering) is included in the substrate.

The marked increase in rate of amylase production with vermiculite, together with other advantages attached to the use of solid cultures make the method very promising for industrial purposes.

Acknowledgments

Some preliminary experiments were carried out at the Dept. of Agricultural Bacteriology and Fermentation, Swiss Federal Institute of Technology, Zürich, where financial support was provided by Dr. H. Müller, Mullerbräu A.-G., Baden, and technical assistance by Mrs. A. Meyrath. The stimulating interest of Professors L. Ettlinger (Zürich) and E. O. Morris (Glasgow) has also been greatly appreciated.

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Received 20 May, 1964

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ON THE FLAVOUR VOLATILES OF FATS AND FAT-CONTAINING FOODS. I.—Degradation of the Peroxides of Autoxidised Sunflower and Linseed Oils

By C. H. LEA and A. HOBSON-FROHOCK

Refined sunflower and linseed oils purified by ' stripping ' in a molecular still, treatment with silicic acid and steam deodorisation were autoxidised at 37° to an oxygen absorption of approximately 100 μmoles/g. The amounts of peroxide, non-volatile carbonyl and volatile carbonyl were determined in the oxidised oils before and after destruction of the peroxides by heating in vacuum.

The peroxides (mainly linolenate) of the linseed oil decomposed more readily than those (mainly linoleate) of the sunflower oil, and produced a higher proportion (60% as compared with 40%) of carbonylic compounds. In both oils nearly half of the total carbonyl groups formed were in volatile compounds. Not more than 1–2% of the volatile carbonyls could have been formed from non-peroxidic precursors.

Introduction

Volatile compounds of objectionable odour and flavour are produced during the autoxidation or catalysed atmospheric oxidation of unsaturated fats and lipids and most of these compounds are carbonylic in nature. Mainly they consist of saturated and unsaturated aliphatic aldehydes of medium molecular weight, with minor proportions of ketones, alcohols and hydrocarbons. The methyl ketones and lactones prominent in butterfat have a different origin.[1]

Substances of these same general types are, however, also identified repeatedly as major contributors to the normal aromas and flavours of undeteriorated and especially of cooked foods. It is clear that many of the techniques required for the concentration, separation and identification of ' off ' flavour constituents and for the assessment of their relative importance by organoleptic procedures will be applicable also to studies of normal food flavours.

In previous papers[2, 3] a simple vacuum distillation procedure was described whereby small quantities, of the order of 0·1 μmoles/g., of aliphatic aldehydes of chain length C_3–C_{12} can be recovered quantitatively from solution in oil and determined colorimetrically as 2,4-dinitrophenylhydrazones, and preliminary results were reported from the application of this method to determination of the volatile carbonylic substances present in autoxidising fats.

In the present paper the production of volatile carbonylic compounds in sunflower and linseed oils, specially treated to remove preformed oxidation products, has been measured after autoxidation of the oil at 37°, and the further formation of carbonyls, both volatile and non-volatile, has been followed during thermal decomposition of the peroxides which are the major oxidation products at low temperatures. Flavour characteristics of the volatile oxidation products formed and further investigations of their nature will be reported in subsequent papers.

Experimental

Composition and purification of the oils

Composition.—Refined sunflower oil, analysed by gas-liquid chromatography of the methyl esters, contained 68·5% of linoleic, 21% of oleic and 10% of saturated (palmitic and stearic) acids, expressed as % of the total fatty acids. No linolenic acid could be detected.

Refined linseed oil was used as a linolenate-rich substrate. It contained 60% of linolenic, 15% of linoleic and 16% of oleic acid, the remaining 8–9% being saturated (palmitic and stearic) acids.

Removal of tocopherols.—The refined oils were ' stripped ' by passage twice through a wiped-wall molecular still, to remove in all about 5% of the oil, the distillates, containing most of the tocopherols, being rejected. Each run required a little less than 2 h. at 250° and 10–15 μ. (We are indebted to Dr. A. Thomas of the Unilever Research Laboratories for carrying out these distillations.)

Removal of preformed oxidation products.—Refined vegetable oils, even after treatment by steam deodorisation to destroy peroxides and remove volatile decomposition products, still contain appreciable quantities of non-volatile autoxidation products which can act as precursors of ' off '-flavour compounds.[4–7] Paul & Roylance[8] and Crossley *et al.*[9] have used alumina to improve the stability of vegetable oils by selective absorption of ' off '-flavour precursors, and silicic acid has been employed by Frankel *et al.*[10, 11] to remove and fractionate oxidation products from autoxidised unsaturated fatty acids or esters.

The stripped sunflower and linseed oils were therefore purified by an adsorption treatment, prior to deodorisation. The oil (300 g.) in redistilled de-aerated light petroleum (900 ml.) was passed through a column of ' Celite '–silicic acid (1 : 1, activated at 120° and cooled under nitrogen). Recovery was about 90% of oil of carbonyl content $<0{\cdot}2$ μmole/g.

Steam deodorisation.—For removal of volatile odorous substances, including the last traces of solvent, the oil was steam deodorised in an apparatus similar to that of Heide-Jensen[12] at a temperature of 180°. All deodorised oil samples were sealed immediately into glass ampoules which were evacuated and stored at −20° until required.

Autoxidation of the oils

Samples of the purified deodorised oils were shaken under oxygen at 37° in flasks fitted with mercury manometers and containing ' Hi-drite ' ($CaSO_4$) drying tubes. When the desired amount of oxygen had been absorbed (usually about 100 μmoles of O_2 per g. of oil) the oxidised oil was packed into small glass-stoppered tubes filled to the top and immediately stored at −20° or −80°. At −20° the peroxide value slowly increased during storage up to a maximum increase of about 3 units, presumably due to continued reaction of dissolved oxygen.

Removal of the volatile products

For removal of the volatile substances produced during autoxidation of the oils, the distillation method previously described[3] was used, with condensation of the distillate on a ' cold finger ' cooled by liquid oxygen. For greater ease of manipulation, the design of the still was modified to utilise a flat flanged joint instead of the cone type previously employed, a silicone rubber O-ring (fitted round a thinner stainless steel supporting washer) providing the vacuum-tight seal. Three sizes of still, based on standard (Quickfit & Quartz Ltd.) flat flanged joints of nominal bore 57, 75 and 100 mm., provided distilling surfaces of approximately 18·9, 39·5 and 78·5 cm.^{2}, respectively. In each case the condensing surface was approximately 2 cm. above the surface of the oil. With this type of still application of a slight internal pressure of nitrogen at the end of the distillation easily separated the condenser from the base, an operation which had sometimes proved difficult with the earlier cone-joint model.

Various times and temperatures of distillation were employed, after the initial stage of restricted pumping used to prevent loss of the more volatile components.[3] In some experiments a metal deactivator (0·01% disodium EDTA) was added to the still (in ethanol, subsequently evaporated off) before the charge of oil, to minimise metal-catalysed decomposition of the fat peroxides during the run.

Determination of the volatile carbonylic compounds

(a) *By the 2,4-dinitrophenylhydrazine colorimetric method.*—As previously described[3] the method of Henick *et al.*,[13] as modified by Chipault *et al.*,[14] was used for preparation of 2,4-dinitrophenylhydrazine (DNPH) derivatives of the volatile carbonyl compounds present in the condensate on the cold finger, and their spectrophotometric estimation in alkaline solution. The quantities, however, were reduced to give a final volume of 25 ml. instead of 50 ml. When the amount of carbonyl present was >1 μmole the condensate was dissolved in carbonyl-free benzene and an aliquot taken ; otherwise the condensate was dissolved directly in the acid 2,4-dinitrophenylhydrazine reagent.

Since the purpose of this procedure was to measure the *total* quantity of volatile carbonylic compounds present the extinction of the test solution was measured at 430 mμ only. It can be seen from curves published by Stitt *et al.*[15] that at this wavelength the absorption of heptanal, hept-2-enal and hepta-2,4-dienal all approximate to $\varepsilon = 20{,}500$. Our own determinations on the corresponding C_6 compounds gave a similar average ε value, although the coincidence of the three absorption spectra at this wavelength was less good, and the corresponding curves for decanal and crotonaldehyde crossed nearer to 425 than to 430 mμ. Rather variable results have been reported in the literature for the molar extinction coefficients of aldehyde 2,4-DNPH derivatives in alkaline solution ; a value of 21,000 has been used in the present paper to convert extinction at 430 mμ to moles of carbonylic substance.

(b) *By direct spectrophotometric assay.*—In an alternative procedure, the volatile compounds were removed from the condenser in a few ml. of cyclohexane (spectroscopic grade) or carbonyl-free ethanol and examined, after appropriate dilution when necessary, by direct measurement of their ultra-violet absorption spectra.

Thermal decomposition of the peroxides

To measure the quantity of carbonylic substances, both volatile and non-volatile, produced by the thermal decomposition of a known amount of fat peroxide, samples of the oxidised oil were heated under vacuum in the still for various periods at a series of temperatures between 100 and 200°, with condensation of the volatile decomposition products on a liquid oxygen-filled cold finger. In this procedure the volatile products resulting from thermal decomposition of the peroxides were distilled off as they were formed, and thereby protected from any further reaction. In an alternative procedure, designed to produce an oil of low peroxide and high carbonyl content, the peroxidised oil sample was sealed under vacuum into a small glass tube and heated for 1 h. at 180° or 200° to decompose the peroxides.

Determination of the peroxide value

Peroxide values of the oxidised oils were determined by an anaerobic iodometric procedure (Lea[16]) using a 100 ml. wide-mouthed conical flask, 20 ml. of glacial acetic acid–chloroform (3 : 2, v/v), 1·2 ml. of saturated aqueous potassium iodide solution and a reaction time of 15 min.

Determination of the non-volatile carbonylic compounds

(a) *By the 2,4-dinitrophenylhydrazine colorimetric method after reduction of the peroxides.*—It has frequently been reported that fat peroxides interfere with carbonyl determination by the 2,4-dinitrophenylhydrazine method, though the extent of the interference does not seem to have been investigated. Carbonyl values determined in the presence of peroxide are therefore likely to be much too high and, in the present work, carbonyl contents of oils of other than very low peroxide content have only been determined after prior reduction of the hydroperoxide by treatment with potassium iodide in acetic acid–chloroform solution. The carbonyl groups themselves were not appreciably affected by the procedure used to reduce the peroxides.

(b) *By the direct spectrophotometric method.*—Holman & Burr,[17] Lundberg & Chipault[18] and others have measured the increase in absorption in the region of 270 or 277·5 mμ during the autoxidation of linoleate, and have attributed it to the probable formation of conjugated diene ketones, such as could be produced by loss of a molecule of water from a hydroperoxide. An

attempt was therefore made to utilise the observed extinction at this wavelength as a measure of non-volatile carbonyl content which would not be subject to interference by peroxides.

Results

Purification of the oils

Typical results of the preliminary purification procedure applied to the sunflower oil used in the autoxidation experiments are given in Table I. Removal of 5% of the oil by molecular distillation was very effective in reducing the apparent tocopherol content, while the silicic acid column removed most of the (largely non-volatile) carbonylic compounds. Finally, steam deodorisation destroyed any residual traces of peroxides that might still be present and removed odorous (volatile) compounds.

Table I

Preliminary purification of refined sunflower oil for use in the autoxidation experiments

		Apparent tocopherol content, μg./g.*	Total carbonyl content, μmole CO/g.
A	Commercially refined oil	330	6·7
B	A stripped by molecular distillation	47	6·8
C	B treated with silicic acid	13	0·2

* Reducing substance by the Emmerie & Engel method

Autoxidation of the oils

At the comparatively low temperature of 37° sunflower oil purified only by silicic acid treatment and deodorisation required 1100 h. to absorb 100 μmoles of oxygen per g. (Fig. 1). Removal of most of the tocopherols by molecular distillation reduced this time to a little over 100 h. and altered the shape of the autoxidation curve to that of an animal-type fat, with a marked induction period. Addition of EDTA approximately doubled the induction period and reduced the 'scatter' between individual runs.

Linseed oil behaved in a generally similar manner although molecular distillation was less effective in removing the natural antioxidants.

Composition of the oxidised oils

Of the oxygen absorbed by sunflower oil at 37° (e.g., 103 μmoles/g.), 94% accumulated as peroxide measurable by the iodometric method. The fact that the sum of the peroxide oxygen (97 μmoles/g.) and of the carbonyl oxygen by direct determination (41 μmoles/g.) greatly exceeded the total amount of oxygen absorbed provided strong evidence that the carbonyl determination was in error, a conclusion confirmed by the very much smaller carbonyl values

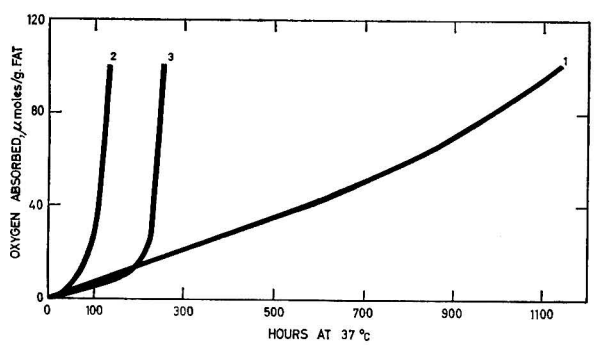

FIG. 1.—*Autoxidation of sunflower oil in oxygen at 37°*
(1) Refined oil treated with silicic acid and deodorised
(2) As (1), but first 'stripped' of tocopherols by molecular distillation
(3) As (2), but with addition of 0·01% disodium EDTA

(6 μmoles/g.) obtained after iodometric reduction of the peroxide. Typical figures for linseed oil were oxygen absorbed 101, peroxide value 81, and total carbonyl values before and after peroxide reduction 65 and 11 μmoles/g., respectively.

Most of the apparent carbonyl present in the oxidised oils was therefore peroxide, which was converted to carbonyl, probably by acid-catalysed decomposition to unsaturated keto-glyceride and water, during the determination. The proportion of the peroxide groups of oxidised sunflower oil reacting in this way was rather over one third, which is similar to the proportion of carbonyl groups formed by thermal decomposition of the peroxides of this oil (p. 23).

It is possible that even the small residual carbonyl value found after peroxide reduction is too high, because Coleman & Swern[19] have reported the formation of as much as 40–50% of carbonyl by a side reaction during the chemical reduction of methyl oleate hydroperoxide. However, in our oils, not more than 6–9% of the hydroperoxide could have decomposed by such a side reaction, and the proportion may well have been less.

Non-volatile carbonyls by the direct spectrophotometric method

The conjugated triene (presumed di-unsaturated carbonyl) absorption peak was measured for samples of the autoxidised oil which had been heated at 175, 185 and 200° (with removal by distillation of the volatile decomposition products) and the values obtained were compared with the carbonyl contents found in the oils by the 2,4-DNPH method after iodometric reduction of the peroxides. The peak, at 270–271 mμ in cyclohexane, was clearly visible and E_{270} increased approximately linearly as the peroxide value and the conjugated diene absorption at 232–233 mμ decreased, and as the chemically determined carbonyl increased. The 270 mμ peak, however, was too poorly separated from the much more powerful absorption at shorter wavelengths and too much influenced by the different background absorption of different oils for satisfactory use as a general method for non-volatile carbonyl assay. All the values for non-volatile carbonyls reported in this paper were therefore obtained by the 2,4-DNPH method, after iodometric reduction of the peroxides when appreciable quantities of these were present.

Volatile carbonyl content of the oxidised oils by the 2,4-DNPH method

In the earlier work,[3] continued slow evolution of volatile carbonyls from oxidised lard or cottonseed oil after vacuum distillation at 50° for more than 2 h. had suggested that some further formation of volatile carbonyls was occurring during the distillation process employed to remove them. Moreover, autoxidised sunflower oil, after heating in a sealed tube at 180° to destroy nearly all of its peroxides, has now been shown to give off virtually all of its volatile carbonyls in less than 45 min. at 38·5 or 50° (Table II), confirming that the slow evolution observed with the unheated fats is probably due to slow decomposition, even at these low temperatures, of labile precursors (presumably peroxides) rather than to slow distillation of carbonyls of high molecular weight.

Table II

Distillation of volatile carbonyls from sunflower oil oxidised at 37° to peroxide value 94 μmoles/g. and subsequently heated for 1 h. at 180° in a sealed tube to destroy the peroxides

Temp. of distillation, °C	Volatile CO (μmoles/g.) after distillation for		
	0·75 h.	1·5 h.	3·0 h.
38·5	12·04	12·17	12·22
38·5	12·16	12·23	12·24
50	11·94	11·96	12·05

Oils that have been oxidised at low temperatures in a free supply of oxygen, so that nearly all their absorbed oxygen has remained in the form of peroxides with very little degradation to carbonyls, represent the most difficult material in which to determine preformed volatile carbonyls with any accuracy. Sunflower oil oxidised under these conditions to a peroxide content of the order of 100 μmoles/g., but containing only <0·2 μmoles/g. of volatile carbonyls, was therefore used in a series of vacuum distillation runs designed to establish the best conditions for removal of preformed carbonyls with minimum further production during the process.

Effect of depth of oil in the still

The surface of the charge of oil in the still was constantly renewed by the magnetic stirrer and variation of the average depth over the range 1–3 mm. had only a small effect on the efficiency of removal of the volatile carbonyls.

For most purposes a depth of 1 mm., corresponding to a charge of 4 ml. of oil in the medium sized still, gave an adequate quantity of condensate, and this depth was standardised in most of the experiments. However, some increase in sensitivity can be obtained, if required, by increasing the diameter of the still and the depth of the oil.

Effect of temperature and time of distillation

The results in Table III show that while there was some apparent decomposition of labile precursors to volatile carbonyls at all the distillation temperatures used the rate was very slow at 20° or 35°, and still slower at 50°, but it increased rapidly as the temperature was further raised.

Table III

Effect of conditions of heating in vacuum on the amount of volatile carbonyls removed from autoxidised sunflower oil (peroxide value 97–100 μmoles/g.) or linseed oil (peroxide value 81 μmoles/g.)

Temperature, °C	Additive (0·01%)	No. of runs	μmole CO/g. oil after 0·75 h.	1·5 h.	3·0 h.	Increase in 1·5 h.–3·0 h.
Sunflower oil						
75°	—	3	0·34	0·51	0·70	0·19
	EDTA	2	0·33	0·47	0·65	0·18
50°	—	5	0·20	0·25	0·32	0·07
	EDTA	6	0·18	0·22	0·27	0·05
50°	Citric acid	1	0·26	0·36	0·43	0·07
	CMS acid*	1	0·28	0·38	0·45	0·07
38·5°	EDTA	1	0·17	0·20	0·25	0·05
35°	EDTA	3	0·14	0·18	0·22	0·03
20°	EDTA	3	0·12	0·16	0·20	0·04
Linseed oil						
50°	EDTA	2	0·21	0·26	0·31	0·05
35°	EDTA	2	0·18	0·22	0·25	0·03

* Carboxymethylmercaptosuccinic acid

The presence of 0·01% of the metal deactivator disodium EDTA just perceptibly reduced this decomposition, but citric acid and carboxymethylmercaptosuccinic (CMS) acid both *increased* it slightly, possibly because of their decidedly acid reaction.

