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CONTENTS

Physical State of Cellulose During Ripening of Peach

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(Manuscript received July, 1960)

SUMMARY

Cell walls of ripening fruits of peach were purified of a large part of their non-cellulosic components and examined with X-ray techniques. Small, but distinct, increases in micellar size, and perhaps in percentages of crystallinity, are a function of the maturation process. These increases are interpreted as evidence of a limited degradation of cellulose (broadly defined).

Softening of a fruit during ripening has been found generally related to alterations in the pectic components in the cell wall (Kertesz, 1951). In the peach, as in other fruits, these alterations involve de-esterification, depolymerization, and solubilization (Nightingale *et al.*, 1930; Reeve, 1959; Sterling and Kalb, 1959). It has also been shown that there is a cell wall lysis in fruits of certain Rosaceae, including peach (Addoms *et al.*, 1930), prune (Sterling, 1953), and pear (Sterling, 1954). In apple, too, the cell walls become markedly thinner during maturation (Carré and Horne, 1927). For the most part, the histological changes have been attributed to pectic breakdown (Branfoot, 1929). Presumably, a breakdown of the gel-like pectic matrix may leave cellulose microfibrils unsupported, with consequent collapse of the cell wall.

In addition to breakdown of the pectic substances, a definite decrease in the amount of cellulose and of hemicelluloses during peach ripening was found by Nightingale *et al.*, (1930). Jermyn and Isherwood (1956) also reported that the content of alpha-cellulose decreased, whereas the content of certain hemicelluloses could increase or decrease in maturing pears. Thus, it is possible that cellulose degradation, presumably hydrolytic, is involved in fruit maturation.

Generally, few cellulases have been observed in higher plants; potato sprouts (Singh *et al.*, 1938) and malting barley grains (Kristiansson, 1950; Ziese, 1931) seem to be the only organs of record in which a cellulase has been reported. No endogenous cellulases have been found in fleshy fruits. However, the lysis of vascular tissues and the occurrence of gummosis in fruits of the genus *Prunus* (Beijerinck, 1914; Reeve, 1959; Sterling, 1953) suggest that cellulose is being broken down in these fruits. The breakdown may be hydrolytic, through the agency of cellulase, or it may occur by means of some other mechanism. In either event, it is of interest to determine the manner in which cellulose might change during ripening.

As a fresh approach, a study of the physical state of cellulose seems desirable. If cellulose is being broken down as a consequence of ripening, degradation should first involve the amorphous molecular regions in preference to the crystalline micellar regions. Thus, differential attack by acid has been shown to result in a relative increase in percentage of crystallinity in the remaining cellulose (Foster and Wardrop, 1951; Hermans and Weidinger, 1949b). As in starch (Sterling and Pangborn, 1960), differential degradation might also be expected to lead to a relative increase in the average micellar diameter. However, if very vigorous degradation continued, both crystallinity and micellar diameter would eventually decrease.

MATERIALS AND METHODS

Five collections of fruit were made from a single Elberta peach tree during the time of ripening. In the first collection the fruit was hard and green, although full-sized. Subsequent collections were made at 4–5-day intervals until the fruit had turned yellow and become extremely soft. Preparation of the fruit and extraction of

the pectic substances are described elsewhere (Sterling and Kalb, 1959). Suffice it to note that the sliced peaches were macerated in alcohol and extracted successively with water, Calgon (sodium phosphate) solution, and hot 0.5% HCl.

After further washing in water, the pulp was boiled two hours in 4% NaOH solution, washed with hot distilled water, and then put in several successive changes of 100% ethanol. After the last change, the pulp was centrifuged, dried in air, and ground to a coarse powder (i.e., passing through a screen with 80 meshes per square inch). The preparatory treatment will generally remove pectic substances and many hemicelluloses from cell walls in higher plants (Mitchell, 1930; Roelofsen, 1959; Schulze and Godet, 1909). It is recognized that some resistant hemicelluloses will remain with the cellulose residues. Nevertheless, as regards the X-ray structure of the residual polysaccharides, the principal crystalline constituent is cellulose I.

Percentage of crystallinity was estimated by the method of Hermans and Weidinger (1949a). Copper radiation was monochromatized by "reflection" from the (002) plane of a large single crystal of pentaerythritol (Sterling, 1959). The beam of X-rays was then collimated by a brass cylinder 9 cm long, with pinholes 0.8 mm in diameter and 2 cm long at each end. Because collimation conditions differed from those used by Hermans and Weidinger (1949a), a crystallinity nomogram was re-calibrated with cotton cellulose and cellophane.

Size of the micelles was determined from line-broadening measurements, obtained with an X-ray powder camera of the Guinier type (Wolff, 1948 a, b). Instrumental line-broadening was ascertained with powdered As_2O_3 and Na_2SO_4. The photometer-film translation ratio was 4.65, and the relationship between optical density and pen travel was linear. The method of calculation has been described (Sterling, 1957). Results were checked with cotton cellulose, whose micellar diameter is reasonably well known (Sterling, 1957).