Thermal decomposition of the peroxides

The effects of heating oxidised sunflower oil of peroxide value 100 μmoles/g. for 1 h. in vacuum at temperatures between 50 and 200°, with and without the addition of EDTA, are shown in Fig. 2. As already indicated the production of volatile carbonyls is detectable even at 20° (Table III) but peroxide destruction, owing to the limitations imposed by the measurement of relatively very small changes in a much larger value, only becomes perceptible at higher temperatures (approximately 1 unit/h. at 100°). In general, the presence of the metal deactivator (EDTA) raised the temperature required for a given amount of decomposition by about 25°.

Corresponding determinations for oxidised linseed oil showed that the mainly linolenate peroxides present in this oil broke down more readily on heating than did the mainly linoleate peroxides of the sunflower oil, and the same conclusion can be drawn from a direct comparison of the two oils during heating in vacuum at 160° (Fig. 3).

When peroxide destruction is plotted against carbonyl production (Fig. 4) it can be seen that each peroxide group destroyed gave rise on the average to about 0·4 carbonyl groups in the sunflower oil and to about 0·6 in the linseed oil and that in both oils nearly half of the total carbonyl groups produced were in the volatile oxidation products.

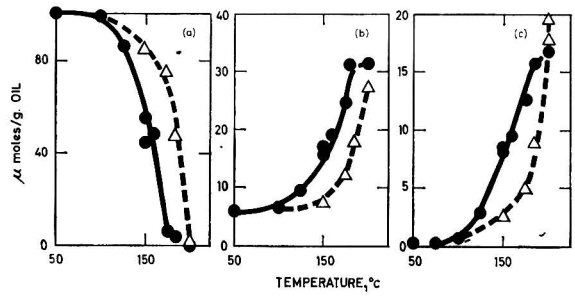

FIG. 2.—*Effect of temperature on the conversion of peroxides to carbonyls in autoxidised sunflower oil*
Samples heated 1 h. in vacuum in presence (△) or absence (●) of EDTA
(*a*) Peroxide value (*b*) Non-volatile CO (*c*) Volatile CO

Production of volatile carbonyls from non-hydroperoxide precursors

To ascertain what proportion, if any, of the volatile carbonylic compounds produced by heating oxidised oils arises from the thermal decomposition of non-hydroperoxidic compounds, e.g., from non-volatile carbonyls (keto-glycerides) or peroxide-free polymers, the peroxides present in a sample of oxidised sunflower oil (peroxide value ~100 μmoles/g.) were destroyed (*a*) by heating under vacuum for 1 h. at 180° or 200°, and (*b*) by reduction with potassium iodide and acetic acid. The resulting ' peroxide-free ' oils were then heated for a further period of 1 h. under vacuum at 175° (or 200° if the oil contained EDTA) with collection on a cold-finger condenser of the volatile carbonyls produced.

The results (Table IV) show that thermal destruction of the peroxides at 180° or 200° left oils with a high non-volatile carbonyl content which showed only a very small, but still easily measurable, production of volatile carbonyls on further heating. Since reduction of iodide-reducible peroxides was probably not quite complete (Fig. 2) it cannot be decided whether the very small volatile carbonyl production remaining derived from traces of residual peroxides or from the slow degradation of non-volatile non-hydroperoxide oxidation products, but the amount, in any case, was only 1–2% of that known to derive from peroxide. It would seem, therefore, that if non-peroxidic oxidation products serve as precursors for volatile carbonyls, as, for example, in the ' flavour reversion ' of edible oils, a further oxidation stage must be involved, heat alone does not suffice.

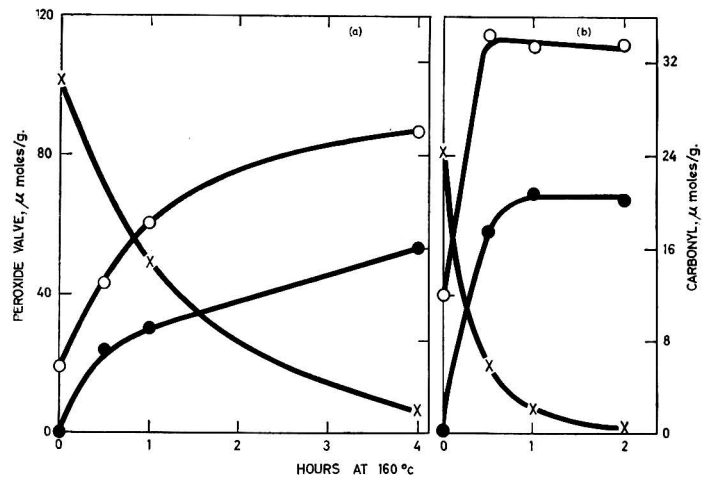

FIG. 3.—*Production of carbonyls by thermal decomposition at 160° of the peroxides of oxidised sunflower* (left) *and linseed oils* (right)
○ non-volatile CO ● volatile CO × peroxide

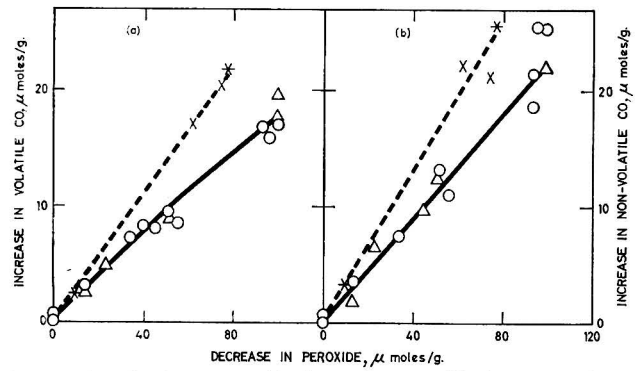

FIG. 4.—*Relation of carbonyl production to peroxide destruction in oxidised sunflower* (———) *and linseed oils* (- - - -) *heated in presence* (△, ✳) *or absence* (○, ×) *of EDTA*

Table IV

Production of volatile carbonyls from non-hydroperoxide precursors

Sample	Analysis (μmoles/g.)			Carbonyls after 1 h. at 175 or 200° in vacuum (μmoles/g.)	
	Peroxide	Non-volatile CO	Volatile CO	Non-volatile	Volatile
Oxidised sunflower oil (A)	100	42*	0·16	24 (175°) 31 (200°)	12·7 (175°) 17·6 (200°)
A treated with acid KI to reduce peroxides	2	6	<0·1	—	0·7 (200°)
A heated 1 h. at 180° to destroy peroxides†	—	31	0·0	28 (175°)	0·2 (175°)
A heated 1 h. at 200° to destroy peroxides†	—	31	0·0	29 (175°)	0·2 (175°)

* Apparent value, mainly due to the peroxides
† And also remove volatile decomposition products

After iodometric reduction of the peroxides of the oil from 100 to 2 μmoles/g. the volatile carbonyls produced on heating at 200° for 1 h. fell from 17·6 to 0·7 μmoles/g., of which about half can probably be attributed to the residual peroxide. This method then also indicates a production of volatile carbonyls from non-peroxidic precursors on heating which is unlikely to be more than 2% of the production from peroxide, and maybe less.

Rapid manipulation and exclusion of air are necessary to obtain a low peroxide value in oil recovered from the washed and dried chloroform layer after determination of the peroxide value: the reduced oil re-oxidises rapidly.

Volatile carbonyls by the direct spectrophotometric method

Volatile compounds were removed from a sample of the sunflower oil, autoxidised at 37° in the presence of EDTA to a peroxide value of approximately 100 μmoles/g., by stirring the oxidised oil in vacuum at 50° for 1 h. and condensing the volatiles on a cold finger. The condensate would consist very largely of the volatile carbonyl compounds already present in the oxidised oil.

A second sample of the same oxidised oil was stirred in vacuum for 1 h. at 200°, to decompose all the peroxides, and the volatile products similarly collected.

When examined spectrophotometrically after appropriate dilution, both condensates showed well-defined peaks at 216–218 and at approximately 270 mμ (265 in cyclohexane, 273 in ethanol), but the total absorption in the 200°-sample was more than 160 times greater than in the other, and the relative intensities of the peaks indicated the presence of a greatly increased proportion of conjugated dienals in the volatiles from the heated sample (Table V). Gaddis *et al.*[20] have previously observed a higher proportion of conjugated dienals in methyl linoleate and methyl linolenate heated to 165° after oxidation, to simulate cooking.

Table V

Direct spectrophotometric determination of unsaturated volatile carbonyls distilled from autoxidised sunflower oil (peroxide value 100 μmoles/g.) at 50° or 200°

Oil heated 1 h. at	$E^{1\%}_{1\,cm.}$ × 10⁴ *		Calculated vol. of CO in oil, μmole/g.	
	217 mμ (2-enal)	270 mμ (2,4-dienal)	2-enal	2,4-dienal
50°	211	62	0·12	0·02
200°	9825	34,250	5·46	10·44

* Based on wt. of oil heated

Swift *et al.*[21] give the absorption spectra of oct-2-enal and deca-2,4-dienal, which they isolated from the volatile autoxidation products of cottonseed oil by fractional crystallisation of their semicarbazones. The major peak (in ethanol) for the monoenal was at 216–17 and for the dienal at 273–4 mμ, but each possessed a smaller peak at approximately the other wavelength. The $E^{1\%}_{1\,cm.}$ of the dienal was given as 1706, corresponding to a molecular extinction coefficient of 26,100.

Commercial samples of hex-2-enal and hexa-2,4-dienal were redistilled. The hexenal then gave an apparent purity of 98·3% when titrated by the hydroxylamine method of Feuell & Skellon,[22] and very little impurity appeared to be present when the sample was run on the gas–liquid chromatograph. The main peak recovered from the chromatograph showed only one major absorption peak, at 217 mμ in cyclohexane, with no sign of a peak at the longer wavelength. The corrected ε value, determined on the redistilled material (preparative GLC was not available), was 18,000.

The hexadienal, similarly treated, also showed one peak only, at 263 mμ in cyclohexane, without any subsidiary peak at the shorter wavelength. The redistilled material gave an apparent purity of 100% by hydroxylamine titration and an ε value of 32,800. These figures have been used to compare the compositions of the distillates obtained at the two temperatures (Table V). Forss *et al.*[23] in a recent paper give the absorption maxima for non-2-enal and nona-2,4-dienal in n-hexane as 215 and 263 mμ respectively: molecular extinctions were not given.

Forrester[24] has shown that aldehydes in solution in (mainly higher) alcohols are converted to equilibrium mixtures with the hemiacetal. Decanal as a 5% solution in decanol, for example, reached equilibrium, with approximately equal proportions of the aldehyde in free and combined form, in about 3 h. at 25°, the change being half complete in about 1 h. No appreciable error from this cause was noticed in the present work, but undue delay should obviously be avoided when extinctions of aldehydes are measured in ethanolic solution.

Saturated aldehydes absorb in the region of 290 mμ but the intensity is too low ($\varepsilon \simeq 24$) to be of value.

Discussion

The chemical changes considered in this paper are small. An oxygen absorption of 100 μmoles/g. of oil corresponds to the reaction of about 0·3% of oxygen by weight, and only one thousandth part of this was present as volatile carbonyl compounds (of the order of 20 p.p.m.) in the freshly autoxidised oils. Heating the oxidised oils to decompose the peroxides present increased their volatile carbonyl contents about 100 times.

Estimation of the amount of preformed flavour substances present in an oxidised fat or fat-containing food is complicated by the fact that many methods of separation, including the use of steam distillation and extraction with such carbonyl-binding reagents as 2,4-dinitrophenylhydrazine or the Girard T reagent, tend to give values that are far too high owing to the conversion of labile precursors to carbonyls during the extraction.[25, 26] Even in the low-temperature vacuum distillation procedure, oxidised oils of high peroxide content did not behave entirely as simple solutions of volatile carbonyls (Table III), although they did after heating to destroy all the peroxides present (Table II). These high peroxide oils, which contained about 500 times as much precursor as preformed volatile carbonyl, however, represent the most unfavourable case possible, and most fats can be expected to fall between the two extremes.

As an alternative apparatus a wiped-wall falling film or centrifugal molecular still would

probably cause less decomposition of precursors than the pot still, by limiting the time required for stripping off the volatiles, but the path to the condenser is often longer in apparatus of this type and removal of the condensate in a sufficiently small volume of solvent may also present more difficulty than with the simple pot still.

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Received 3 April, 1964; amended manuscript 1 June, 1964

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DIGESTION. III.*—Faecal Analyses and Digestibility

By NILS HELLSTRÖM and MARGARETHE AAMISEPP

A study is made of five fractions (separated by a technique described) from the faeces of sheep fed on ryegrass and cocksfoot cut at different periods of growth. The correlation between the feed, the protein contents of the faeces fractions and the metabolic product is briefly discussed.

Introduction

In a previous paper[1] faeces from sheep were separated by ultrasonic irradiation and stepwise sedimentation into five fractions (B, C, F, Q and U). Fraction F contained the fibrous part and nearly all of the birefringent material of the faeces; after irradiation of faeces at a frequency of 20 kc/s, its content of crude protein was small (2–4%). Other fractions (C and U) contained about 20% or more of crude protein. Fraction B had a lower content of crude protein together with a very small amount of birefringent material and was regarded as an intermediate between F

* Part II: *Ann. R. agric. Coll. Sweden*, 1958, **24,** 49

and the other fractions. These results demonstrated a new approach to faecal analysis, in which undigested structural units are determined directly without any mechanical or chemical treatment.

It seemed important to investigate faeces from grasses harvested at different dates in order to obtain a measure of the changes in quality during growing, such as were observed, for instance, in 1833 by Thaer.[2] The present authors, in 1959, obtained two such series of faeces from the Grassland Research Institute, Hurley, Berks., England ; the samples were, of course, not ground (a necessary condition for the method so far).

Experimental

The feeds used were ryegrass (variety S.24) and cocksfoot (variety S.37). The faeces were collected and the samples rapidly mixed in a bakery mixer at the Grassland Research Institute and were frozen until they were required. The feed and sheep numbers, cutting dates and digestibility of the dry matter are given in Table I.

Table I

Particulars of the feeds and the digestibility of their dry matter

The cutting no. (left-hand column) gives the interval (days) from the first cutting. These figures are used to designate the faeces samples. Further results on these samples are given by Minson *et al.*[3]

Cutting no.	Cutting date	Feed no.	Sheep	Digestibility, %	Cutting no.	Cutting date	Feed no.	Sheep	Digestibility, %
		Ryegrass					Cocksfoot		
0	23·4	240	F11	82·5	0	21·4	238	F8	74·2
15	8·5	250	F25	77·8	13	4·5	245	F16	75·4
26	19·5	261	L7	71·1	17	8·5	249	F24	72·5
36	29·5	275	G27	65·0	38	29·5	274	G22	64·3
43	5·6	282	I9	65·0	56	16·6	291	I21	54·5
60	22·6	293	I22	64·7	80	10·7	305	K25	51·2

The experimental method was identical with that previously described.[1]

Faeces (0·5 g.) were mixed with water (20 ml.) in a beaker and irradiated at a frequency of 20 kc/s for 15 min. The mixture was then treated according to the scheme in Fig. 1. The fibrous part (F) was filtered off and then dried at 105°, as were also the sediments B and C. The liquids Q and U were evaporated on the steambath and also dried at 105°.

Nitrogen was determined by the Kjeldahl process and crude protein calculated as N × 6·25.

Results

The amounts of fractions F, B, C, U and Q as a proportion of faecal dry matter, their contents of crude protein, and the proportion of faecal protein in each fraction, are given in Tables II–IV. The figures given in Table II are the means of two or three determinations, each of which was within ±2% units of the mean value.

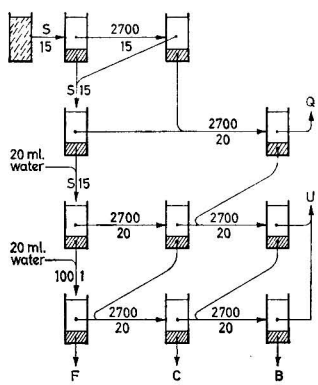


FIG. 1.—*Treatment of faeces after irradiation, by sedimentation (S) and centrifugation at 2700 r.p.m.* (800 g) numerals on arrows are rate of centrifugation and time (1, 15, 20) in min.

Table II

Fractions of faeces in % of faeces dry matter

Cutting* no. / Fraction	0	15	26	36	43	60
Ryegrass						
F	16	33	47	56	60	61
C	7	5	1	4	4	4
B	25	13	17	11	8	9
U	15	11	11	10	12	9
Q	37	39	25	19	16	17
Cocksfoot						
F	31	33	42	51	63	59
C	7	7	4	6	4	10
B	17	11	14	8	9	7
U	15	13	11	12	7	6
Q	30	36	31	24	18	18

* See Table I

Table II shows that, as the grasses matured, there was a pronounced increase in the amount of fraction F, but a decrease in fraction Q; there was also a slight decrease in fraction B. Fraction C was the smallest fraction, and amounted to not more than 10% of faeces, the mean value being about 5%.

Table III

Content of crude protein in faeces fractions as % of dry matter

Cutting* no. / Fraction	0	15	26	36	43	60
Ryegrass						
F	4·0	2·0	2·0	2·5	2·5	1·5
C	10·0	9·5	—	8·5	10·5	9·0
B	23·0	26·5	15·0	23·0	19·5	21·0
U	32·5	41·0	29·5	29·0	28·0	26·6
Q	33·0	33·0	31·0	29·5	28·0	29·5
Cocksfoot						
F	2·0	3·0	—	1·5	1·5	1·0
C	—	9·5	—	7·0	6·0	4·0
B	—	28·0	—	20·5	14·0	14·5
U	—	30·0	—	26·5	23·5	20·5
Q	—	30·5	—	33·5	25·5	22·0

* See Table I

(Results for cocksfoot 17 are missing owing to an accident: those for cocksfoot 0 are not included for 4 fractions as the replicate determinations gave divergent results.)

Table III shows that there was little difference between the protein contents of the U and Q fractions; this is to be expected as they are both supernatant solutions (cf. [1]). The crude protein contents for both were high, over 20%. Fraction F contained only between 1 and 4% of crude protein. Fraction C had a rather low content of crude protein but decidedly more than the F fraction. Fraction B contained more crude protein than fraction C, but decidedly less than the U and Q fractions. Until further chemical examinations are made, it seems preferable to simplify the discussion by combining either fractions F + C and U + Q, or fractions C + B and U + Q. The latter combination gives separation by sedimentation

Table IV

Distribution (%) of total crude protein in faeces for fractions F–Q

Ryegrass

Cutting* no. / Fraction	0	15	26	36	43	60
F	2·5	3·0	5·5	10·5	13·5	9·5
C	3·0	2·5	—	3·0	3·5	3·0
B	24·0	15·5	17·5	19·0	14·0	18·0
U	20·5	20·5	22·5	24·0	28·5	21·5
Q	50·0	59·0	54·0	43·5	40·5	48·0
U + Q	71	80	77	68	69	70

Cocksfoot

Cutting* no. / Fraction	0†	13	17†	38	56	80
F	—	5·0	—	6·5	10·0	9·5
C	—	3·5	—	3·0	2·5	5·5
B	—	15·5	—	12·0	14·5	15·0
U	—	19·5	—	22·0	18·5	17·5
Q	—	56·5	—	57·0	55·0	53·0
U + Q	—	76	—	79	73	70

* See Table I † See footnote to Table III

without centrifugation until the centrifugation stage at 100 r.p.m. to give sediment fraction F ; subsequent separation by centrifugation at 2700 r.p.m. (800 *g*) gives sediment fractions C and B ; and supernatant solution fractions U and Q.

Table IV gives the distribution of crude protein in regard to the fractions F to Q. The fractions U + Q contain the main part (about 70–80%) of the total crude protein. Fraction C contains only 2–6%. Although fraction F contains a fairly constant content of crude protein (Table III), the percentage of the total crude protein present in faeces in this fraction increases with the date of cutting, because the amount of fraction itself increases (Table II).

Some F fractions were examined microscopically by Dr. Hemming Virgin in regard to fragments of tissues and separated cells (Table V).

Table V

Microscopical examination of some F-fractions, giving the relative amounts of tissue fragments and separated cells

(results in parentheses are actually lower than is indicated)

	Ryegrass			Cocksfoot	
	0	26	60	0	80
Fragments of tissues	+	++	++++	+++	++
Epidermis	((+))	+++	++++	++++	(+)
Vascular bundles with fibre sheath	((+))	++	++++	++	(+)
Vascular bundles without fibre sheath	+	++	(+)	+++	+++
Parenchyma	–	((+))	(+)		
Separated cells	++++	+++	+	+++	+++
Epidermis	+++	+	+	(+)	+++
Vessel	++++	+++	(+)	+	++
Parenchyma	(+)	(+)	+	++	++
Fibre	+++	+	(+)	++	+

Discussion

A previous study[1] discussed a method to separate faeces into fractions with decidedly different contents of crude protein. Since then, Trémolières *et al.*[4] have used stepwise centrifugations of mixtures of faeces with various solvents, such as salt solution, and mixtures of organic solvents and water. Paloheimo[5] has investigated this and older methods for separation of a bacteria fraction from faeces and concluded ' that they scarcely can be considered very reliable '. In order to elucidate the nature of this fraction Paloheimo prepared, in a way not specified, a suspension for further separation trials. The separation was performed by filtration

through coarse filter paper and a number of filtrates were ' thrown out ' before the separation was concluded. This and other filtration methods are very arbitrary with regard to preparation of a useful suspension, filtration and collection of fraction samples. Therefore the method used here is preferable as it gives a complete set of fractions, easily prepared for further investigation. Fraction F represents wholly or mostly unaltered structural parts of the feed, while fractions U, Q and B are composed of endogenous material, residues of the fauna and flora of the digestive tract, and undigested parts of the feed such as lignin.

The samples of fraction F had the lowest content of crude protein, similar to that of the product from hay extracted under mild conditions and digested with pepsin.[6] These findings indicate that further investigations should be made to determine whether the crude protein in fraction F is a residue of the crude protein present in the feed.

Tables II–IV indicate some correlation between the digestibility of herbages and the composition of faeces as regards the amounts of fractions F–Q and also their contents of crude protein. The changes in composition in regard to the time of harvest is demonstrated in Fig. 2. Evidently the experimental error with young plants is great, but nevertheless the correlation is obvious. Further, Fig. 3 demonstrates the correlation between composition and digestibility.

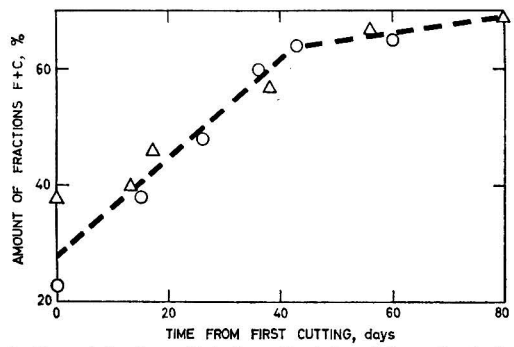


FIG. 2.—*Proportion of fractions F + C vs the interval from the first cutting (days)*

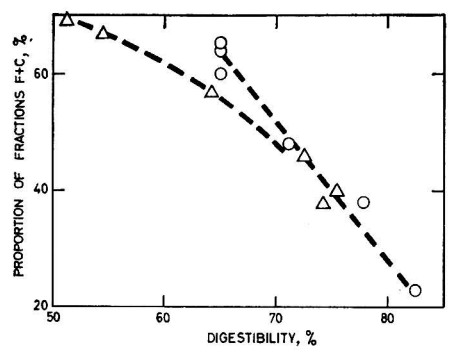
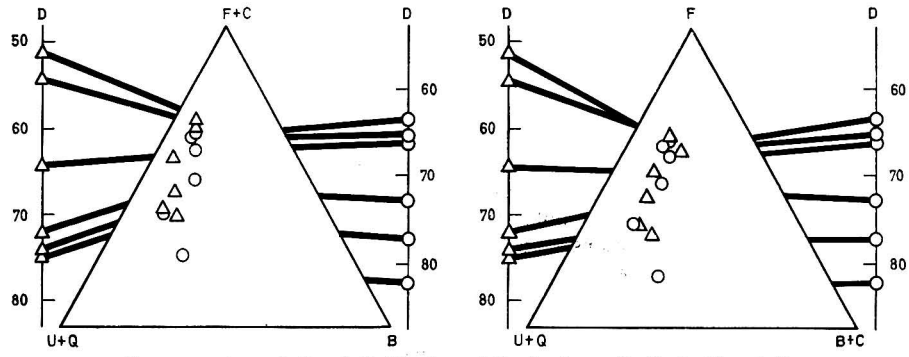


FIG. 3.—*Proportions of fractions F + Q vs digestibility (%) of dry matter*
○ ryegrass △ cocksfoot

It was stated above that the five fractions can be combined to give three main components. Fig. 4 gives a survey of the correlations discussed, fractions F and C being taken together. The other possibility, the combination of B and C, gives the pattern shown in Fig. 5. In both cases, the pattern is similar, because the amount of fraction C is small. The strong correlation between the position of the values for the samples in the diagrams and the digestibility is evident. In this and subsequent investigations into this new approach, this more detailed representation of the values in this manner is more useful than diagrams such as those in Figs. 2 and 3.

In view of recent papers[7, 8] on the possibility of calculating the intake of herbage from the



FIGS. 4 and 5.—*Internal distribution of the fractions, F, C, B, U and Q*
The lines from the axes D (the experimentally determined digestibility) point towards the actual point in the triangle
○ ryegrass △ cocksfoot

amount and the crude protein content of faeces it is worth while to draw attention also to Fig. 6, which gives values for digestibility (D) plotted against the amount of fraction F (second quadrant) and the percentage of faecal crude protein in fractions U and Q (uq) (in the first quadrant) and amount of fractions F plotted against uq (fourth quadrant).

The results in Figs. 4–6 indicate that it may be possible to investigate in this way which parts of a grass feed are digested. At present a representation in a triangular diagram gives the best correlation with digestibility (Figs. 4 and 5). Further, which parts of the grass are digested can be further studied in this way by a chemical investigation and by microscopical examination, as indicated in Table V.

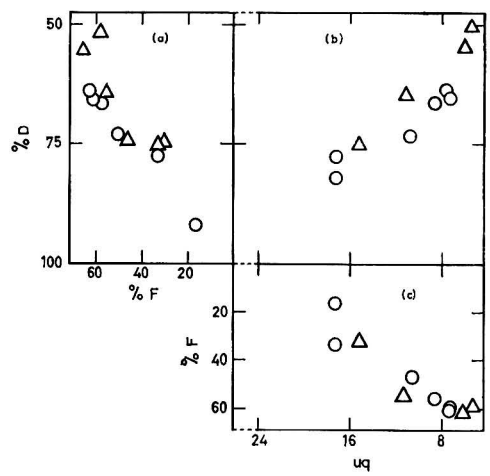


FIG. 6.—*Diagrams giving the figures for digestibility (D) versus those for the percentage of fraction F* (quadrant a); *digestibility (D) versus the percentage of total faecal crude protein appearing in the fractions U + Q (uq)* (quadrant b) and *F versus uq* (quadrant c)
○ ryegrass △ cocksfoot

The microscopical investigation of fraction F shows, according to Table VI, that in the case of ryegrass the relative amount of separated cells in the faeces is greater when the plants are younger and the reverse is true for fragments of tissues. For ryegrass 60 it was noted that the tissues were squeezed, but the middle lamella was mostly unaltered. The cocksfoot sample gave a different picture; the relative amount of epidermis as fragments is greater than the

separated cells in younger plants, but the reverse in older ones. Otherwise there was no pronounced difference in respect to age of the plant in this case. It should be possible to disintegrate the feed and the faeces fraction F into free cells and, by counting the different types of cell, their digestibility could be determined. This, in combination with chemical investigations, may open a new way in digestibility studies.

Acknowledgments

The authors express their thanks to the Grassland Research Institute for the samples of faeces and particulars of the feeds used ; to Prof. H. Virgin for the microscopical examination of some F fractions from the faeces, and to Dr. J. M. A. Tilley for help in the compilation of the manuscript.

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Received 14 February, 1964 ; amended manuscript 28 May, 1964

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LEAF ANALYSIS AS A GUIDE TO THE NUTRITION OF FRUIT CROPS. VI.*—Determination of Magnesium, Zinc and Copper by Atomic Absorption Spectroscopy

By E. G. BRADFIELD and D. SPINCER†

With the appropriate instrumental settings magnesium may be determined in solutions containing 0·01–2·0 μg. of Mg/ml., zinc in solutions containing 0·02–4·0 μg. of Zn/ml. and copper in solutions containing 0·1–15·0 μg. of Cu/ml. The effect of phosphate and calcium on the absorption of magnesium in the coal gas/air flame has been investigated in relation to the position in the flame at which measurements are made ; the effect of combinations of magnesium and sulphate on the absorption of zinc has also been studied.

Introduction

Since the original paper by Walsh[1] in 1955, much interest has been shown in atomic absorption spectroscopy as a simple analytical technique, and its application to agricultural analysis has been the subject of several papers, principally by Allan[2–4] and David.[5, 6] The technique has been employed at Long Ashton over the last 3 years and this paper describes some findings on its use for the determination of magnesium, zinc and copper in plant materials and soil extracts.

Apparatus

This was similar to that previously described[7] except that a scale expansion device (David[8]) was constructed and used in conjunction with the output measuring unit.

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Factors which affect the sensitivity of determination

(i) *Lamp current.*—As reported by previous workers, maximum sensitivity was achieved by using the lowest possible lamp current compatible with adequate stability.

(ii) *Slit width.*—This was not found to be critical for magnesium and zinc and widths of 0·3 and 1·0 mm. respectively were used for these elements. For copper, a width of 0·3 mm. should not be exceeded since the 3247 Å and 3274 Å lines are then not completely resolved and some loss in sensitivity results.

(iii) *Fuel gases and burner height.*—The slit burner could be used with either coal gas or acetylene as fuel. The sensitivity attainable with either gas was dependent on gas flow rate, air flow rate and the distance of the burner top below the centre of the hollow cathode lamp. In practice, the air and gas flow rates were fixed to give a slightly lean flame and the burner height was adjusted to give maximum sensitivity. Using a coal gas flame, absorption by magnesium atoms was at a maximum when the burner top was 3·5 mm. below the centre of the hollow cathode. Deviations, of 1 mm. in either direction, from this position resulted in a pronounced decrease (approx. 20%) in absorption. Absorption by zinc atoms was little affected by the position of the burner top, whether 5 or 15 mm. below the centre of the hollow cathode lamp. However, the stability of the response decreased markedly as the burner was lowered; thus the optimum position for maximum absorption was 5 mm. below the lamp. In the acetylene/air flame, absorption by magnesium atoms was less dependent on burner height and was constant over the range 4–7 mm.; the stability of response was greatest when the burner top was 5·5 mm. below the centre of the hollow cathode lamp. Variation of zinc absorption with height in the acetylene/air flame was similar to that in the coal gas flame. Copper gave similar results to zinc with both types of flame. With the above burner heights, the instrumental settings shown in Table I were used.

Table I

Instrumental settings

	Magnesium	Zinc	Copper
Wavelength (Å)	2852	2138	3247
Hollow cathode current (mA)	4	25	20
Air flow (l./min.)	3	3	3
Coal gas flow (l./min.)	2·2	2·2	2·2
Acetylene flow (ml./min.)	670	670	670
Photomultiplier H.T. (V)	350	500	350
Sensitivity (optical density of 2 p.p.m. solution)			
Acetylene/air flame	0·750	0·318	0·076
Coal gas flame	0·120	0·300	0·070

(iv) *Partially organic solution.*—Useful increases in sensitivity can be obtained by spraying from an aqueous/organic solution.[9, 10] For isopropanol/water solution, a 20% isopropanol content was optimum for maximum increases in the absorption of magnesium, zinc and copper. Since introduction of an organic solvent increases the size of the flame, the burner height for optimum response must be redetermined. For the coal gas flame the top of the burner should be 6 mm. below the centre of the hollow cathode for magnesium, and 5 mm. for zinc and copper, while the values for the acetylene/air flame are 7·5 mm. and 5 mm. respectively. Amounts of magnesium, zinc and copper (p.p.m.) in 20% isopropanol solution which give the same optical density as 2 μg./ml. in aqueous solution for the acetylene/air and coal gas flames, respectively, are: magnesium 1·1, 1·0; zinc 1·2, 1·05; copper 1·2, 1·0.

An increase in sensitivity of approximately 100% is therefore obtained by using a 20% isopropanol solution, although in practice this is partially offset by the dilution of the sample involved in the addition of the organic solvent.

(v) *Effect of other elements added as interference suppressors.*—When magnesium solutions containing strontium are aspirated into a coal gas flame a marked increase in sensitivity results due to a change in the $Mg \rightleftharpoons MgO$ equilibrium, as suggested by Willis.[11] This equilibrium

may be so far displaced that the sensitivity approaches that attainable in the acetylene flame ; with the latter flame no significant increase in sensitivity is observed in the presence of strontium.

(vi) *Scale expansion.*—The scale expansion unit described by David[8] gave the increases in sensitivity shown in Table II ; the method of assessment of the limit of determination was that described by Wilson.[12] All values are expressed as μg./ml. : the acetylene/air flame was used throughout.

Table II

Increase in sensitivity by use of scale expansion unit[8]

Element	Aqueous solution				20% isopropanol			
	Normal use		Scale expansion		Normal use		Scale expansion	
	50% absorption	Limit of determination	50% absorption	Limit of determination	50% absorption	Limit of determination	50% absorption	Limit of determination
Mg	0·75	0·025	0·22	0·01	0·50	0·02	0·15	0·01
Zn	1·90	0·08	0·56	0·03	1·10	0·05	0·36	0·02
Cu	7·80	0·30	2·50	0·15	4·40	0·24	1·80	0·10

Interferences

(*a*) *Magnesium.*—The results obtained were similar to those reported by previous workers. When acetylene was used as fuel gas, and measurements were carried out at the optimum height in the flame, interference was observed only with aluminium and silicate ; these decreased the measured absorption. In the cooler coal gas flame, interference was observed with phosphate, calcium, aluminium, sulphate and silicate. These interferences were completely eliminated by the addition of 1000 μg./ml. of strontium.

A study of the effect of phosphate and calcium on the absorption of magnesium was carried out in some detail with acetylene and coal gas flames, in the presence and absence of isopropanol. The results obtained in the presence of phosphate are shown in Fig. 1 where % Mg recovered in the presence of the interfering element is plotted against the height in the flame at which the absorption was measured.

In the coal gas flame, the effect of phosphate is more pronounced in the lower portions of the flame and is less in the presence of isopropanol. Since the effect of phosphate on magnesium absorption is generally ascribed to the stability of magnesium phosphate in the flame, it would be expected that the effect would be less the longer the particles were in the flame before the absorption was measured. In the presence of an organic solvent, the droplet size entering the flame is reduced (Dean & Carnes[13]), the surface area is increased and dissociation of magnesium phosphate can occur more easily. Although the influence of phosphate is less in the higher portions of the flame, the overall concentration of magnesium atoms is very much reduced due to a combination of dilution (due to expansion of flame gases) and of magnesium

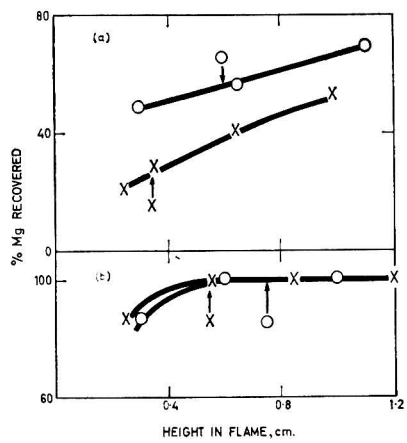

FIG. 1.—*Effect of phosphate on magnesium measured at different heights in the flame*

(*a*) Coal gas/air flame ; 4 p.p.m. Mg ; 10 p.p.m. P
(*b*) Acetylene/air flame ; 1·5 p.p.m. Mg, 2·5 p.p.m. P
× aqueous solution ○ 20% isopropanol

Arrows indicate position in the flame at which the absorption by magnesium is a maximum in the absence of phosphate

oxide formation. The height in the flame where the maximum concentration of magnesium atoms occurs is indicated in Fig. 1.

Phosphate has no influence on magnesium absorption in an acetylene flame provided measurements are carried out above the fuel gas reaction zone. In the area of unburnt gases just above the burner top, depression of magnesium absorption by phosphate can again be demonstrated.

In the cooler coal gas flame, calcium considerably enhances the absorption of magnesium. According to Willis[11] this effect is perhaps due to competition between calcium and magnesium for oxygen in the flame and displacement of the $Mg \rightleftharpoons MgO$ equilibrium towards increased formation of magnesium atoms; the effect is slightly more pronounced in the presence of isopropanol. The magnitude of the enhancement decreases in the higher regions of the flame, presumably due to partial re-establishment of the above equilibrium. In the hotter acetylene flame, the influence of calcium is much reduced although a measureable effect is still noted in the region immediately above the burner top.

In both coal gas and acetylene flames, the effects of phosphate and calcium were negligible in the presence of 1000 μg./ml. of strontium.

(*b*) *Zinc.*—Previous workers have reported that elements present in plant material do not interfere with the determination of zinc by atomic absorption spectroscopy. This was confirmed when the interfering elements were tested singly but when magnesium and sulphate were present together it was noticed that absorption of zinc was reduced at certain Mg/S ratios. With a coal gas flame, in both aqueous and 20% isopropanol solutions, the reduction in zinc absorption is most marked (Table III). In the acetylene flame, it is negligible in aqueous solution but becomes more pronounced in 20% isopropanol. The maximum reduction in absorption always occurs at a molar ratio of Mg to S of 2 : 3, indicating formation in the flame of some compound, containing magnesium and sulphur in these ratios, which incorporates zinc and prevents its complete dissociation into atoms. The effect may be overcome by addition of 1000 μg./ml. of magnesium to both samples and standards.