RESULTS AND DISCUSSION

Table 1 gives the data on changes in the micellar size of cellulose and the percentage of crystallinity (relative amount of cellulose organized into crystalline micelles). As is implicit in the description of the method of isolation, "cellulose" is taken here in the sense of Myers and Preston (1959): that fraction of the cell wall which is insoluble after treatment with hot dilute acid and hot dilute alkali (i.e., approximately alpha-cellulose). Thus defined, the cellulose micelles

Table 1. Half-breadth widths, micellar diameters, and per cent crystallinity for peach and cotton cellulose.

Sample	Collection date	Half-breadth width [a] (mm)	Micelle diameter (Å)	Crystallinity (%)
Na_2SO_4		2.0	—	
As_2O_3		2.5	—	
Peach (hard, green)	July 28	26.6	28	50
Peach (firm, yellow-green)	Aug. 2	24.8	31	50
Peach (firm, yellow)	6	24.4	31	52
Peach (soft, yellow)	11	23.6	32	51
Peach (very soft, yellow)	15	21.8	34	52
Cotton cellulose		12.6	61	70

[a] Width on film. (Effective camera radius, 114.6 mm; photometer translation, 4.65:1; θ, 11.43°.)

definitely enlarge in diameter in the course of ripening. Likewise, there seems to be a small increase in the crystalline fraction of the cellulose as a function of time. However, the amount of that increase lies within the limits of precision of the analytical method and may not be real.

The enlargement of micellar diameter cannot reasonably be ascribed to formation of new cellulose microfibrils with larger micelles. This enlargement can be explained by cellulosic degradation. Several possible mechanisms may be involved: 1) better organization of molecules (which are already in quasi-micellar configurations) because of freer movement following cutting of their outer ends; 2) reorientation of previously amorphous molecular segments, for the same reason; 3) removal of amorphous molecules and of smaller, more accessible micelles, leaving the larger crystallites in proportionally greater abundance.

It does not appear probable that changes in the other polysaccharide components of the cell wall would affect cellulose significantly. All available electron microscopic evidence (Roelofsen, 1959) shows that cellulose in higher plants is organized exclusively in the form of rather dense microfibrils, which are embedded in an unorganized matrix of other cell-wall components. The penetration of other macromolecules

into a cellulosic microfibril will be greatly restricted. Consequently, the removal of pectic substances and of hemicelluloses would have but a limited effect on the reorganization of amorphous zones within a cellulose microfibril. (For further discussion of this point, *cf.* Sterling, 1957.)

Thus, the X-ray evidence confirms microscopic and chemical evidence that ripening can involve a limited, though noticeable, degradation of cellulose (*sensu lato*). [However, note that the computations of Nightingale *et al.* (1930) were based on total "green matter" of the fruit. In another connection, Smith (1935) pointed out that there may be pitfalls in such computations. Jermyn and Isherwood (1956) followed the technique of Smith (1935) when finding that the quantity of cellulose decreased in pears upon ripening.] It is important to note that this limited degradation of cellulose cannot contribute greatly to the dramatic softening that is a function of fruit maturation.

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Rheology of Cocoa Butter. IV. Further Studies of "Omega" Crystallinity

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(Manuscript received September, 1960)

SUMMARY

An X-ray diffraction study was made of several plastic fats, including lard, butterfat, oleomargarine, and hydrogenated vegetable shortenings. Their liquid contents were varied by altering the holding temperature. Separate mixtures were made of cocoa butter or tristearin with corn oil, tributyrin, or trioctanoin, and their diffraction patterns were obtained. The crystal form (omega), previously described for oxidized cocoa butter, was found in the first group of fats and in the corn oil–tristearin mixture. Omega crystallinity was not related to the solid-liquid ratio of the fats or to differences in molecular length in the constituent fatty acid radicals. Omega crystallinity seemed to be related to a general difficulty in crystallization, probably due to steric hindrances.

In the third paper (Sterling, 1960) of this series, the author suggested that the crystalline structure of oxidized cocoa butter is sufficiently distinctive to be designated by the term "omega." He further postulated that the dimer organization of its oxidized fat molecules prevents the triple-chain configuration, which is characteristic of the β crystal of native cocoa butter. (Of course, other steric hindrances will also be the consequence of oxidation.) The omega crystal of cocoa butter has a double-chain length.

The presumptive ω crystal is not as well formed as the β crystal: there are far fewer spacings in the former. Moreover, the spacings are generally broader—an indication of rather small crystalline domains—and their intensity is weaker. Seemingly, omega crystallinity is a function of a much reduced crystallizability of the fat molecules, a large amorphous ("liquid") fraction being present. The gel-like structure and plastic properties of oxidized cocoa butter are further tokens of a significant amorphous fraction.