Applications in plant and soil analysis

Magnesium

The application of atomic absorption spectroscopy to the determination of magnesium in plant material after dry ashing or nitric/perchloric acid digestion is well known[2] as also is its use in the determination of magnesium in ammonium acetate and ammonium chloride extracts of soils.[6]

In addition, investigational work at Long Ashton has shown the usefulness of the technique for the determination of magnesium in the sodium sulphate/selenium/sulphuric acid digest which Bould *et al.*[14] used in their method for the determination of nitrogen, phosphorus and potassium in plant material. In this procedure, strontium can no longer be used as interference suppressor because of precipitation of strontium sulphate, but it may satisfactorily be replaced by 2000 μg./ml. of lanthanum. Table IV shows some results for magnesium obtained by this

Table III

Apparent recovery of zinc when solutions containing 2 μg. of Zn/ml. and combinations of magnesium and sulphate are atomised from aqueous solution into a coal gas flame

Mg, μg./ml.	Sulphur, μg./ml.				
	0	100	200	400	800
0	2·00	2·00	2·00	2·00	2·00
25	2·00	1·55	1·50	1·60	1·75
50	2·00	1·35	1·20	1·30	1·45
100	2·00	1·65	1·00	0·95	1·05
200	2·00	2·00	2·00	0·80	0·85
300	2·00	2·00	2·00	2·00	0·85
400	2·00	2·00	2·00	2·00	0·85
600				2·00	2·00
800					2·00
1000					2·00

Table IV

Comparison of magnesium concentration in plant materials determined by different methods

Method / Material	A		B		C		D	
	Mean % Mg	σ	Mean % Mg	σ	Mean % Mg	σ	Mean % Mg	σ
Strawberry leaf	0·261	0·005	0·260	0·005	0·279	0·016	0·282	0·016
Apple leaf	0·201	0·003	0·204	0·003	0·209	0·003	0·223	0·013
Black currant leaf	0·433	0·010	0·422	0·006	0·423	0·009	0·441	0·019
Raspberry leaf	0·352	0·006	0·355	0·004	0·362	0·007	0·368	0·019
Plum leaf	0·478	0·005	0·483	0·007	0·458	0·008	0·486	0·017

A Dry ashing of plant material, followed by atomic absorption spectroscopy using an acetylene/air flame (1000 μg./ml. of strontium added)
B As A but using a coal gas/air flame
C Sodium sulphate/selenium/sulphuric acid wet digestion of plant material, followed by atomic absorption spectroscopy using an acetylene/air flame
D Titan Yellow colorimetric method[17]

method. Each result quoted is the mean of ten determinations. Agreement is good between the two methods (A and C) although the standard deviation of results obtained from the sodium sulphate/selenium/sulphuric acid digest are somewhat greater.

The use of the scale expansion unit increases still further the sensitivity of the technique and appears to provide a method for the determination of magnesium in very small amounts of plant material (in the region of 200 μg. of sample). This could provide a suitable analytical method for detailed investigations of the distribution of magnesium throughout a plant.

A further application of atomic absorption spectroscopy to soil investigation has been found in the determination of the small amounts of magnesium present in equilibrium soil solutions prepared as described by Arnold[15] and Matthews & Beckett.[16]

In the presence of 1000 μg./ml. of strontium, results for magnesium determinations in the coal gas flame are in good agreement with those obtained in the acetylene flame (Table I) although the sensitivity of the determination is not quite so high. Results obtained by a Titan Yellow colorimetric procedure[17] are also included. Values obtained by this method are significantly higher ($P = 0{\cdot}001$) for samples of strawberry and apple leaves and the standard deviation of the method is approximately twice that of the atomic absorption procedure.

Zinc and copper

The application of the technique to the determination of zinc in plant material has already been described.[4, 5, 7] With scale expansion, zinc can probably be satisfactorily determined in plant samples weighing in the region of 40 mg. Copper is not usually determined directly in plant material by atomic absorption spectroscopy because of lack of sensitivity; a preliminary concentration stage is usually employed.[8] However, by use of the scale expansion unit it seemed probable that copper could be determined directly in a plant digest. In Table V are shown some results for the direct determination of copper and zinc in plant material. Digest solutions of 1 g. in 20 ml. were prepared as previously described[7] and, with no further treatment, aspirated into the flame. Zinc was determined using standards to cover the range 0–4 μg. of Zn/ml. and copper (using the scale expansion unit) with standards to cover the range 0–1 μg. Cu/ml. The coefficient of variation for the determination of copper is approximately twice

Table V

Values for copper and zinc concentrations in plant material, determined by two methods

Material	Mean p.p.m. Cu	σ	Coefficient of variance, %	Mean p.p.m. Zn	σ	Coefficient of variance, %
Strawberry leaf	12·3	1·0	8·1	22·7	0·88	3·9
Black currant leaf	15·9	1·3	8·3	112	2·4	2·1
By previous method[7]						
Strawberry leaf	12·5	0·60	4·8	23·6	1·25	5·3
Black currant leaf	14·9	0·36	2·4	113	1·7	1·5

that of the method previously described[7] but the precision is still sufficient for the rapid determination of the small amounts of copper present in plant material. By reduction of the final volume of digest solution to 10 ml., results of similar precision could be obtained for samples containing 5 p.p.m. of copper.

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Received 26 May, 1964

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THE NITROGEN METABOLISM OF THE YOUNG PIG. II.*—Effect of Heat Treatment on the 'Available' Lysine Content of Fish Meal and the Performance of Pigs

By A. S. JONES and A. CADENHEAD

Young male pigs were given diets containing barley and fish meal with added minerals and vitamins. The fish meal was Peruvian or white fish meal of similar 'available' lysine content but different total lysine content, heated white fish meal with the same total but reduced 'available' lysine content, or heated white fish meal with supplementary lysine. There was a reduction in the nitrogen retention, growth rate and feed conversion of the pigs when the heated meal was given, but there were no significant differences in these criteria for pigs given the Peruvian or the white fish meal. Some of the reduced performance on the heated meal was due to a lowering of the digestibility of dry matter and the addition of synthetic lysine to the diet containing heated fish meal only partly restored nitrogen retention, growth rate and feed conversion.

Introduction

Improvements in rates of liveweight gain by pigs have been reported by many workers when supplements of L-lysine, alone or with methionine, were added to pig diets. For diets based on barley containing white fish meal, soya-bean meal or groundnut meal, lysine is generally the first limiting amino-acid. Estimates of the lysine requirement for optimum growth of the young pig[1–8] have varied between 0·5% and 1% of diet. Jones *et al.*[9] found increases in nitrogen retention by pigs as lysine in the diet was increased to 1·01%.

* Part I: *J. Sci. Fd Agric.*, 1961, **12**, 353

At least part of the variability in the estimates of lysine requirement of the pig may be due to differences in the protein quality of the protein concentrates used. This variation in the protein quality is due in part to variation of heat treatment of the concentrate which has the effect of reducing the availability of the amino-acids, in particular lysine. A ' Maillard reaction '[10] takes place between pentose sugars and free ε-amino-groups[11] and the change is not apparent from an amino-acid assay of the acid hydrolysate.[12] The availability of lysine (ALV) in animal by-products may be estimated by the method of Carpenter.[13]

The ALV is the number of ε-amino-groups of lysine that will combine with 2,4-dinitrofluorobenzene to give stable dinitrophenyl-lysine.[14] A highly significant relationship has been found between the ALV and the gross protein value determined using chicks for animal by-products by Carpenter & Ellinger.[15]

The present study was undertaken to determine the effect of heat treatment of white fish meal on the ALV and the effect of any change in the fish meal on performance of young pigs.

Determination of the ' available lysine ' content of a large number of fish meals in the laboratory has shown the greatest variation to be in products from Peru. In the current study a comparison is made between white fish meal and Peruvian fish meal, both of similar ' available lysine ' content.

Experimental

Animals and design

Six litters of five Large White male pigs which had been castrated at 3 weeks of age were weaned at 8 weeks of age and gradually transferred from a creep feed to the appropriate test diet. When each pig weighed approximately 21 kg. they were randomly allotted to the experimental diets and individual test feeding commenced. The test period was 33 days and over the last 5 days measurements were made of nitrogen retention.

Preparation and selection of the fish meals

Partial analyses of the three fish meals used are given in Table I. The ' available ' lysine was determined by the method of Carpenter,[13] and total lysine by Moore & Stein ion-exchange chromatography.[16] The white fish meal was purchased locally and was carefully mixed to give a homogeneous product.

Half of the mixed white fish meal was moistened with water and heated in an air oven at 150° for 36 h., which reduced the ' available ' lysine by approximately 50%. Previously, in pilot trials, attempts were made to reduce the ' available ' lysine content of the meal by varying the drying temperature and the degree of moistening. Dry heat led to charring and considerable loss of nitrogen.

The Peruvian fish meal was selected on the basis of the ' available ' lysine content so that it contained the same concentration of ' available ' lysine as the unheated white fish meal.

Diets

The composition of the experimental diets are shown in Table II. They were based on a mixture of barley meal and fish meal and provided approximately 14·5% crude protein and 67% total digestible nutrients. The white fish meal diet (FM) contained 10% of white fish meal, the amounts of fish meal added to the other diets being adjusted so that each diet contained the same amount of fish meal protein. Tricalcium phosphate was added to each diet

Table I

Composition of fish meals used

	Crude protein, %	Total lysine, g./16 g. N	Available lysine, g./16 g. N	Ca, %	P, %
White fish meal	62·6	7·5	6·40	6·22	1·78
Damaged white fish meal	63·7	7·6	3·40	6·31	1·75
Peruvian fish meal	66·7	8·2	6·32	3·59	1·03

so that all diets contained allowances for calcium and phosphorus as recommended by the National Research Council:[17] because of the lower mineral content of the Peruvian fish meal, considerably more tricalcium phosphate was needed in the diet containing Peruvian fish meal (PFM). Supplements of vitamins and trace elements were added as indicated in Table II so that all diets provided the mineral requirements and all known vitamins in adequate amounts.

The L-lysine monohydrochloride used contained 5% of the D-isomer (which is not available) so that the availability of the material used was deemed to be 95%. A supplement of L-lysine monohydrochloride (0·26%) was added to the diet containing damaged white fish meal (DFM) to give a diet, DFML, in which the concentration of 'available' lysine was equal to that calculated for the diet FM. A similar amount of L-lysine monohydrochloride was used in the FML to provide a positive control.

Each diet contained the same amount of barley so that the contribution of the cereal portion of the diet towards the total 'available' dietary lysine was the same in each case. Maize starch was used as necessary to make the diets of equal proportions.

Method

Except during the determination of nitrogen balance when metabolism cages were used, all pigs were housed together in wooden huts, but individually fed. The daily feed allowance was an amount equal to 5% of liveweight and this was adjusted weekly except that no increase was allowed for the period prior to the pigs being placed in metabolism cages. The method used for the collection of urine and faeces was identical with that described in an earlier publication.[9]

Table II

Composition of diets

	1 FM	2 FML	3 PFM	4 DFM	5 DFML
Barley meal	87·7	87·7	87·7	87·7	87·7
White fish meal	10·0	10·0	—	9·83	9·83
Peruvian fish meal	—	—	9·38	—	—
Starch (maize)	0·5	0·24	0·62	0·67	0·43
Tricalcium phosphate	0·5	0·5	1·00	0·5	0·5
L-Lysine monohydrochloride	—	0·26	—	—	0·24
Analysis					
Total crude protein[a]	14·5	14·6	14·7	14·4	14·5
Total lysine[a]	0·70	0·89	0·74	0·71	0·91
Available lysine[b] from protein concentrate and synthetic lysine	0·40	0·50	0·40	0·21	0·40
T.D.N.[c]	69·0	68·9	69·2	69·1	68·9

In addition all diets contained Adisco[d] + vitamin B_2 0·5% (providing, per g., vitamin A 1000 i.u., vitamin D 200 i.u., vitamin B_2 500 μg.), Aurofac 2A 0·3% (providing 7·9 g. of Aureomycin hydrochloride per kg.), salt (NaCl) 0·5%, zinc carbonate 66 g./100 kg. of diet.

[a] By analysis

[b] By calculation from the actual analytical value for the 'available' lysine of the protein concentrate and the % of concentrate in the diet, but the availability of the L-isomer was assumed to be 100%.

[c] By calculation

[d] Isaac Spenser & Co., Aberdeen

Results

Table III shows the nitrogen metabolism data, growth rates and feed conversion ratios. The reduction of the 'available' lysine content of the protein concentrate significantly reduced the rate of gain and feed utilisation, and reduced both apparent nitrogen digestibility and nitrogen retention (cf. FM and DFM). The addition of synthetic lysine to DFM significantly improved daily gain, feed conversion and nitrogen retention (cf. DFM and DFML). However, while nitrogen retention on DFML and FM was not significantly different, growth rate and feed conversion were significantly poorer on DFML than on FM. Lysine supplementation did not affect the apparent nitrogen digestibility.

Addition of synthetic lysine to the FM diet had no significant effect on performance although there were slight improvements (cf. FM and FML). Feeding two different types of fish meal

Table III

Nitrogen metabolism data, feed conversion ratios and daily liveweight increase

	Treatment					Overall significance of differences between treatments	S.E. of difference between means
	1 FM (control)	2 FML	3 PFM	4 DFM	5 DFML		
Nitrogen metabolism data							
Dietary N retained, %	41·1	44·9	46·0	27·2	37·2	***	±1·30
N-retention, g./5 days	63·5	72·4	70·8	39·9	59·3	***	±4·30
N-retention, g./kg. live-weight/5 days	2·10	2·19	2·24	1·40	1·90	***	±0·22
Apparent N digestibility, %	79·2	80·6	79·8	67·2	68·5	***	±0·91
Feed conversion (kg. feed/kg. liveweight gain)	2·61	2·44	2·42	3·63	3·02	***	±0·089
Daily liveweight increase, g./day	494	528	511	336	416	**	±38
		*** P < 0·001		** P < 0·01			

(Peruvian and white fish meal) of similar ' available ' lysine content gave performances by pigs which were not significantly different (cf. FM and PFM).

Heating the white fish meal reduced the digestibility of dry matter, as shown in Table IV. The difference in conversion of digestible dry matter on diets FM and DFM was significant and differences in the conversion of digestible dry matter were of the same order as for feed conversion kg. feed/kg. liveweight gain.

Table IV

Digestibility of dry matter (DM) and conversion of digested dry matter

	Treatment					S.E. of mean
	1 FM	2 FML	3 PFM	4 DFM	5 DFML	
DM digestibility, %	86·7	87·3	85·7	81·7	81·3	±2·2*
DM intake, kg./5 days	6·44	6·44	6·19	6·28	6·10	
Digested DM, kg./5 days	5·59	5·62	5·30	5·13	4·96	
Conversion of digested DM (kg. digested DM per kg. liveweight gain)	2·26	2·13	2·08	2·96	2·46	

* Difference between damaged and undamaged meals significant at 5% level

Discussion

The results of this experiment show that heat treatment of white fish meal reduced the ' available ' lysine content, and significantly reduced liveweight gain, feed conversion and nitrogen digestibility and retention in pigs when the heated meal was included in the diet at a rate of 10%.

The depression in liveweight gain and feed conversion was not due entirely to the lowering of the ' available ' lysine content of the fish meal. There were, as shown in Table IV, changes in the digestibility of dry matter, but when allowances were made for these changes, there were still significant differences in conversion of digested dry matter. Thus the lower performance of the pigs given the damaged fish meal was due to a lowering of the ' available ' lysine, and possibly other amino-acids, and also to a lowering of dry-matter digestibility.

The significant decreases in nitrogen digestibility cannot be accounted for by changes in the lysine alone, since the nitrogen content of lysine is only about 19%, and it must be assumed that there were significant changes in the digestibility of the other amino-acids or protein fractions. It is known that other amino-acids, for example, methionine, can also undergo a Maillard-type reaction.

The reduction in digestibility of the nitrogen caused by heat treatment of the fish meal was approximately 15%. The protein content of the diet was about 17% crude protein on a dry-matter basis so that a 15% change in digestibility would cause a change in dry-matter

digestibility of about 2·6% ; thus the change in digestibility of the protein accounted for only half of the change in digestibility of the dry matter.

The addition of synthetic lysine to the fish meal diet (FM) did not significantly improve the nitrogen retention, growth rate or feed conversion, although these were slightly improved ; thus lysine concentration was not seriously limiting growth rate or N-retention in that diet. These results are at variance with the earlier observation[9] where improvements in performance were noted up to 1·01% lysine in diet. However, the pigs used in the earlier study were younger (11–22 kg.) and they were given diets of higher energy content, and both these factors would tend to increase the optimum dietary lysine level. Likewise the performances of pigs on diets PFM and FM were not significantly different ; these diets had similar contents of ' available ' lysine, but differed in their total lysine content.

The addition of synthetic lysine to the damaged fish meal significantly improved retention, so that this diet was then not significantly different from the diet containing undamaged fish meal (DFML and FM), while growth rate and feed conversion, although significantly improved, were still significantly poorer than on the FM diet. Diets FM and DFML provided the same amounts of ' available ' lysine if it is assumed that L-lysine monohydrochloride is 100% available. The fact that nitrogen retention was almost the same on DFML and FM would indicate that, although the lowered growth rate and feed conversion could not be accounted for by damage to lysine alone, lysine must have been by far the most limiting amino-acid and was probably the amino-acid which underwent the greatest change during heat treatment. Since the retention of nitrogen on DFML and FM were similar, the difference in growth rate which was recorded might well have been due primarily to the lowering of the digestibility of dry matter. If an allowance is made for the lower digestibility of dry matter, then the growth on DFML would be expected to be halfway between that on FM and DFM diets which was actually the case. Difference of conversion of digested dry matter were also similar on DFML and FM although poorer on the DFM diet. These results suggest that the factor limiting growth rate on DFML was primarily digestibility of dry matter.

Conclusion

The results of this experiment indicate that, while performance and nitrogen retention in young pigs is related to differences in the ' available' lysine content of the diet rather than to total lysine, part of the lowering in performance of pigs given heated fish meal as compared with unheated fish meal is due to change in the digestibility of dry matter other than that accounted for by differences in the digestibility of protein.

Acknowledgments

The authors are indebted to Mr. R. Smart for assistance with chemical analyses and Mr. I. Philip for management of the pigs.

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Received 4 June, 1964; amended manuscript 17 August, 1964

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ACTION OF HEAT ON PYRETHRUM EXTRACT: THE ISOMERISATION OF PYRETHRINS TO ISOPYRETHRINS

By A. A. GOLDBERG, S. HEAD and (Mrs.) P. JOHNSTON

The thermal isomerisation of the pyrethrins to isopyrethrins has been studied, the progress of the reaction being followed by the rise in optical density at 2700 Å and the fall at 2300 Å. The isomerisation is a first-order reaction with 10^6k equal to 4·58 and 481 at 125° and 195° respectively; these values give $E^* = 24{,}610$ cal. mol.$^{-1}$; log $A = 8{\cdot}17$; $\Delta S^* = -21{\cdot}8$ cal. deg.$^{-1}$.

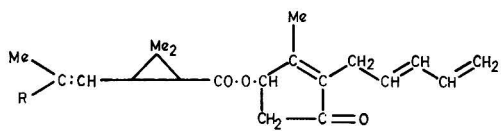
In normal (unheated) pyrethrum extract the ratio of the optical densities at 2700 Å and 2300 Å is 0·08; in pyrethrum extract which has been fully isomerised by heat this ratio is 0·62.

The biological activities of normal and of partially and completely isomerised pyrethrum extract have been examined by three methods (two on flies and one on grain weevils). Extract in which the pyrethrins have been completely isomerised to isopyrethrins has approximately one-half of the lethal, and one-quarter of the knockdown, activity of normal extract on houseflies.

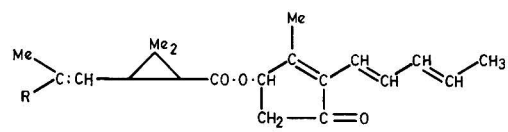
Pyrethrum extract which has been submitted to heat treatment shows no loss in biological activity if the ratio of the optical density at 2700 Å to that at 2300 Å has not risen above 0·095.

Introduction

The structural alteration of the pyrethrins effected by heat was first observed by Staudinger & Ruczicka,[1] and confirmed by Haller & LaForge[2] who reported that the biological activity of pyrethrum extract decreased when the material was subjected to prolonged heating. Brown *et al.*[3] submitted pyrethrum extract which had been heated to 200° for ½ h. to displacement chromatography and separated, in addition to the four known original constituents, two new compounds which they termed isopyrethrins I and II for which structures c and d were advanced from spectrophotometric evidence.



(*a*, Pyrethrin I, R=Me)
(*b*, Pyrethrin II, R=CO_2Me)



(*c*, Isopyrethrin I, R=Me)
(*d*, Isopyrethrin II, R=CO_2Me)

The new isomers are formed by a shift of the double bonds of the pentadienyl side-chain into conjugation with the double bond of the cyclopentenolone ring. It was assumed that cinerin I and cinerin II are unchanged by the heat treatment. Elliott *et al.*[4] confirmed this structure and reported that isopyrethrin I was only one-sixteenth as toxic to mustard beetles (*Phoedon cochleariae*) as pyrethrin I.

The thermal isomerisation of the pyrethrins to the isopyrethrins is considered to be a free-radical reaction in which the rate-determining process is the removal of a hydrogen atom from the linolenic methylene group. In the free radicals the π-electrons in the side-chain become mobile and will find the lowest energy level; this will be the assembly of maximum conjugation, viz., the isopyrethrin structure.

The present work was carried out in order to obtain information on the rate of transformation of the pyrethrins to isopyrethrins at different temperatures. Such information is

of value in the problems encountered in distilling pyrethrum oleoresin in order to produce a virtually colourless extract without loss of biological activity.

Experimental and results

A solution of pyrethrum extract in kerosene, sealed in ampoules, was heated in a bath of refluxing amyl alcohol or kerosene at 125° and 195° respectively (atmospheric pressure at Nakuru 620 mm. Hg). After a given time an ampoule was removed, opened, the contents analysed and the ultra-violet absorption spectrum in ethanol solution plotted from 2100 to 2900 Å.

Table I (Fig. 1) and Table IA (Fig. 2) show the effect of heating pyrethrum extract at 125° and 195° respectively; Tables II and IIA and Tables III and IIIA are the results obtained with substantially pure pyrethrin I/cinerin I and pyrethrin II/cinerin II prepared[5] by fractional distillation of pyrethrum oleoresin in a 2-in. wiped-wall molecular still. The Tables show that as the heating continues there is a progressive fall in optical density at 2300 Å and rise at 2700 Å.

Table I

Analyses of pyrethrum extract heated at 125°

Heating time, h.	DNP analysis* Py I, %	Py II, %	Total Py, %	PBK analysis Py I, %	Py II, %	Total Py, %	Spectroscopic analysis (total Py)	Ratio d_{2700}/d_{2300}	% Isomerised
0	4·67	2·91	7·58	5·74	3·58	9·32	9·78	0·081	0
8	4·56	2·84	7·40	5·68	3·16	8·84	9·52	0·125	14
21	4·49	2·80	7·29	5·44	3·22	8·66	9·22	0·188	31
48	4·44	2·47	6·91	5·01	2·02	7·03	8·34	0·294	54
74	4·40	2·31	6·71	4·55	2·06	6·61	7·60	0·348	64
96	4·08	2·25	6·33	4·21	1·92	6·13	7·20	0·424	76

Table IA

Analyses of pyrethrum extract heated at 195°

0	5·37	3·39	8·76	7·08	3·90	10·98	11·0	0·081	0
¼	5·03	3·02	8·05	7·01	3·81	10·82	9·5	0·196	33
½	4·44	2·51	6·95	6·60	3·87	10·47	7·9	0·326	60
1	3·82	2·01	5·83	6·41	3·74	10·15	6·6	0·489	85
2	3·74	1·85	5·59	6·00	3·58	9·58	6·5	0·579	96
2½	3·97	1·91	5·88	5·81	3·55	9·36	6·1	0·620	100
3	3·82	1·79	5·61	5·24	2·96	8·20	6·0	0·630	100

d_{2700}/d_{2300} is ratio of optical densities at 2700 and 2300 Å Py = Pyrethrin

* A pyrethrum extract analysing at 25·0% pyrethrins by the PBK method (Official Method of the Pyrethrum Board of Kenya, September, 1954) analyses at 22·3% pyrethrins by the A.O.A.C. method ('Official Methods of Analysis of the Association of Official Agricultural Chemists', 1950, 7th Edn) and 20·0% pyrethrins by the DNP method (Smith[6a]). For this reason the method of assay should be quoted when stating specific extinction coefficients. The spectrophotometric analyses in all the tables is obtained by using $E^{1\%\ PBK}_{1\ cm.} = 900$ (at 2300 Å). (It should be noticed that it is normal practice for the Pyrethrum Board of Kenya to determine optical densities at 2300 instead of the usual 2270 Å.)

In normal unheated pyrethrum extract $E^{1\%\ PBK*}_{1\ cm.} = 900$ (at 2300 Å) and 72 (at 2700 Å), the ratio of the optical density at 2700 Å to that at 2300 Å being 0·08. In pyrethrum extract which has been heated until this ratio attains its maximum value, viz., 0·62 after 2½ h. at 195°, $E^{1\%\ PBK}_{1\ cm.} = 500$ (at 2300 Å) and 310 (at 2700 Å); it is assumed that *all* the pyrethrins initially present have isomerised to isopyrethrins (see footnote to Table IA). Accordingly in any heat-treated extract in which x is the proportion of the initial pyrethrins which have isomerised to isopyrethrins it follows that

$$r = \frac{d_{2700}}{d_{2300}} = \frac{310x + 72(1 - x)}{500x + 900(1 - x)}$$

Hence

$$x = \frac{900r - 72}{400r + 238}$$

This gives the extent of the isomerisation in terms of the d_{2700}/d_{2300} ratio.

* 1% solution of pyrethrins as determined by the PBK method

Table II

Analyses of pyrethrin I/cinerin I heated at 125°

Heating time, h.	DNP analysis Py I, %	Py II, %	Total Py, %	Ratio d_{2700}/d_{2300}
0	4·76	0·44	5·20	0·070
20	4·20	0·39	4·59	0·125
48	4·18	0·40	4·58	0·198
75	4·01	0·40	4·41	0·248
92	3·87	0·40	4·27	0·280

Table IIA

Pyrethrin I/cinerin I heated at 195°

Heating time, h.	DNP analysis Py I, %	Py II, %	Total Py, %	Ratio d_{2700}/d_{2300}
0	4·76	0·44	5·20	0·070
½	4·34	0·41	4·75	0·193
1	3·88	0·39	4·27	0·361
2	3·44	0·39	3·83	0·512
4	3·09	0·40	3·49	0·518

Table III

Analyses of pyrethrin II/cinerin II heated at 125°

Heating time, h.	DNP analysis Py I, %	Py II, %	Total Py, %	Ratio d_{2700}/d_{2300}
0	0·31	2·33	2·64	0·100
8	0·37	2·06	2·43	0·156
23	0·42	1·86	2·28	0·218
49	0·50	1·71	2·23	0·288
72	0·52	1·69	2·21	0·367
96	0·54	1·34	1·88	0·433

Table IIIA

Pyrethrin II/cinerin II heated at 195°

Heating time, h.	DNP analysis Py I, %	Py II, %	Total Py, %	Ratio d_{2700}/d_{2300}
0	0·31	2·33	2·64	0·100
¼	0·41	2·01	2·42	0·218
½	0·49	1·53	2·02	0·382
1	0·60	1·15	1·75	0·578
3	0·59	0·77	1·36	0·634
4	0·61	0·72	1·33	0·640

In the six families of ultra-violet absorption curves relating to Tables I–III all the component curves intersect at 2440 Å (see Figs. 1 and 2). The specific extinction coefficient at this wavelength therefore has the same value irrespective of whether the pyrethrins have undergone partial or complete isomerisation to isopyrethrins. From this it follows that, for an extract which has or has not been damaged by heat: $E^{1\%\ PBK}_{1\ cm.}$ at 2440 Å = 430.

For the isomerisation of pyrethrum extract the proportion of the initial pyrethrins converted to isopyrethrins has been calculated from the optical densities at 2700 Å and entered in Tables I and IA. The first-order reaction equation $kt = \ln \alpha(\alpha - x)$, where α is the initial concentration of pyrethrins and x the concentration of isopyrethrins at time t, to give 10^6k, for reaction times 8, 24, 48, 72 and 92 h. at 125° respectively, equal to 5·2, 4·9, 4·5, 4·0 and 4·3 (average 4·58 sec.$^{-1}$). At 195° the values for 10^6k, for reaction times ¼, ½, 1 and 2 h., are, respectively, 443, 508, 526 and 446 (average 481 sec.$^{-1}$). If the Arrhenius and Eyring equations

$$k = A \exp(-E^*/RT) \quad \text{and} \quad k = (RT/Nh) \exp(-E^*/RT).\exp(\Delta S^*/R)$$

where R = 1·987 cal. deg.$^{-1}$, R/N (Boltzmann constant) = 1·3805 × 10^{-16} erg deg.$^{-1}$, h (Planck constant) = 6·624 × 10^{-27} erg. sec.$^{-1}$, are applied to these values for k, it follows that E^* = 24,610 cal mol.$^{-1}$, log A = 8·17; and ΔS^* = −21·8 cal. deg.$^{-1}$. The negative ΔS^* reflects the loss in rotational freedom about the bond between the 2-position in the cyclopentenolone ring and the 1′-position of the pentadienyl side-chain when the transition structure is formed. In the mesomeric transition structure this bond must assume partial double-bond character with consequent loss of rotational and flexing freedom of the whole side-chain with respect to the plane of the cyclopentenolone ring.

In another series of experiments, 20% pyrethrum extract containing 6% of ethyl alcohol (a wax co-solvent) was diluted with an equal volume of xylene and this solution vigorously refluxed under a double surface condenser. The vapours above the boiling liquid protected the pyrethrins from atmospheric oxygen. The internal temperature, initially 122°, slowly rose to 128°/620 mm., the solution doubtless losing ethyl alcohol slowly through the condenser. The solution was sampled at intervals for analysis. It was observed in these experiments that there was considerable time lag before the optical density at 2300 Å began to fall and that at 2700 Å began to rise. Eventually, after heating for 100 h., the ratio of optical densities at 2700 Å and 2300 Å rose to its normal maximum of 0·62. Table IV is typical of a number of such sets of experiments; all the ultra-violet absorption curves intersect at 2440 Å. Although the evidence is not conclusive, it would appear that the ethyl alcohol initially present inhibits the isomerisation, since the same formulation which did not contain alcohol showed the normal

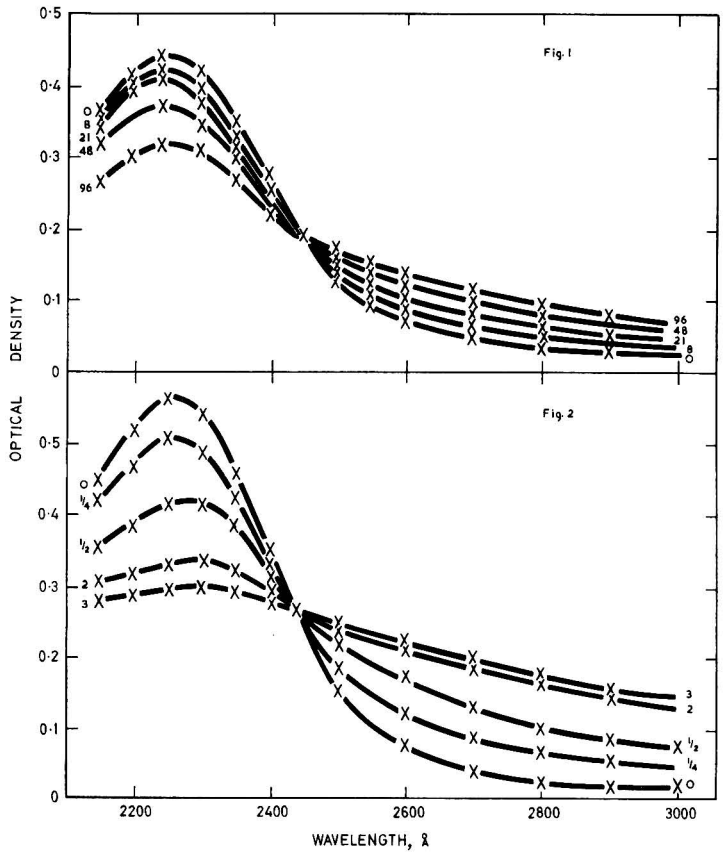


FIG. 1.—*Spectrum of pyrethrum extract* (9·32% *pyrethrins PBK*) *heated for* 0, 8, 21, 48 *and* 96 *h. at* 125°
(50 mg. of extract per l. in ethyl alcohol; 1 cm. cell)

FIG. 2.—*Spectrum of pyrethrum extract* (10·98% *pyrethrins PBK*) *heated for* 0, ¼, ½, 2 *and* 3 *h. at* 195°
(60 mg. of extract per l. in ethyl alcohol; 1 cm. cell)

progressive rise in the ratio of the optical densities at 2700 Å and 2300 Å (Table V). (Compare the protective influence of piperonyl butoxide[7] on the photolysis of the pyrethrins.)

Effect of heat on the chemical components of pyrethrum extracts

It can be seen from Table I that the total pyrethrins (PBK method) in a solution of pyrethrum extract in kerosene fall from 9·32% to 8·66% (a fall of 7% of the initial value) when the extract is heated for 8 h. at 125°; the d_{2700}/d_{2300} ratio rises from 0·081 to 0·125. In contrast to this, heating at 125° for 8 h. in a solvent consisting of 48·5% of kerosene, 48·5% of xylene and 3% of ethyl alcohol (Table IV) causes only an insignificant fall in total pyrethrins (PBK method) and an insignificant rise in the d_{2700}/d_{2300} ratio.

At 195° there is a severe pyrolysis and loss of pyrethrins; after 3 h. at this temperature the PBK- and DNP-pyrethrins are only 75% and 64% of their respective initial values. The figures for the DNP analyses indicate that the products of the pyrolysis of pyrethrin II travel down the chromatographic column with the pyrethrin I band; a similar observation has been made after partial hydrolysis[8] of pyrethrum extract with dilute acid.

It was of interest to determine whether there is any loss in pyrethrins, as measured by the PBK method, when pyrethrum oleoresin is submitted for short periods to temperatures of the order of 70–100°. (During the final stages of manufacture, oleoresin is normally heated in

Table IV

Pyrethrum extract containing 10% *pyrethrins,* 50% *xylene and* 3% *ethyl alcohol refluxed at* 122–128°

Time, h.	Method of analysis							Ratio d_{2700}/d_{2300}
	DNP			PBK			Spectroscopic total Py, %	
	Py I, %	Py II, %	Total Py, %	Py I, %	Py II, %	Total Py, %		
0	4·87	3·57	8·44	6·21	4·43	10·64	10·9	0·088
2	4·67	3·59	8·26	6·27	4·42	10·69	10·9	0·083
4	4·64	3·55	8·19	6·29	4·09	10·38	10·8	0·082
6	4·52	3·45	8·07	6·61	4·27	10·80	10·9	0·082
10	4·69	3·35	8·04	6·19	4·22	10·41	10·9	0·093
16	4·42	3·27	7·69	5·98	4·20	10·18	10·1	0·155
22	4·26	2·92	7·18	6·32	4·09	10·41	9·5	0·243
28	4·04	2·92	6·94	6·06	4·33	10·39	8·3	0·351
75	4·15	2·54	6·69	5·78	4·09	9·87	8·1	0·450
100	3·48	2·18	5·66	5·72	4·01	9·73	7·5	0·590

Table V

Pyrethrum extract containing 10% *pyrethrum,* 50% *xylene (no alcohol) refluxed at* 128°

0	4·86	3·42	8·28	6·47	3·99	10·46	11·0	0·088
4	4·49	3·03	7·52	6·28	3·84	10·12	10·1	0·165
8	4·48	2·75	7·23	6·19	3·91	10·10	9·4	0·186
16	4·44	2·64	7·08	6·06	3·86	9·92	8·9	0·400

the falling-film evaporator to ~75°/25 mm. Hg for a few seconds in order to remove traces of isohexane.) Accordingly, a sample of 25% oleoresin was sealed in twelve 5-c.c. ampoules. Six of these were heated in an oven at 105° for 1½ h., cooled, opened and analysed; the other six were analysed without any heat treatment. Each analysis was made in duplicate and the average of the duplicates recorded in Table VI.

For the observed mean difference (MD) the statistical parameters are: variance 0·20; standard deviation 0·45; standard error 0·183; 95% confidence limits −0·69 to +0·25; 99% confidence limits −0·96 to +0·52. There is therefore no statistically significant difference between the means of the analyses of the heated and unheated extract and it must be concluded that there is no loss of PBK pyrethrins when oleoresin is stripped of residual solvent at 80–100°/25 mm. in a falling-film evaporator during the final stages of manufacture.

The stability of chrysanthemic acid to heating

The only change in the pyrethrin molecule, other than isomerisation to isopyrethrin, which might occur on heating is inversion of optical configuration at one or more of the three asymmetric centres. LaForge & Green[9] reported the toxicity of (−)*cis*-cineronyl (+)*trans*-chrysanthemate to flies (determined by the Campbell turntable method) to be 1·8 times that of the natural (+)*cis*-cineronyl (+)*trans*-chrysanthemate; Gersdorff[10] found no difference in the activities of (+)*cis*-pyrethronyl (+)*trans*-chrysanthemate and (*rac.*)*cis*-pyrethronyl (+)*trans*-chrysanthemate. It must be accepted upon this evidence that inversion at C_4 of the cyclopentenolone ring would either not effect or would cause a slight increase in the biological activity.

Table VI

Effect of heating oleoresin at 105° *for* 1½ *h. on the PBK analysis*

% Pyrethrins		Difference	(X − MD)	$(X - MD)^2$
Unheated	Heated	X		
26·58	25·97	−0·61	-39×10^{-2}	1521×10^{-4}
26·45	25·83	−0·62	−40	1600
25·71	25·74	+0·03	+25	625
25·79	26·07	+0·28	+50	2500
26·16	25·52	−0·64	−42	1764
25·11	25·32	+0·21	+45	2025
Mean 25·97	Mean 25·75	Mean (MD) −0·22		S, 10035×10^{-4}

Inversion at C_1 and C_3 of the cyclopropane ring, however, would substantially reduce toxicity since the relative activities of a given pyrethrolone esterified with (+)*trans*-, (+)*cis*-, (−)*trans*- and (−)*cis*-chrysanthemic[11] acids are (+)*trans* > (+)*cis* ≫ (−)*trans* > (−)*cis*. It was therefore important to ascertain the stability of (the natural) (+)*trans*-chrysanthemic acid when subjected to temperatures of the order of 200–220°.