Whether ω crystals are real entities or simply mixtures of β, β', and α crystals with amorphous fat is not known. If the latter be the case, it might be expected that the liquid fat plays a disorganizing role in crystal structure, preventing the normally complete development of the β form. It seemed reasonable to test this assumption with X-ray diffraction studies of other native and synthetic fats and mixtures of these fats, with particular reference to their plastic properties and semisolid structure. This report describes the results of such studies.

MATERIALS AND METHODS

The following fats were used: tristearin, trioctanoin, tributyrin, cocoa butter, corn oil, butterfat (bovine), lard, oleomargarine fat, and two hydrogenated vegetable shortenings ("Crisco" and "Snowdrift"). In addition, the fats that are liquid at room temperature (trioctanoin, tributyrin, and corn oil) were used to dissolve tristearin and cocoa butter, respectively, with gentle warming. The mixtures were allowed to stand until recrystallization, if any, occurred at room temperature. In the proportion of 4:1, the six liquid fat–solid fat mixtures were calculated to present a liquid-solid ratio common to many plastic fats (Bailey, 1950; Hofgaard, 1938; Singleton and Bailey, 1945). The minimum time of standing before X-ray examination was five days.

While being held in a short, open capillary, the fats were exposed to an X-ray beam. In some cases, a series of different holding temperatures, from $-15°$ to $+50°$C, was imposed on the specimens to vary the relative amounts of liquids and solid portions (Bailey, 1950; Singleton and Bailey,

1945). These temperatures were maintained for three hours before an additional three-hour X-ray exposure. Copper radiation was filtered through nickel foil (or monochromatized via crystal reflection) and collimated with a brass capillary. Diffraction patterns were recorded on film, the specimen-to-film distance being uniformly 4.0 cm.

RESULTS

The results are presented in the X-ray diffraction patterns of Figs. 1–24. A most striking finding is that there are nearly identical (outer) short spacings (*ca.* 3.78, 4.16 Å) and (inner) long spacings (*ca.* 14, 22, and 44 Å) for butterfat, oleomargarine fat, and the two vegetable shortenings (Figs. 1–4). Although these spacings are about 2% smaller than those for mildly oxidized cocoa butter (held 90 days at 60°C), the appearance of the X-ray diagrams is closely similar (*cf.* Fig. 5, Sterling, 1960). Thus, these fats all show the omega pattern.

The pattern for lard (Fig. 7) is like these others but has an additional short spacing of about 4.6 Å (Chapman *et al.*, 1957). Note that more severely oxidized cocoa butter (held 270 days at 60°C) also has this added short spacing. In the lard diagram, a long spacing of 33 Å, rather than 44 Å, is present, indicating a triple-chain length organization in that fat.

These crystalline patterns are further characterized by a broad, rather intense "halo," which extends from about 4.1 to 4.7 Å. Therefore, the 4.16 Å ring is not completely distinct at 20°C nor is the 4.6 Å ring, when it occurs, clearly set off from the background halo. The halo is affected by temperature. When the temperature is lowered to about 0° C the halo tends to become somewhat less intense (Fig. 15). At a temperature of about −15°C, the halo virtually disappears (Fig. 24).

The X-ray pattern (β) of native cocoa butter at 25°C is presented in Fig. 9. Closer to the β melting point of 33–34°C (Vaeck, 1951), the weaker short spacings gradually begin to fade away. Fig. 10 shows the cocoa butter pattern at 30°C, and Fig. 11 the pattern at 33°C. [At 33°C, the solids content of cocoa butter is about 26% (Hofgaard, 1938)]. Note that these patterns are not omega and that the strong spacing here is 4.6 rather than 4.16 Å. (The sharp, intense 4.6 Å spacing is uniquely characteristic of the β form of a fat.) At 35°C (Fig. 12), cocoa butter is completely molten. Its X-ray pattern has become abruptly that of liquid hydrocarbon materials: a broad, intense ring at about 4.6 Å and a broad, intense inner ring whose spacing cannot easily be determined but is a function of the chain length (Morrow, 1928; Warren, 1933).

When the vegetable fat shortenings are gradually warmed, little significant change occurs in the X-ray patterns short of the melting point (50°C). Up to 45°C (Fig. 5), there is only a gradual decrease in intensity of some of the spacings. However, the 4.1–4.7 Å halo remains unchanged. At 50°C, a liquid pattern suddenly appears, characterized by a shift in the midpoint of the halo from about 4.4 Å to 4.6 Å (Fig. 6). Similar loss of intensity accompanies the warming of lard: at 25°C, there already occurs also a shift in intensity, so that the 4.6 Å ring is more intense than the 4.16 Å ring (*cf.* Fig. 7 and 8). At 50°C, lard shows the typical liquid pattern.