A sample of (+)*trans*-chrysanthemic acid (Found $[\alpha]_D^{20}$ + 14·32° (ethanol); lit.[12] + 14·2°) was heated from 25° to 200° during 1 min., held at 215–225° for 2 min., and cooled to room temperature during 1 min. The specific rotation of the products was then $[\alpha]_D^{20}$ + 14·31° (ethanol) showing that the configurational integrity of the molecule had not been affected by the heat treatment. Chrysanthemic acid obtained by hydrolysis of oleoresin which had been distilled at 230°/5 μ in a 12-in. industrial molecular still[5] had b.p. 114–116°/1 mm. and $[\alpha]_D^{20}$ + 14·31°.

The biological activity of heat-treated pyrethrum extract

Various heat-treated pyrethrum extracts together with their unheated controls were subjected to bioassay in order to determine the extent of any impairment of biological activity due to heat treatment and to relate the loss in activity to the d_{2700}/d_{2300} ratio.

The standard techniques used were: (i) Kearns & March method[13] for knockdown of flies; (ii) the measured drop method for kill of flies;[14] (iii) the dusted wheat method[15] for kill of *Calandria oryzae*. For the preparation of the required dilutions for the bioassays the pyrethrin content of the heated concentrate was taken to be the same as the initial concentration before heating: this enables damage to be directly assessed.

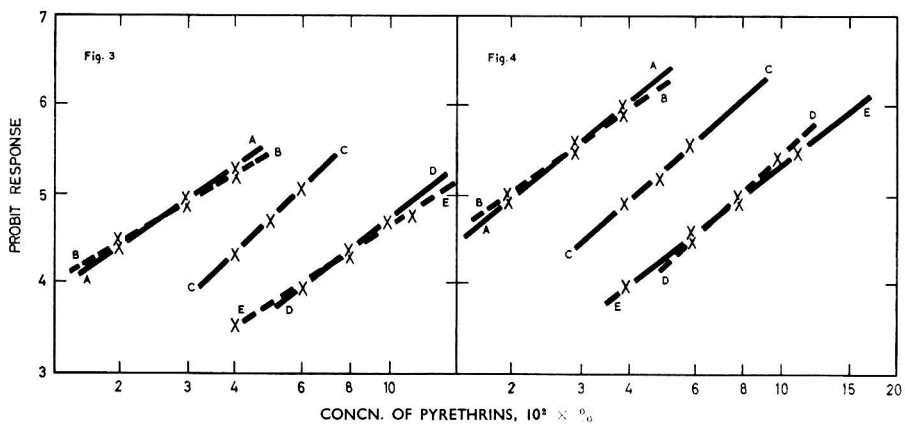
The pyrethrum extracts used were:

(A) unheated control: d_{2700}/d_{2300} = 0·081; (B) (A) after being heated 10 h. at 125° in 50% xylene + 3% ethanol solution (d_{2700}/d_{2300} = 0·093); (C) (A) after heating 28 h. at 125° in 50% xylene at 3% ethanol solution (d_{2700}/d_{2300} = 0·351); (D) (A) after heating 100 h. at 125° in 50% xylene + 3% ethanol solution (d_{2700}/d_{2300} = 0·590); (E) (A) after heating 3 h. at 195° in kerosene solution (d_{2700}/d_{2300} = 0·62).

The results obtained are shown in Figs. 3–7 and the KD_{50} and LD_{80} values derived from these figures are given in Tables VII–IX.

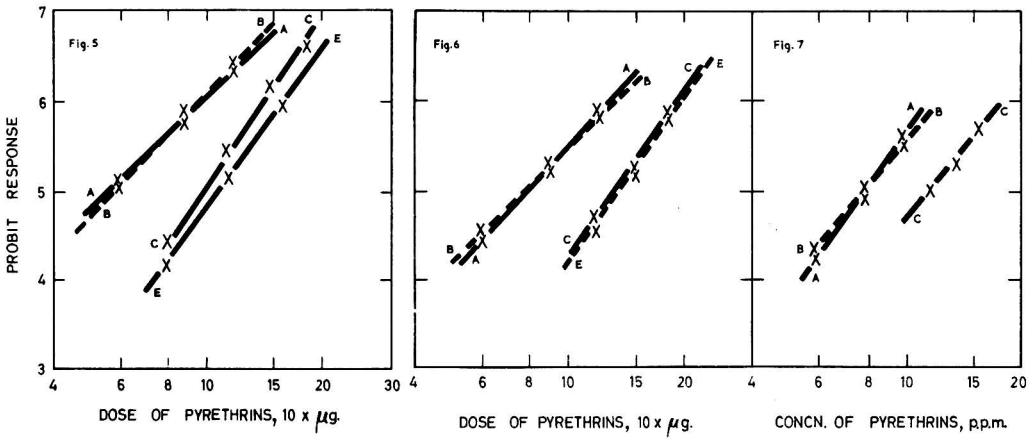
Discussion

The results given above show that the biological activity of pyrethrum extract which has been subjected to heat treatment is a function of the ratio of the optical density of the extract at 2700 Å to that at 2300 Å. This ratio is a measure of the isopyrethrin content of the extract



Results obtained by Kearns & March method on heated pyrethrum extracts (A–E) for (FIG. 3) 5 *min.*, (FIG. 4) 10 *min. knock-down of houseflies*

5-day-old flies: 80–120 flies per batch. Solutions prepared containing 0·01%–0·12% pyrethrins: 0·2 c.c. sprayed from each nozzle during 5 sec. at 12·5 p.s.i. at 28°: 3 doses: 4–6 replicates on each sample



Results by measured drop technique on heated pyrethrum extracts applied to male (FIG. 5) *and female* (FIG. 6) *houseflies*

5-day-old flies: 30 flies per dose: 4 replicates on each sample. Flies dosed topically with solution of pyrethrins in kerosene and then placed in jars at 27° and supplied with 5% sucrose solution for 24 h.

FIG. 7.—*Results by dusted wheat technique*[15] *on heated pyrethrum extracts (kill of* C. oryzae)

The extracts were mixed with B.P. talc to give dusts containing 1% of pyrethrins (calc. on unheated extract) and aliquots weighed into and mixed in bottles with 50 g. of wheat. Each bottle was infected with 50 *C. oryzae* spores and kept for 7 days

and, provided this ratio does not exceed a value of 0·095, the heat treatment has caused no damage. This information is of significance in the problems encountered in the distillation of pyrethrum extract and in the design of thermal fogging machines for dispensing pyrethrum mists.

In normal unheated extracts the ratio of the optical densities at 2700 Å and 2300 Å is 0·08, whereas in an extract in which the pyrethrins have been completely isomerised to isopyrethrins the ratio is 0·62; such extract has one-half of the lethal, but only one-quarter of the knockdown, activity of normal extract on houseflies. It is of interest that in the mesomeric structure of the isopyrethrins the bond between $C_{(2)}$ of the cyclopentenolone ring and $C_{(1)}$ of the pentadienyl side-chain will have partial double-bond character which will cause substantial loss of rotational and flexing freedom of the whole side-chain with respect to the plane of the cyclopentenolone ring. The remarkably high knockdown activity of the pyrethrins on insects implies a multiplicity of rapidly effected attachments of the pyrethrin molecule to receptor sites in the nerve substrate. This restraint imposed upon the flex and rotation of the side-chain may be the reason for the much greater loss in knockdown activity than in lethal activity when the pyrethrins are converted to isopyrethrins.

It is of interest to consider the thermodynamic aspects of the conversion of pyrethrins to isopyrethrins. The energy and entropy of activation in the process are respectively 24,610 cal. mol.$^{-1}$ and $-21{\cdot}8$ cal. deg.$^{-1}$. Since $\Delta F^* = \Delta H^* - T.\Delta S^*$, the free energy of activation ΔF^* at 225° (the normal temperature for distillation of pyrethrum extract) is 35,460 cal. mol.$^{-1}$. With a free-energy barrier of this magnitude, the transformation cannot take place

Table VII

KD_{50} values (Kearns & March method) derived from Figs. 3 and 4

Extract	d_{2700}/d_{2300} ratio	KD_{50} (5 min.)	KD_{50} (10 min.)	Relative activity
A (unheated control)	0·081	0·032%	0·018%	100
B	0·093	0·031	0·019	100
C	0·350	0·059	0·042	54–43
D	0·590	0·130	0·082	25–21
E	0·620	0·140	0·086	23–21

Table VIII

LD_{50} values derived from the results shown in Figs. 5 and 6

Extract	d_{2700}/d_{2300} ratio	LD_{50} μg./fly male	LD_{50} μg./fly female	Relative potency
A (unheated control)	0·081	0·56	0·80	100
B	0·093	0·58	0·80	100
C	0·350	1·00	1·36	c. 58
E	0·620	1·12	1·40	c. 54

Table IX

LD_{50} values derived from the results shown in Fig. 7

Extract	d_{2700}/d_{2300} ratio	LD_{50} (p.p.m.)	Relative potency
A	0·081	8	100
B	0·093	8	100
C	0·350	12	66

with any high degree of spontaneity. (For example, for the conversion[16] of the comparatively stable diphenyl ether-2-carboxylic acid to xanthone, the energy and entropy of activation are respectively 20,900 cal. mol.$^{-1}$ and −13 cal. deg.$^{-1}$.)

Accordingly it is to be expected that in a wiped-wall falling-film molecular still, in which the pyrethrins are subjected to the high temperature for a very brief period, distillation can be accomplished with no measurable conversion of pyrethrins to isopyrethrins. This has been proved in practice. Elliott *et al.*[17] distilled neat 25% extract in a 2-in. wiped-wall still and found no loss in toxicity to mustard beetles. Goldberg *et al.*[5] have fractionally distilled pyrethrum extract diluted with light liquid paraffin B.P. in the same type of still and have effectively separated pyrethrin I and pyrethrin II. In this connexion it is significant that the pyrethrin II recorded in Table V above had been passed through the still eight times at temperatures ascending from 160 to 220° and the ratio d_{2700}/d_{2300} had not risen above 0·10. It was also shown[5] that it had four times the knockdown activity of pyrethrin I against houseflies and, when synergised 5 : 1 with piperonyl butoxide, twice the knockdown activity of similarly synergised pyrethrin I which had only passed through the still once at 140°. During the last few years pyrethrum extract has been co-distilled[18] with piperonyl butoxide on an industrial scale at temperatures above 200° in falling-film, short-contact stills. Furthermore, in the period 1960–1962 some 200,000 lb. of 25% pyrethrum extract have been co-distilled[5] in Nakuru with light liquid paraffin B.P. in a 12-in. Edwards' molecular still to give a 92·5% yield of decolorised extract all of which had a d_{2700}/d_{2300} ratio of 0·08–0·085, showing it to be free from isopyrethrins. Examination for KD_{50} and LD_{50} against *Musca domestica* and LD_{50} against *Tribolium castaneum* and *C. oryzae* showed[5] no difference between the distilled material, the undistilled oleoresin and nitromethane decolorised extract.

Acknowledgments

The authors thank Miss Hardaker for the PBK analyses and the Chairman of the Pyrethrum Board for his stimulating interest.

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Received 1 June, 1964; amended manuscript 24 August, 1964

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THE RÔLE OF WHEAT FLOUR PENTOSANS IN BAKING. III.*—Enzymic Degradation of Pentosan Fractions

By PAMELA M. WRENCH

Enzymes in snail digestive juice degrade two of the five fractions obtained when flour pentosans are chromatographed on DEAE cellulose. These fractions are both glycoproteins containing arabinose and galactose and one also contains xylose. Solutions of the latter fraction gel on the addition of oxidising agents, but this ability is lost after incubation with snail juice enzymes. Loaves baked from doughs in which the pentosans have been degraded by snail juice enzymes are inferior in volume, but this effect is not observed in the presence of oxidising agents. The effect of oxidising agents, however, does not appear to be directly related to that of the pentosans.

Introduction

The possible importance of the pentosans of wheat flour in contributing to the rheological and baking properties of a dough has been reviewed in the first paper of this series.[1] Tracey[1] used the technique of enzymic degradation of dough components *in situ* to establish that pentosans play a significant rôle in determining dough properties. Cawley[2] later described the rheological and baking behaviour of gluten–starch doughs reconstituted by the addition of native flour pentosan† or one of a range of polysaccharides of known composition. The present paper describes the fractionation of pentosans extracted from flour and the further use of enzymes to identify the components responsible for their effects in dough. The relation between the formation of gels by pentosans when oxidised to the effect of oxidising agents on doughs has also been studied briefly.

Experimental

Flour

A typical Australian baker's flour (protein 11·9% on a 14% moisture basis) was used throughout.

Pentosan preparations

Flour solubles prepared as described by Cawley[2] were used as a source of crude pentosan. For some experiments these were purified by removing the soluble starch with α-amylase as described by Kundig *et al.*[3]

* Part II: *J. Sci. Fd Agric.*, 1964, **15**, 834.

† For the purpose of this work, flour pentosans are defined as the non-dialysable polysaccharides contained in a heated water extract of flour. Such preparations contain approximately 55% α-glucosan as well as some galactose and protein.[3]

Enzyme treatment

Enzymes already present in a freshly prepared water extract of flour were allowed to act on the pentosans by incubating the whole extract at 37° for 6 days in the presence of toluene. The digestive juice of the garden snail (*Helix aspersa*) was used as an external source of hydrolytic enzymes. This was prepared as described by Bawden & Pirie[4] and diluted to the equivalent of the juice from one snail per ml. Diluted juice was added in the ratio of 0·02 ml. per ml. of crude flour extract, or pentosan solution, containing from 15 to 30 mg. of total carbohydrate; and the mixture was incubated at 37° for 6 days. Toluene was added as a microbial inhibitor, and enzymes were inactivated by boiling before chromatography of the reaction mixtures.

Sephadex chromatography

Five-ml. samples containing 100–150 mg. of total carbohydrate were applied to a 3 × 100 cm. column of Sephadex G-25 and eluted with water at a constant rate of approximately 20 ml. per h. Five-ml. fractions were collected automatically and analysed for total and reducing sugar.

DEAE-Cellulose chromatography

The method of Kundig *et al.*[3] was used as follows: DEAE cellulose (Whatman, exchange capacity 1·0 mequiv. per g.) was washed, converted to the borate form, washed with water and packed into a 1·4 × 54 cm. column. Five ml. of pentosan solution containing about 100–150 mg. of carbohydrate were applied to the column and eluted stepwise with (1) water, (2) 0·0025M-sodium tetraborate, (3) 0·025M-sodium tetraborate, (4) 0·125M-sodium tetraborate, and (5) 0·5M-sodium hydroxide. The flow rate was 1·0–1·2 ml. per min. Five-ml. fractions were collected and automatically analysed for total sugar and protein.

Carbon column chromatography

Ten-ml. columns of a 1 : 1 (w/w) mixture of activated charcoal powder (British Drug Houses) and Hyflo Supercel (Johns Manville Products) were prepared and washed with concentrated hydrochloric acid, then with water. A new column was prepared for each sample. The low-molecular-weight carbohydrates eluted from Sephadex G-25 (see Fig. 1) were freeze-dried and 40–50 mg. in 1 ml. of water applied to the charcoal column. The sample was eluted at 0·5 ml./min. first with 20 ml. of water, then with a gradient to 25% ethanol in 100 ml. One-ml. fractions were collected and analysed for total sugar.

Chromatography of sugars

Sugars were chromatographed on thin layers of Kieselgel G (Merck) buffered with 0·02M-sodium acetate. Chromatoplates were run twice to 10 cm. in ethyl acetate/isopropanol/water 78 : 52 : 21 and sugars detected by spraying the plates with anisaldehyde–sulphuric acid and heating. Pentosan fractions were hydrolysed with 1·0N-nitric acid for 8 h. at 105° and deionised before chromatography. One or two μg. of sugar dissolved in 10% isopropanol were applied with a microsyringe. A trace of pyridine was added to the sugar solutions to reduce streaking effects caused by salts. Good separations of pentoses, hexoses and di- and tri-saccharides were obtained by this technique. The determinations were made approximately quantitative by the algebraic method of Purdy & Truter.[5]

Baking test

This was as described by Tracey.[1] Additions of snail juice and/or bromate were made in a volume of 6 ml. which replaced 6 ml. of the water added to the control dough.

Dough extracts

Doughs of 120 g. prepared as for baking were allowed to prove and were then extracted with 162 ml. of water in a Waring blendor at half speed for 2·5 min. Extracts were centrifuged and the viscosity of the supernatants measured.

Viscosity measurements

Viscosities were measured in Ostwald viscometers in a water bath at 20°. Five-ml. samples

were used, and the results expressed as relative viscosities (η_r), or viscosity increments ($\eta_r - 1$) for comparison of the viscosity of the same solution after different treatments.

Assay methods

Total sugars were measured by the orcin method and reducing sugars by Somogyi's modification[6] of the colorimetric method of Nelson. Protein was estimated by the method of Lowry *et al.*[7] Pentosan fractions eluted from DEAE were analysed automatically for protein and total carbohydrate using respectively the Lowry and orcin methods adapted to a Technicon Auto-analyser.

Results

The fractionation of wheat flour pentosans achieved by chromatography of a crude water extract of flour on Sephadex G-25 is shown in Fig. 1C. Fig. 1A shows the effect of snail enzyme action on the elution patterns of total and reducing sugars and Fig. 1B shows the elution patterns of a crude flour extract which had been incubated without snail juice, i.e., in which the changes observed were due to native flour enzymes. In comparing the elution patterns, it should be noted that different amounts of total carbohydrate were chromatographed in each experiment. When the small-molecular-weight fractions of each of these extracts (sugars eluted after 380 ml.) were further fractionated on carbon columns, they gave the patterns shown in Figs. 2A–C. It can be seen that the snail-treated extract (Fig. 2A) contained practically no di- or tri-saccharide, nearly all the low-molecular-weight fraction being in the form of monosaccharides. The unincubated extract (Fig. 2C) had very little monosaccharide, and about twice as much di- as tri-saccharide, while the extract incubated without added enzyme (Fig. 2B) showed a pattern intermediate between Figs. 2A and 2C. Thin-layer chromatography of the sugars after separation on charcoal showed that the increase in monosaccharides after treatment with snail juice was due to a marked increase in free glucose and fructose and also to the appearance of free arabinose and galactose and, to a lesser extent, xylose. Fructose and traces of glucose occurred free in all three extracts, and arabinose and galactose also in the extract incubated alone. Free xylose could not be detected in the latter preparation. The disaccharide fraction (Figs. 2B and C) contained maltose and a trace of sucrose, and the ' trisaccharide ' fraction of Fig. 2C contained raffinose and three other sugars, possibly larger oligosaccharides, which were not identified. Hydrolysis of these di- and tri-saccharides must be partly responsible for the increase in glucose and fructose in the incubated extracts.

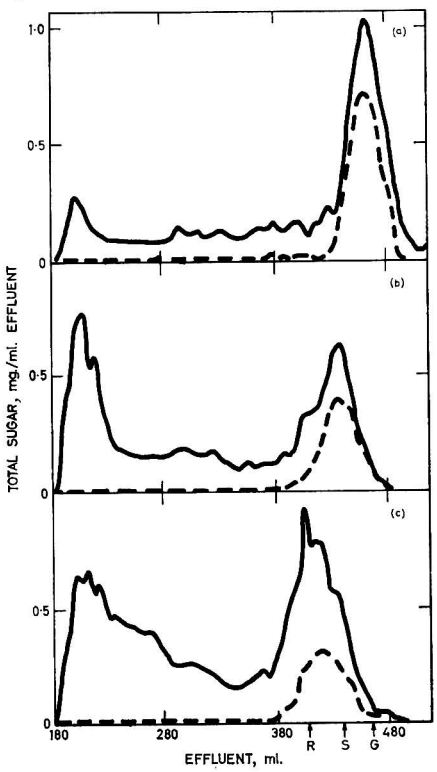

FIG. 1.—*Chromatography of a water extract of flour on Sephadex G-25*

A. Flour extract after incubation with snail juice for 6 days at 37° (70 mg. of total carbohydrate loaded)
B. The same extract after incubation alone for 6 days at 37° (85 mg. of total carbohydrate loaded)
C. The freshly prepared extract (105 mg. of total carbohydrate loaded)

The position of elution of standard raffinose (R), sucrose (S) and glucose (G) are shown for comparison

——— total sugar - - - - - reducing sugar

The effect of treatment with snail juice on the DEAE fractionation of pentosans prepared from flour by Kundig's method[3] (removal of starch by α-amylase treatment of a heated water extract) is shown in Figs. 3A and B. The main changes in the pattern of elution of total sugar are in fractions B and C, and in the trailing edge of fraction A. (The naming of fractions is that used by Neukom & Kundig.[8]) Both the total sugar and protein (Lowry) patterns showed an increase in fraction D, otherwise there was no marked change in the elution of protein. Reducing sugar was also estimated in fraction A of both runs, and plotting the sum of the reducing sugar figures after snail juice treatment and total sugars before treatment gave a fraction very similar to the total sugars of fraction A after treatment. The main changes would thus appear to be explained by the partial hydrolysis of the polysaccharide constituents of fractions B and C and the trailing edge of fraction A to give material which is eluted with fraction A under the conditions of chromatography. Quantitative thin-layer chromatography of the hydrolysed fractions gave approximate values for their percentage composition of arabinose, galactose and xylose (Table I). Fraction E was not analysed as it contained large amounts of material yielding glucose on hydrolysis eluted from the cellulose of the column with 0·5N-sodium hydroxide.

After treatment with snail juice, only arabinose and galactose could be detected in fraction B.

It has been observed in this laboratory that the deleterious action of snail juice on doughs can be overcome on baking by the addition of bromate. This prompted several experiments on the relation between the effect of oxidising agents, and that of snail enzymes, on flour pentosans. Fig. 4A shows the elution pattern obtained when the pentosans from a commercial chlorine dioxide-treated flour are chromatographed on DEAE cellulose, and Fig. 4B the pattern obtained after incubating the extract with snail juice. It can be seen that oxidation of the flour appears to lead to a great increase in fraction A, in agreement with the results of Neukom & Kundig.[8] Oxidation of the flour, however, did not alter the effect of the snail juice on fractions B and C, and the latter half of fraction A.

Water extracts or Kundig pentosan preparations from the flour used gave characteristic 'gelling' reactions (as measured by changes in viscosity increment) with hydrogen peroxide and sodium chlorite, but viscosities dropped rapidly on addition of snail juice to the oxidised solution (Table II). Pentosan solutions which had been incubated with snail juice overnight could not be gelled by the addition of hydrogen peroxide, sodium chlorite, potassium bromate or potassium iodate. Table III shows the results of an experiment in which snail juice and/or bromate were added to doughs. Half of each dough was extracted with water and the viscosity

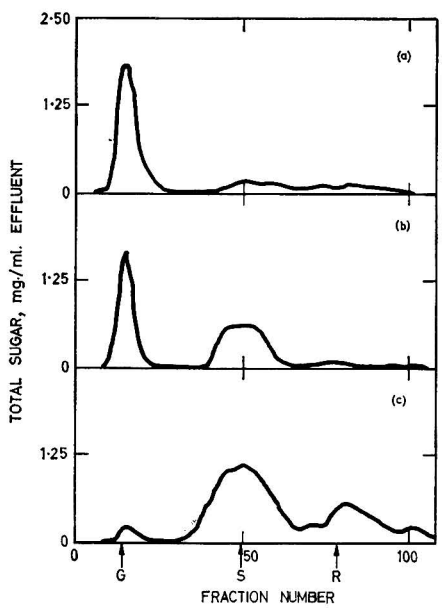

FIG. 2.—*Carbon column chromatography of the low-molecular-weight fraction of flour extracts separated on Sephadex G-25 as in Fig.* 1

A, B and C as in Fig. 1

The positions of elution of standard raffinose (R), sucrose (S) and glucose (G) are indicated

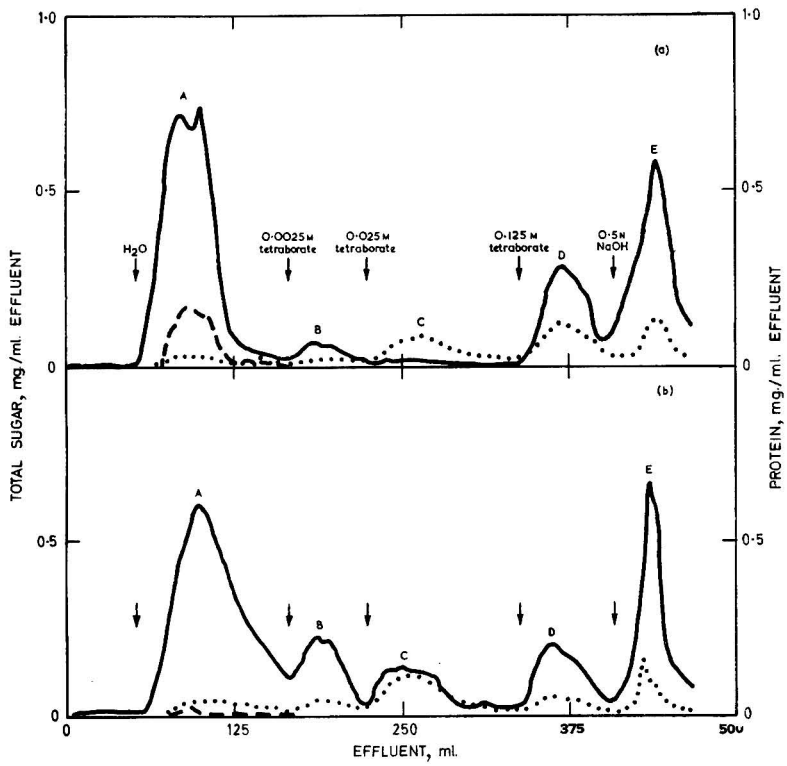

FIG. 3.—*DEAE chromatography of Kundig flour pentosan preparations*

A. 5 ml of pentosan containing 67 mg. of total carbohydrate after incubation with 0·1 ml. of snail juice for 6 days at 37°
B. 5 ml. of pentosan solution containing 69 mg. of total carbohydrate

——— total sugar - - - - - reducing sugar ..... protein

of the extract measured, and the other half was baked. Although the dough to which snail juice *and* bromate were added gave a loaf of good volume, the viscosity of the dough extract was as low as that to which no bromate but only snail juice was added.

Conclusions

Chromatography of the water-soluble pentosans of an Australian flour on DEAE cellulose yielded five fractions of composition very similar to those prepared by Kundig *et al.*[3] The slight differences observed may be varietal; no work has yet been done on this. When the pentosans are incubated with snail juice, one fraction (C) is completely degraded and another (B) is partially digested. Fractions B and C are respectively a galactose–arabinose–xylose polymer and an arabogalactan, and each also contains some protein. A partially resolved second component of peak A also appears to be degraded by snail juice enzymes, but fractions D and E

Table I

Composition (%) of fractions A, B, C and D of the water-soluble pentosans extracted from an Australian flour

Figures based on the results of two separate determinations each in duplicate

(Figures obtained by Kundig *et al.*[3] for pentosans extracted from a Manitoba II wheat flour are shown in brackets for comparison)

Fraction	Arabinose	Xylose	Galactose
A	43 (45)	49 (55)	8 (0)
B	41 (36)	42 (38)	17 (26)
C	35 (46)	0 (0)	65 (54)
D	43 (43)	0 (8)	57 (49)

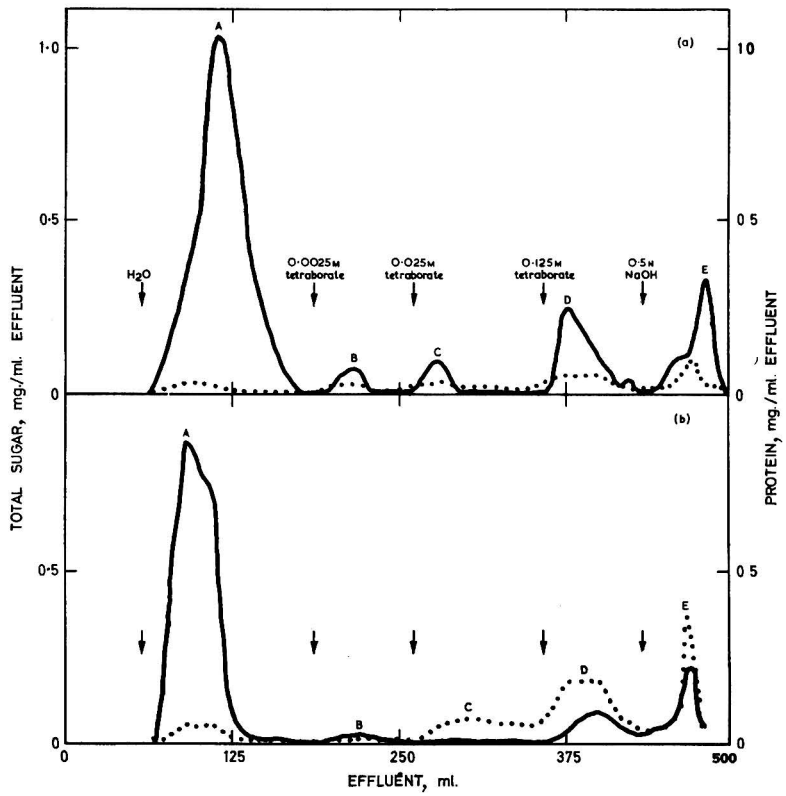

FIG. 4.—*DEAE chromatography of a Kundig pentosan preparation from a chlorine dioxide-treated flour*

A. 5 ml. of pentosan containing 50 mg. of total carbohydrate
B. 5 ml. of pentosan containing 44 mg. of total carbohydrate after incubation with 0·1 ml. of snail juice for 11 days at 37°

——— total sugar ····· protein

are not attacked. The protein content of each of the fractions remains unaffected by the enzymic treatment, agreeing with the absence of detectable protease in snail juice.[9] It is possible that the protein components of fractions D and E may protect the polysaccharide from enzymic action. An attempt was made to digest the protein from fraction D with Pronase before treating

Table II

Typical results of experiments on the effect of oxidising agents and snail digestive juice on the viscosity of flour pentosan solutions

Two or three drops of a 0·1% solution of the oxidising agent were added, and 0·1 ml. of snail juice, to 5 ml. of pentosan solution

Oxidising agent	Viscosity increment		
	Before adding oxidising agent	After adding oxidising agent	After adding 0·1 ml. of snail juice
0·1% Hydrogen peroxide	5·2	7·5	0·6
0·1% Sodium chlorite	7·5	10·9	1·4

Table III

Effect of bromate and snail juice on loaf volume and on the viscosity of dough extracts

	Control	Bromate, 10 p.p.m.	Snail juice, 6 ml.	Bromate, 10 p.p.m. + snail juice, 6 ml.
Loaf volume, c.c.	590	630	530	620
Relative viscosity of dough extract	8·9	9·7	2·9	2·8

it with snail juice, but a large proportion of the fraction still remained resistant to attack by snail enzymes.

The results of the Sephadex fractionations of crude undialysed flour extracts showed that snail juice enzymes also hydrolyse the smaller oligosaccharides present to their constituent sugars. The effect of snail juice on dough cannot be explained by the hydrolysis of these small sugars, however, as dialysed pentosan preparations are still fully effective in improving reconstituted gluten–starch doughs.[2] Water extracts of flour and sprouted wheat also contain enzymes capable of degrading flour pentosans, although not as actively as the snail juice enzymes. Chromatography of the sugars released by the action of the flour enzymes showed no free xylose to be present. This suggests that the pentosans eluted from DEAE as fractions C and D, which contain little or no xylose, may possibly be substrates for the flour pentosanases. Enzymes in sprouted wheat extracts appear to act mainly on fraction B, but activities are too low to say whether this is a real difference in specificity between enzymes from the two sources.

The glycoprotein known as ' fraction B ' has been shown to consist of two polysaccharide fractions of different composition, possibly linked by a polypeptide bridge.[10] Ferulic acid and probably other phenols are linked to the polysaccharide and take part in the oxidative gelling reaction.[10] The present work has shown that this reaction requires the integrity of the complex and that, although oxidation and cross-linking may occur in the degraded molecule, there is no gel formation. The fact that the loss of volume on adding snail juice to dough may be almost overcome by the addition of bromate does not therefore mean that the two effects are directly related, and probably reflects the action of bromate on components other than pentosans. Even so the pentosans are significant in determining the physical properties of dough ; their phenolic content makes them also a factor to be considered in interpreting the effect of oxidising agents on dough.

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Received 5 June, 1964

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STUDIES OF THE QUALITY AND FLAVOUR OF CEYLON TEA

By R. L. WICKREMASINGHE* and T. SWAIN

The amounts of total phenols, flavans, leuco-anthocyanins, theaflavins, thearubigins, and certain amino-acids in methanolic extracts of Ceylon and other teas have been determined, together with the individual low-boiling volatile compounds which are produced on infusion of the teas in boiling water. The correlation between the quantities of phenolic compound and commercial valuation, and the contribution of the volatiles to flavour, is discussed. *N*-Ethylasparagine has been tentatively identified in tea leaf.

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Introduction

The polyphenols present in fresh tea leaf juice and black tea have been extensively studied by Roberts and his co-workers,[1] whilst the polyphenols present in ethanolic extracts of a Ceylon tea leaf sample have been fractionated by Vuataz *et al.*[2] Both groups of workers showed the occurrence in their material of flavanols and their gallic acid esters, flavonols and their 3-glucosides, polyphenolic acids and leuco-anthocyanins. Oxidation and polymerisation products were present only in small quantities in unprocessed green tea leaves, but formed the major polyphenolic fraction of black tea.[3] Among the products of fermentation of tea leaf, Roberts & Myers[4] isolated and characterised theaflavin, theaflavin gallate and a group of substances referred to as ' thearubigins '. The amounts of these were shown to determine the strength, colour and briskness of black tea liquors and to play an important part in the assessment of quality. Quality, however, is also dependent on other factors including the volatile constituents responsible for the flavour of tea. These substances were studied by Yamanishi and co-workers,[5] who separated and identified 27 components in the essential oil of green tea leaf. Gas-chromatographic methods were also used for the separation and identification of the volatile fatty acids of black tea,[6] and the earlier work on tea volatiles has been reviewed by Stahl.[7] Apart from the essential oil, amino-acids have also been shown to contribute to the aroma complex of black tea. Thus Bokuchava & Popov[8] showed that addition of different amino-acids to hot aqueous infusions of tea led to the production of different aromas, and that no aroma developed if a carbonyl-binding reagent was added. Based on these results, Popov[9] suggested a scheme for the formation of carbon dioxide, ammonia and aldehydes from amino-acids.

The present paper presents the results of comparative qualitative and quantitative studies of the polyphenols, amino-acids and low-boiling volatile compounds in two specially prepared samples of Ceylon tea, and those in 20 commercial samples of black tea, 8 of which were manufactured from Ceylon-grown tea. Two commercial brands of instant tea have also been examined. The correlation between commercial valuation and quality on the one hand and polyphenol content on the other, as well as the contribution of low-boiling volatiles to the flavour, is discussed.

Experimental

Materials

Green tea leaf.—As soon as possible after being plucked from selected bushes at two tea estates of the Tea Research Institute (Ceylon), samples of the two batches of fresh green leaf (the terminal bud with two leaves attached) were packed in sealed polythene bags and flown from Ceylon to London. The samples were kept at 3–5° during transit and used in the laboratory about 3 days after being plucked. Hence the term ' unprocessed leaf ' used here (Table II) is not strictly applicable in view of the fact that ' chemical withering ' begins immediately after the leaf is separated from the parent bush (G. W. Sanderson[9a]).

' Fermented ' tea.—' Fermented ' tea was made in the laboratory after ' withering ' a selected part of the green leaf samples at room temperature (21°) for 5 h. on a perforated tray through which air was passed continuously. The ' withered ' leaf was then minced, the macerate spread on a tray, and allowed to ' ferment ' at 25° for 2½ h. It was then kept at −20° until required.

Black tea.—Samples 1 and 2 (Table I) of black tea were manufactured in Ceylon from the remainder of two batches of green leaf mentioned above. Particulars regarding these and the 20 other black tea samples tested (numbered 3–22) are given in Table I.

Instant tea.—Two commercial brands of instant tea were examined, one produced in England and one in Ceylon.

Methods

Extracts.—For the qualitative and quantitative studies of *total phenols* and *total amino-acids* in unprocessed green leaf, bud, fermented leaf (leaf and bud together) and black tea, extracts were made first with boiling absolute methanol (3 × 25 ml./g.) and the residue was then re-extracted with boiling 80% (v/v) methanol (3 × 25 ml./g.). For the quantitative estimations

of *individual amino-acids*, black tea samples were extracted only with boiling 80% methanol (5 × 25 ml./g.). *Ammonia* estimations were made on the infusion obtained by adding 25 ml. of boiling water to 2·5 g. of black tea in capped boiling tubes and leaving the tubes in a boiling water bath for 10 min.