Liquid configurations are also present, at room temperature, for trioctanoin (Fig. 13) and tributyrin (Fig. 14). While both these synthetic fats have an outer spacing of roughly 4.5–4.6 Å, the inner rings differ. The inner ring of tributyrin represents a spacing of about 10.7 Å, and that of trioctanoin a spacing of about 16.7 Å. Note that these inner spacings reflect the differences in chain length of the fatty acid moieties of these triglycerides. A liquid pattern is characteristic of the X-ray diagram of corn oil at room temperature.

When the liquid fats are mixed with the solid fats in 4:1 proportion, subsequent crystallization is related to the composition of the mixture. The tristearin mixtures are all plastic fats within a short time of cooling to room temperature. Under the polarizing microscope, no regions of free liquid can be seen. Although tristearin is initially crystalline in the β form (Fig. 16), the crystalline patterns of its mixtures vary with the second component. The pattern of corn

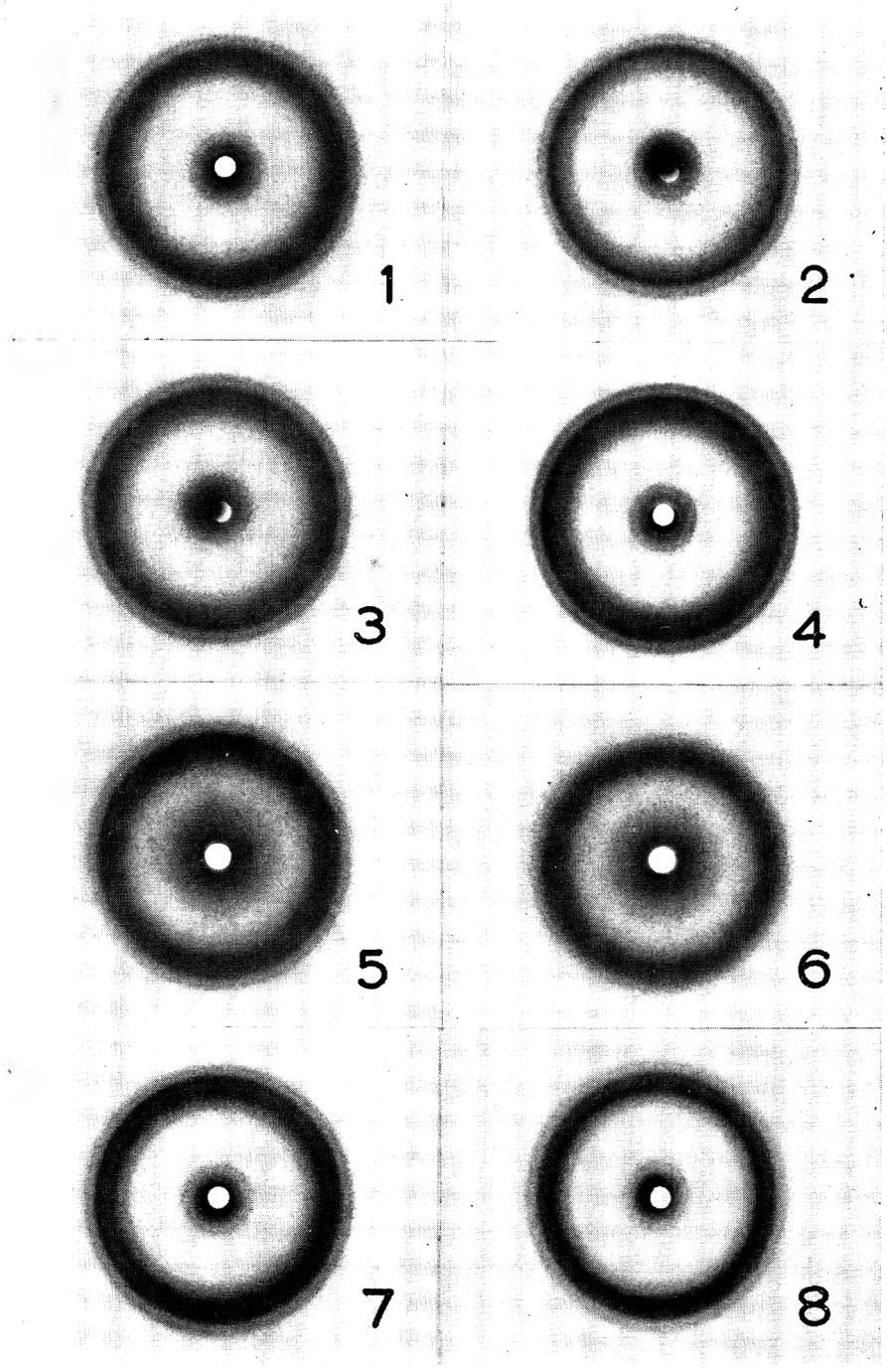

FIG. 1–8. All figures made with Cu-Kα radiation at specimen-film distance of 4.0 cm. 1) Butterfat (20°C). 2) Oleomargarine fat (20°C). 3) Crisco (20°C). 4) Snowdrift (20°C). 5) Snowdrift (45°C). 6) Snowdrift (50°C). 7) Lard (20°C). 8) Lard (25°C).

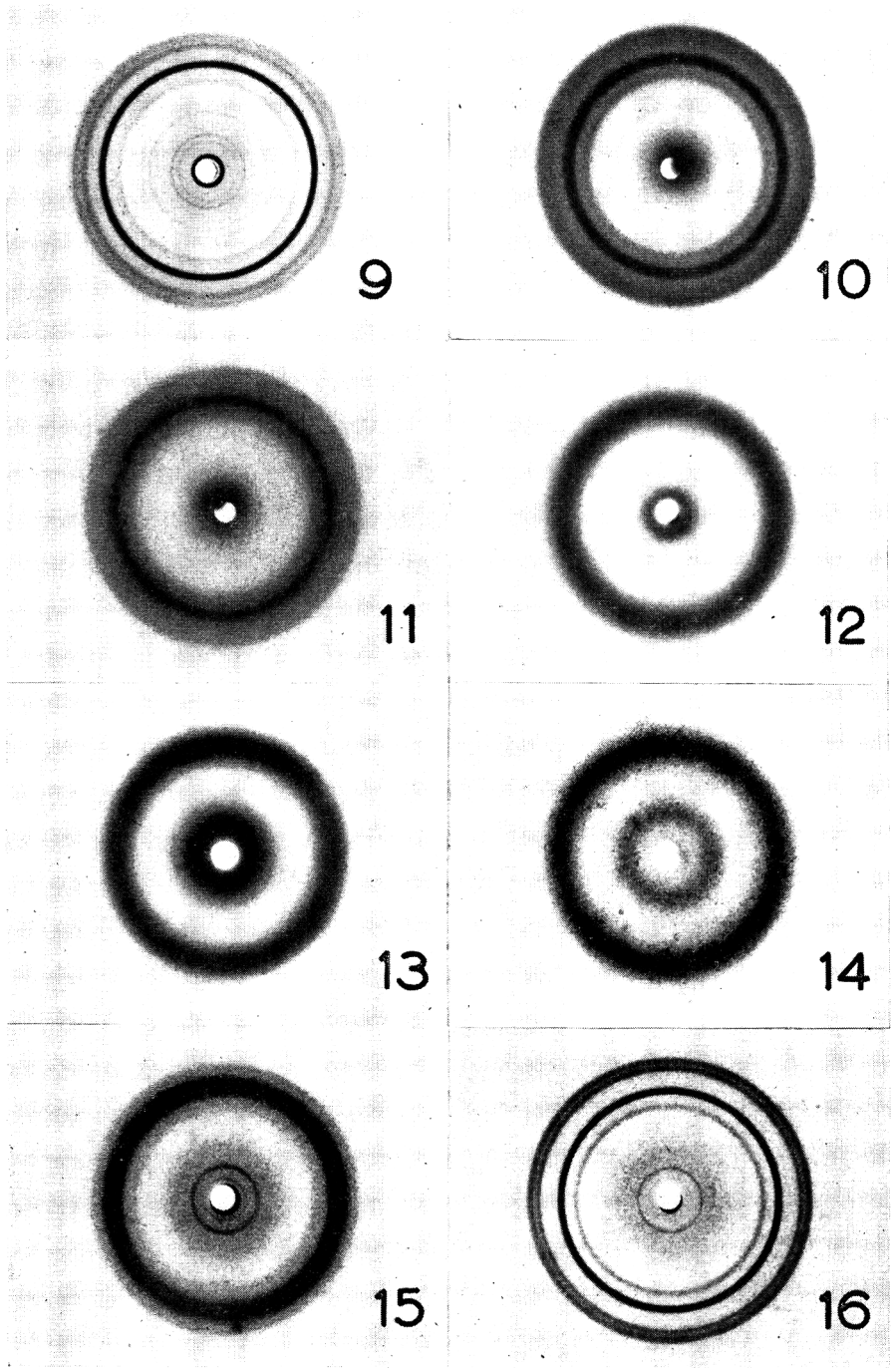

FIG. 9–16. All figures made with Cu-Kα radiation at specimen-film distance of 4.0 cm. 9) Cocoa butter (25°C). 10) Cocoa butter (30°C). 11) Cocoa butter (33°C). 12) Cocoa butter (35°C). 13) Trioctanoin (23°C). 14) Tributyrin (23°C). 15) Lard (0°C). 16) Tristearin (23°C).