Polyphenols.—Identification of polyphenols was made by two-dimensional paper chromatography.[10] Quantitative estimations of total phenols were made with the Folin–Denis, vanillin and acidified butanol (leuco-anthocyanin) reagents,[11] and the sodium molybdate reagent.[12] Cacao leuco-anthocyanin was used as a standard for the acidified butanol reagent and epigallocatechin gallate for the other three. Theaflavins and thearubigins were estimated essentially as described by Roberts & Smith.[13]

Amino-acids.—Identification of the amino-acids was made by two-dimensional chromatography of the amino-acid fraction obtained by use of an ion-exchange column.[14] Quantitative estimations of total amino-acids were made according to Moore & Stein,[15] phenylalanine being used as standard. Determinations of leucine + isoleucine, valine, and theanine + γ-aminobutyric acid were made after separation by paper chromatography, by measuring the absorptivity of the individual spots after dipping in ninhydrin (0·2% in acetone) using the EEL Scanning Densitometer fitted with a green filter.[16]

Low-boiling volatile compounds.—*Ammonia* was estimated on 1-ml. aliquots of infusion by the microdiffusion method:[17] 1 ml. of 10% (w/v) sodium carbonate and 40% (w/v) sodium hydroxide respectively were used to liberate the ammonia (and amines), which was absorbed in boric acid containing indicator, and estimated by titration with 0·01N-hydrochloric acid. Other low-boiling volatile compounds obtained by head-space analysis from 0·5 g. samples of tea boiled in 3 ml. of freshly distilled water were separated by capillary column gas-chromatography[18] and identified by retention times, mixed chromatography and odour. From the top of the condenser 0·5-ml. samples of the head space vapour were collected into a stainless steel trap cooled in liquid oxygen. Three different capillary columns, viz., nylon (50 ft. long) coated with silicone oil, and two stainless steel (each 50 ft. long) coated with PEG 200 and di-2-cyanoethyl ether respectively), were used together with flame ionisation and micro-argon detectors. Quantitative estimations of some of the volatile compounds were made by the method described by Swain & Self.[19]

Table I

Particulars of samples of black tea

No.	Country of origin	Elevation*	Colour	Strength	Quality	Flavour	Valuation	Remarks†
1	Ceylon	4500	Fair	Fair	Bright	—	5/3	B.O.P.
2	,,	300	Useful	Useful	Bright	—	4/4	Big bulk
3	,,	3398	Coloury brown	Little	Plain	—	3/11	B.O.P.
4	,,	600	Poor	Poor	Plain	—	3/8	B.O.P.F.
5	,,	4400	Good	Good	Bright	Attractive	5/6	Flowery B.O.P.
6	,,	4300	Good	Good	Little brightness	—	4/8	B.O.P.F.
7	,,	2500	Some	Little	Little brightness	—	3/6	Big bulk
8	,,	1000	Fair	Fair	Plain	—	3/4	B.O.P.
9	Assam	—	Good, bright	Fair	Bright	—	4/6–4/9	End of season tea
10	Dooars	—	Quite good, dull	Little	Plain	—	3/6–3/7	—
11	Kenya	—	Quite good, dull	Quite good	Little	—	3/8–3/8½	—
12	Uganda	—	Quite good, brightish	Fairly strong	Plain	—	3/6	—
13	Tanganyika	—	Fair, dull	Very little	Plain	—	3/2	—
14	Nyasaland	—	Fair	Fair	Little	—	2/10–2/11	—
15	Portuguese E. Africa	—	Fair	Fair	Fair	—	2/6½–2/7½	—
16	Rhodesia	—	Quite good	Quite good	Bright	—	3/6	—
17	Mauritius	—	Fair, brightish	Fair	Little	—	3/7½	—
18	Argentine	—	Coloury, dull	Little	Plain	—	2/5	—
19	Vietnam	—	Not much	Not much	Bright	A little flavour	2/10½	—
20	S. India	—	Fair	Fair	Bright	Has flavour	4/0–4/3	—
21	Malaya	—	Quite fair, bright	Quite good	Bright	—	3/4–3/5	—
22	Kenya	—	Quite good, bright	Quite good	Bright	A little flavour	5/0–5/2	CTC manufacture, pungent

* Elevation in feet above mean sea level of estate in which tea was grown

† B.O.P. = Broken Orange Pekoe; B.O.P.F. = B.O.P. Fannings; Big Bulk = Grade containing large particles of leaf

CTC = Cutting, Tearing and Curling process

Results

Polyphenols

The main polyphenolic compounds described by Roberts[20] were identified in the green tea leaf and instant tea extracts—kaempferol 3-glucoside, isoquercitrin, myricetin-3-glucoside, rutin, epicatechin, epicatechin gallate, epi-gallocatechin, epi-gallocatechin gallate, chlorogenic acid, isochlorogenic acid, neochlorogenic acid, *p*-coumarylquinic acids and theogallin. On fermentation of the green tea leaf, the intensity of all the flavanol spots diminished and the streak corresponding to theaflavins and thearubigins appeared. It was also observed that the chlorophyll of green leaf underwent some change on fermentation. Chromatograms of black tea were qualitatively similar to those of fermented tea.

Table II shows the results of quantitative estimations of total polyphenols (measured by the Folin–Denis reagent) and of different phenolic nuclei (cf. King & White[21]), 5,7-dihydroxyflavans, measured by the vanillin method, *o*-dihydroxy groups by molybdate, and leuco-anthocyanins by the acid–butanol reagents,[11, 12] the theaflavin and thearubigin contents, and the E_{380}/E_{460} ratios in the different tea samples. The results show that although there is considerable variation in the amounts of polyphenols in the *absolute* methanol extracts as measured by any of the reagents used, the quantities are remarkably constant in the *aqueous* methanol extracts of the 15 black tea samples tested. It is also evident that the polyphenol content of the unprocessed bud and stalk is higher than that of the leaf in both samples, and that fermentation of the leaf and subsequent fining leads to an observed decrease of methanol-extractable polyphenols, presumably due to polymerisation and condensation, since the relative proportion extracted by aqueous methanol as compared to absolute methanol increases (cf. Goldstein & Swain[12]). In the two samples of instant tea, this relative proportion is considerably less, indicating that either extraction of the polymeric polyphenols is far from complete in the preparations themselves or that the absence of other leaf components (e.g., cellulose and proteins) makes for easier extraction of the polymers in absolute methanol. The amounts of *theaflavin* in the black tea samples show no direct correlation to the amounts of polyphenols extracted by absolute methanol although they almost all fall on a relatively smooth curve (Fig. 1). The fact that the more or less linear relationship between theaflavins and total phenols ceases when the tea contains more than 25 mg./g. of total phenols is perhaps due to the increased formation of other polymers. The valuations of the black tea samples are indicated by the figures in brackets (Fig. 1) and are seen to depend mainly on the theaflavin content—as has already been shown by Roberts & Smith.[13] Table II shows that in the instant tea samples the proportion of thearubigins to theaflavin is considerably greater than in the black teas and this is surprising since little thearubigin-like material was observed by paper-chromatography. The values of the E_{380}/E_{460} readings, which Roberts & Smith[13] introduced as a measurement of both colour and thearubigin content, do not appear to correlate with the actual amount of thearubigins or the commercial evaluation of the teas examined here.

Amino-acids and ammonia

The following amino-acids were detected in the absolute methanol extracts of unprocessed tea leaf and bud, fermented tea and black tea (samples 1 and 2, Table I) : α-alanine, β-alanine, γ-aminobutyric acid, asparagine, aspartic acid, cysteic acid, *N*-ethylasparagine, glutamic acid, glutamine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, theanine, threonine, tyrosine and valine. The intensity of the spots was observed to be less in fermented and black tea than in unprocessed tea, although the amino-acid pattern was the same. *N*-Ethylasparagine, which has not hitherto been detected in tea, was identified by its R_F values compared with those quoted by Gray & Fowden[22] and by the formation of aspartic acid only on elution and acid hydrolysis. The results of quantitative analysis of the total amino-acid content of unprocessed, fermented, black and instant tea are given in Table III. It will be seen that : (1) bud + stalk has a higher amino-acid content than green leaf ; (2) fermentation leads to an appreciable decrease in the total amino-acid content ; (3) black tea has an amino-acid content higher than that of fermented, but less than that of unprocessed, tea ; and (4) the instant teas have a low amino-acid content.

Table II

Total polyphenols, theaflavin and thearubigin contents of tea samples

(All values expressed as mg./g. dry weight of tea ; Folin–Denis, vanillin and molybdate values expressed in terms of epigallocatechin gallate ; leuco-anthocyanin values in terms of cacao leuco-anthocyanin)

	Absolute methanol extracts				80% methanol extracts						
	Folin-Denis	Vanillin	Molybdate	Leuco-anthocyanins	Folin–Denis*	Vanillin	Molybdate	Leuco-anthocyanins	Theaflavin	Thearubigins	E_{380}/E_{460}
Unprocessed											
1. Leaf	68	70		5	16 (24)	15		5			
Bud + stalk	79	84		30	27 (34)	26		17			
2. Leaf	130	104		3	13 (10)	11		4			
Bud + stalk	218	143		10	51 (23)	39		20			
Fermented											
1	22	15		13	9 (41)	07		9			
2	57	45		3	17 (30)	16		20			
Black											
1	33	31	42	10	30 (91)	28	28	13	11·2	19·5	5·2
2	53	36	66	10	31 (59)	26	31	12	7·9	11·1	5·45
3	20	21	24	7	38	30	26	12	9·9	13·6	6·55
4	12	12	23	6	38	28	29	11	5·6	14·5	4·7
5	26	29	31	10	35	27	27	13	12·4	14·8	6·05
6	38	26	29	10	33	27	29	13	9·0	13·3	4·5
7	23	17	23	10	32	26	29	13	4·7	8·2	3·9
8	15	11	16	5	35	27	32	12	5·6	13·3	4·5
9	23	10			37	28			9·0	14	4·9
10	14	7			39	28			8·5	11·1	4·3
11	34	23			35	28			10·1	15·8	5·3
12	37	22			35	28			9·0	15·8	4·6
20	24	22			38	29			12·4	13·4	6·2
21	9	5			35	30			5·9	12	4·8
22	26	29			35	28			11·3	15·8	3·5
Instant											
A	127	109	88	38	13 (10)	14	12	7	2·7	42·5	4·4
B	65	61	51	18	12 (18)	13	11	6	7·9	33·8	9·2

* Values in brackets are % of the amount in absolute methanol extracts

The results of quantitative analyses of leucine and isoleucine, valine, and threonine + γ-aminobutyric acid and of ammonia are also shown in Table III. The figures for ' bound ammonia ' include ethylamine which will be liberated from theanine and *N*-ethylasparagine by 40% sodium hydroxide. The variation in the amounts of amino-acids and ammonia may

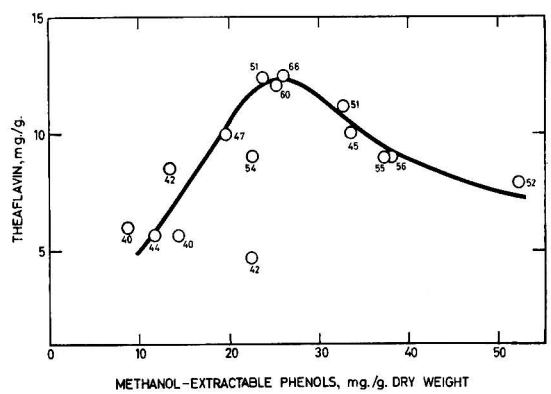

FIG. 1. - *Relation between theaflavin, methanol-extractable phenols and valuation of black tea samples*
Numbers refer to valuations pence/lb.

have some correlation with the flavour of tea as will be discussed later. The results of ammonia estimations on unprocessed green leaf were carried out on samples stored at −10° for about 5 months and are included only for purposes of comparison—it is evident that the amounts of both free and ' bound ' ammonia are lower in unprocessed than black tea.

Gas–liquid chromatography of low-boiling volatile compounds

Qualitative gas–liquid chromatography of unprocessed tea leaf showed the presence of hydrogen sulphide, methanethiol, dimethyl sulphide, acetaldehyde, propionaldehyde, isobutyraldehyde and very large amounts of methanol and ethanol. On fermentation, the amounts of methanol and ethanol decreased and additional peaks corresponding to the formation of 2- and 3-methylbutyraldehyde, n-valeraldehyde and biacetyl appeared. In black tea, the amounts of methanol and ethanol were very small, and further peaks for acrolein, acetone, n-butyraldehyde and methyl ethyl ketone appeared. The results of quantitative analyses of some of the volatile compounds in the black tea samples are shown in Table IV. The concentrations of the methylbutyraldehydes were high and showed wide variations in the different tea samples, generally being low in the ' flavoury ' teas and high in the teas without much flavour (Table I). There were also considerable variations in the absolute and relative amounts of the other compounds and this may contribute to the difference between the ' bouquets ' of tea samples. The instant tea samples showed very low amounts of all volatiles except dimethyl sulphide, acetaldehyde and 3-methylbutyraldehyde.

Discussion

The *total* amount of extractable polyphenols was highest in the unprocessed leaf, decreased on fermentation, and rose slightly on ' firing ' (Table II). The phenols reacting with vanillin,

Table III

Amino-acids and ammonia in tea samples

(All values expressed as μmoles/g. dry weight of tea ; total amino-acids expressed in terms of β-phenylalanine)

Sample*	Total amino-acids		Leucine + isoleucine	Valine	Threonine + γ-amino-butyric acid	Ammonia	
	Absolute methanol extract	Aqueous methanol extract				Free	' Bound '
Unprocessed							
1. Leaf	145	218					
Bud + stalk	382	170				1·4	2·7
2. Leaf	121	73				0·8	2·1
Bud + stalk	212	108					
Fermented							
1	73	32					
2	85	18					
Black							
1	200	79	5·5	7·85	20·4	2·8	17·0
2	106	55	5·4	5·65	15·3	2·9	19·2
3	115	67	4·95	5·7	15·6	2·2	13·6
4	121	67	4·2	3·66	19	2·9	11·2
5	127	55	3·2	4·85	14·5	2·0	9·5
6	79	49	4·05	3·74	13·6	2·0	
7	85	73	6·55	8·95	20·4	3·5	20·8
8	91	91	6·0	6·3	11·4	5·5	14·9
9						3·7	11·0
10			3·42	5·35	25·1	3·0	11·8
11						3·4	11·9
12						2·9	15·1
20	103	85	2·52	5·6	17·5	1·6	7
21						2·8	11·6
22	139	103	3·12	6·35	26·6	1·6	9·6
Instant tea							
A	40·3	7·6				4·55	19·8
B	51	7·6				5·05	17·3

* Numbers refer to sample numbers in Table I

on the other hand, decreased in the absolute methanol extract and increased in the aqueous methanol extract, and this reflects the effects of polymerisation.[12] A similar decrease in Lowenthal titre during the manufacture of tea was also observed by Roberts,[1] who suggested that this was due to the formation of insoluble products as well as the lower reactivity of theaflavins and thearubigins with permanganate. The changes in leuco-anthocyanins are not so marked, but they form a more substantial proportion of the total extractable phenols than other workers appear to have realised.[1] The observation that black tea has a significantly higher content of phenols extractable with aqueous methanol than does fermented tea may be due to lowered binding capacity of denatured protein and cell wall polysaccharides after the ' firing ' process. In this connexion it may be significant that the level of amino-acids follows the same pattern as that of polyphenols (Table III) suggesting that the proteins may be partly broken down.

The results on the black tea samples (Table II) show that, whereas there are appreciable differences in the amounts of polyphenols extractable by absolute methanol, the amounts extractable by aqueous methanol are remarkably constant. The levels of polyphenols in the absolute methanol extract show some relationship to the theaflavin content and taster's valuation (Fig. 1). Samples containing between 25 and 30 mg./g. (on dry weight of tea) of polyphenols extractable by absolute methanol show both the highest theaflavin content and commercial value. Since the amount of polyphenols in black tea depends on the amount originally present in the unprocessed leaf (cf. samples 1 and 2, Table II), it may be possible to predict the quality of a black tea from an estimation of the polyphenol content before processing.

The relative constancy of the polyphenol content of the aqueous methanol extracts may indicate that the enzymic polymerisation of the polyphenols in different teas results in the formation of a relatively fixed quantity of a similar or closely related group of compounds, which is sufficient to inhibit the enzyme systems responsible for their production. Inhibition of tyrosinase activity by the condensation products obtained by the autoxidation of (+)-catechin has been demonstrated in other experiments (Wickremasinghe & Swain, to be published ; cf.

Table IV

Low-boiling volatile compounds in tea

(All values expressed as μg./100 g. of tea)

Sample*	Dimethyl sulphide	Acetaldehyde	Propionaldehyde	Isobutyraldehyde	Acrolein	Methanol	Acetone	Ethanol	3-Methylbutyraldehyde	2-Methylbutyraldehyde	Biacetyl
Black tea											
1	7	23	0·4	15	0·2	2	0·4	15	15	18	2
2	6	28	8	19	7	10	2	38	15	18	13
3	7	21	10	18	3	2	2	1	92	99	7
4	10	27	12	26	0·2	3	1	1	20	25	12
5	8	25	5	20	3	15	1	16	65	75	3
7	8	27	9	26	3	2	0·6	Trace	120	141	10
8	7	25	7	28	0·2	3	2	2·5	140	159	3
9	7	28	16	27	13	Trace	Trace	Trace	43	51	6
10	7	22	14	18	3	3	Trace	Trace	52	60	6
11	6	19	13	18	0·1	3	1	16	27	33	6
12	7	26	14	21	0·2	3	1	23	108	129	6
13	7	28	11	26	5	178	2	Trace	22	25	6
14	6	21	26	16	5	76	4	Trace	54	63	10
15	7	25	18	25	0·2	19	2	Trace	88	105	10
16	5	16	7	13	0·2	1	0·8	Trace	20	24	1
17	5	26	8	19	0·1	3	2	Trace	17	20	1
18	5	18	11	10	0·2	2	1	Trace	14	17	1
19	5	17	13	15	0·1	25	2	2	32	38	22
20	5	17	10	15	5	2	Trace	Trace	30	36	17
21	9	24	5	24	3	25	0·8	Trace	25	30	6
22	7	23	11	15	0·1	2	1	Trace	16	19	10
Instant tea											
A	12	29	5	16	Trace	Trace	0·9	Trace	25	30	0
B	8	19	Trace	8	Trace	Trace	Trace	Trace	14	17	0

* Numbered as Table I

Byrde, Fielding & Williams[23]). This hypothesis would account for the observation that there is a linear relation between the level of polyphenols and theaflavin with the lower levels of phenols (see Fig. 1), since it can be assumed that the enzyme systems are not a limiting factor. Thus, a low initial substrate concentration would *per se* limit the theaflavin level, whilst a high concentration would favour the rapid formation of enzyme inhibiting compounds, which then interfere with the further formation of theaflavin.

Of the amino-acids, *N*-ethylasparagine (Gray & Fowden[22]), the aspartic acid analogue of theamine, is reported for the first time in tea. The total amino-acid content decreased during fermentation concomitant with the production of aldehydes, whilst appreciable amounts of ammonia were found in black tea.

Among the low-boiling volatile compounds, the presence of acrolein, n-valeraldehyde, methyl ethyl ketone, acetone and biacetyl is reported for the first time. The other compounds have been detected by Yamanishi *et al.*,[5] but no attempts have been made at comparing the amounts present in different tea samples. The amounts of the methylbutyraldehydes in the high-grown Ceylon teas were lower than those in low-grown Ceylon teas. Furthermore the leucine and isoleucine, free ammonia and methylbutyraldehydes were all relatively low in the flavoury teas (Nos. 5, 20 and 22) and provide some evidence that aldehydes in the bouquet of tea are derived by deamination of amino-acids.[8] However this correlation did not apply to the other tea samples tested, nor was there any quantitative agreement between the amounts of isobutyraldehyde and valine, although Roberts[1] has shown that the addition of valine to actively fermenting tea leaf leads to an increased production of isobutyraldehyde. The detection of n-butyraldehyde and n-valeraldehyde cannot be explained as being derived from amino-acids since norvaline and norleucine were not detected. Furthermore biacetyl, acetone and methyl ethyl ketone also probably arise from precursors other than amino-acids.

Acknowledgments

The authors are grateful to Messrs. Wm. Jas. & Hy. Thompson and Messrs. Kerr, Hope & Sons, both of London, for reporting on the tea samples and for supplying some of the commercial samples of black tea. They also express thanks to Messrs. R. Self and J. C. Casey for advice. Miss Isobel Jewson and Miss Kit Lutford carried out part of the analyses. One of them (R. L. W.) thanks the Director, Tea Research Institute of Ceylon, for permission to publish.

Low Temperature Research Station
Cambridge

Received 9 June, 1964

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Printed in Great Britain by Butler & Tanner Ltd, Frome and London