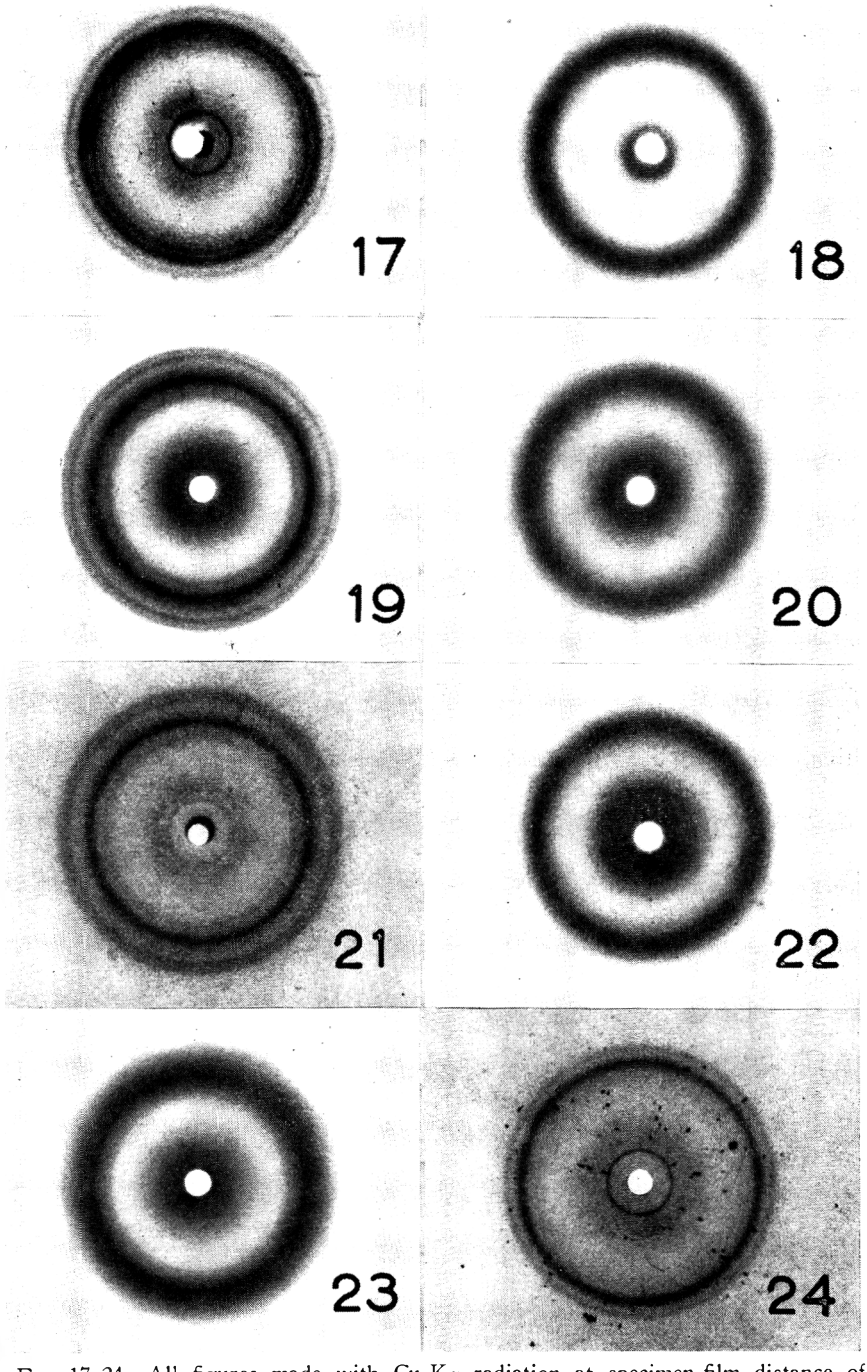

FIG. 17–24. All figures made with Cu-Kα radiation at specimen-film distance of 4.0 cm. 17) Corn oil–tristearin (23°C). 18) Corn oil–cocoa butter (20°C). 19) Trioctanoin–tristearin (23°C). 20) Trioctanoin–cocoa butter (23°C). 21) Tributyrin–tristearin (23°C). 22) Tributyrin–cocoa butter (23°C). 23) Corn oil–cocoa butter (5°C). 24) Oleomargarine (–15°C). Extra diffraction spots due to ice crystals.

oil–tristearin (Fig. 17) is almost indistinguishable from the near-omega pattern of lard. The strong 4.6 Å spacing of lard is to be compared with the strong 4.54 Å spacing of the mixture; and the 33 Å spacing of lard is to be compared with the 44 Å spacing of the mixture. On the other hand, the mixtures of trioctanoin with tristearin (Fig. 19) and of tributyrin with tristearin (Fig. 21) give distinctive β patterns despite their very great liquid composition and plastic properties.

The mixture of cocoa butter with the liquid fats remained non-crystalline for several weeks (Figs. 18, 20, 22). When crystallization did occur (at room temperature), it involved discrete particles in the solid phase, which separated from a liquid phase over a long period (particularly corn oil–cocoa butter and tributyrin–cocoa butter). If the temperature of corn oil–cocoa butter is lowered to 5°C, the whole mixture becomes crystalline (as in a plastic fat), and its X-ray pattern (Fig. 23) is somewhat intermediate between the lard pattern at 25°C (Fig. 8) and that of cocoa butter at 33°C (Fig. 11). At room temperature, the crystallized masses in tributyrin–cocoa butter are rather firm; their X-ray pattern is β.

DISCUSSION

Although omega crystallinity is definitely associated with plastic fats, it is not invariably related to their high content of liquid. The liquid lipoidal pattern, with two broad, intense rings, is not clearly represented in the omega pattern. Although liquid fats may have local "cybotactic" (Morrow, 1928; Stewart and Morrow, 1927; Warren, 1933) regions, the degree of order reflected in these regions is lower than that of omega. The omega pattern is not shown by any fats in the fluid state, nor is it developed when the liquid content of a fat is increased by warming. Thus, in cocoa butter, the original β pattern simply becomes less intense. In warmed vegetable-fat shortening, the omega pattern becomes less, rather than more, intense.

Despite their high liquid content and plastic properties, the 4 : 1 combinations of tributyrin or trioctanoin with tristearin give a very definite β pattern. The combination of corn oil with tristearin, however, has a lard-like, near-omega pattern. In all these cases, there is no free liquid that is microscopically visible.

It is interesting to note that the fats with omega patterns are characterized by a mixed composition of fatty acids. These fats contain both saturated and unsaturated fatty acids, probably in rather random distribution among the 1, 2, and 3 positions of the glycerol molecule. Thus, the principal fatty acids of butterfat are myristic, palmitic, stearic, and oleic; of vegetable oils, palmitic, oleic, linoleic, and linolenic; of lard, palmitic, stearic, oleic, and linoleic—all in varying proportions (Bailey, 1951) and, probably, positions.

It may be expected, then, that there will be difficulty in precise molecule-molecule association, particularly in view of the steric barrier between straight-chain saturated fatty acids and kinked-chain unsaturated fatty acids. Oleic acid radicals will have one kink; linoleic acid radicals will have two kinks, which will serve as even more pronounced steric hindrances. Moreover, if isomers of these unsaturated acids are present, further difficulties in association will exist.

Thus, in order to crystallize in the β form, two fat molecules presumably must have the same distribution of saturated and unsaturated acid radicals in the 1, 2, and 3 positions of the glycerol molecule, and the unsaturated acid radicals must be identical. The question of chain length seems not to be important (*cf.* the β-form combinations of tributyrin or trioctanoin with tristearin). In the case of cocoa butter, the oleic acid radical is in the 2 position in the major fats (POS and SOS). Oleic acid radicals can associate with each other, and the saturated acid moieties can likewise associate readily. For this reason, cocoa butter has a relatively sharp melting point, shows the characteristics of a brittle solid to within a few degrees of the melting point (Bailey, 1951), and can give a β X-ray pattern.

The omega form will then represent the limited, perhaps only two-dimensional, crystallizability of mixed triglycerides. Some fraction of β-form crystals will possibly be present if sufficient like molecules occur in

the native fat. Perhaps this fractional β-crystallization explains the 4.6 Å line that appears in lard, in the corn oil–tristearin mixture, and in the corn oil–cocoa butter mixture held at 5°C. The separation of crystalline material from a mother liquor in the cocoa butter mixtures is presumably due to the preferential union of the POS and SOS molecules of cocoa butter with each other at room temperature.

The 4.1–4.7 Å halo of the omega crystal would then represent a varying average interplanar distance. Most of the molecules will be about 4.16 Å apart, as in typical β' and α crystals. Probably that spacing in the omega form reflects a poorer crystallizability, due to pairing of unlike chains: a saturated adjoining an unsaturated moiety, or two different kinds of unsaturated moieties lying adjacent to each other. The near-liquid properties indicate that these chains are not held very rigidly in ω but are in active motion, perhaps with bonding at relatively few points. Lowering the temperature of the ω form leads to a virtual β' pattern as the 4.1–4.7 Å halo disappears. Thus, ω crystallinity has a lower degree of order than β', omega fats owing their characteristic plastic properties to a partial crystallization of triglyceride chains (*cf.* also Sterling, 1960.)

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The Xanthophylls of Tomatoes

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SUMMARY

The carotenoids of ripe tomatoes were found to contain about 6% xanthophylls; the composition of the latter was about 15% monols, 49% diols, 4% monoepoxide diols, 22% diepoxide diols, and 11% polyols. The diol and polyol xanthophylls were much like those of green leaves, with lutein the major pigment, somewhat smaller amounts of violaxanthin and neoxanthin, and much smaller amounts of zeaxanthin, lutein 5,6-epoxide, and several others.

The monol fraction contained lycoxanthin and "monol 487" (which may be 3-hydroxy-delta-carotene), together with substances tentatively identified as poly*cis* isomers of lycoxanthin, rubixanthin, and "monol 487."

The carotenoids of the tomato are predominantly hydrocarbons (Kuhn and Grundmann, 1932), of which the major component is lycopene. The composition of the hydrocarbon fraction has been investigated thoroughly, in particular by workers at Purdue University. Trombly and Porter (1953) listed 19 components of this fraction, several of them of incompletely known structure. The xanthophylls, which amount to only a few per cent of the total carotenoids, are much less well known. Kuhn and Grundmann (1932) reported that this fraction was mainly lutein and zeaxanthin, and noted the absence of violaxanthin. Zechmeister and Cholnoky (1936) reported the presence of lycoxanthin in small amounts, and of lycophyll is very small amounts.

The present work concerns itself with a careful examination of the xanthophyll fraction of tomatoes. The xanthophylls were separated from the hydrocarbons by countercurrent distribution (Curl, 1953), and fractionated by the same means into monols, diols, monoepoxide diols, diepoxide diols, and polyols. Chromatographic examination of the various fractions showed the presence of a considerable number of carotenoids. Except for the monol fraction, the xanthophylls found were similar to those found in leaves (Strain, 1938; Curl and Bailey, 1957b); lutein was by far the most abundant xanthophyll, with lesser amounts of violaxanthin and neoaxanthin, and much smaller amounts of zeaxanthin, lutein 5,6-epoxide, luteoxanthins, and several others. The monol fraction consisted mainly of lycoxanthin and another component (monol 487), which may be 3-hydroxy-delta-carotene, together with substances tentatively identified as poly*cis* isomers of lycoxanthin, rubixanthin, and "monol 487."

EXPERIMENTAL

The two batches of 2000 g and 2250 g of tomatoes used were obtained in a local market in mid-June and mid-October, respectively. The fruit selected was fully ripe, with no green apparent on the outside.

The tomatoes were blended in 250-g batches with 250 ml of water and 3 g of magnesium carbonate. Filter aid (Celite 545, 6½% by weight of the fruit used) was added, and the mixture filtered on Buchner funnels precoated with filter aid. The cake, which contained the carotenoids, was blended briefly with acetone and filtered on a sintered-glass funnel, and the filter cake was washed with acetone until the washings were colorless. The combined acetone filtrates were evaporated until the acetone was nearly all removed. The residue was transferred to a separatory funnel with water and ether, about 2 g of sodium chloride and 50 ml of methanol were added, and the aqueous solution was extracted with ether (U.S.P.) until the extracts were practically colorless. In general, addition of methanol to about 10% of the volume of the aqueous solution results in considerably more rapid and clean separations. The ether extract was treated in detail and saponified as previously described (Curl and Bailey, 1959). All experiments were carried out with saponified carotenoids. The carotenoid fraction was recovered and dissolved in benzene. It was then filtered through

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a pad of filter aid on a small sintered-glass funnel to remove some colorless precipitate.

Countercurrent distribution in a Craig apparatus with system I (petroleum ether–99% methanol, 1.8 to 1 by volume) was used (Curl, 1953) to separate the carotenoids into 4 fractions (Fig. 1). The combined diol and polyol fraction was then further fractionated by countercurrent distribution. Two solvent systems were used for this purpose. With system IV (petroleum ether–73½% methanol, 1 to 1 by volume) the diols and polyols are cleanly resolved into diols, monoepoxide diols, diepoxide diols, and polyols in 100 transfers (Curl, 1960 c). With the previously used system II (petroleum ether–benzene–87% methanol, 1 to 1 to 1.15 by volume) (Curl, 1953) the separation in 200 transfers is not as good as with system IV in 100 transfers, but considerably larger quantities of material can be used and no preliminary separation of the hydrocarbons and monols is necessary. The N_{100} value [tube number of the maximum per 100 transfers (Curl, 1953)] of several polyol constituents was determined in solvent system III (petroleum ether–acetone–methanol–water, 1.25 to 1 to 0.1 to 0.65 by volume) (Curl, 1960 c).

The various carotenoid fractions were then recovered and chromatographed on columns of magnesia (Sea Sorb 43), 14 by ca 80 mm. Sea Sorb 43, unlike magnesia 2642, can be conveniently used without a diluent. The eluants used for the various fractions were the same as with magnesia 2642 (Curl, 1959), but the bands on the column were considerably sharper.

Spectral absorption curves on the various fractions separated by chromatography were obtained by Beckman DK 2 or Cary model 14 recording spectrophotometers.

RESULTS AND DISCUSSION

The total carotenoids, measured in an Evelyn photoelectric colorimeter at 440 mμ and calculated as β-carotene by means of a conversion table, were 51 and 63 mg per kg for the June and October fruit, respectively. The spectral absorption maxima of lycopene are at considerably higher wavelengths than those of β-carotene; the extinction (1%, 1 cm) of lycopene is much greater than that of β-carotene, so that color measurements with lycopene solutions in an Evelyn colorimeter, using the 440 filter and the conversion table for β-carotene, yield values that are about 11% too low. Hence the values given for total carotenoids above may be a few mg too low.

Countercurrent distribution. Distribution of the carotenoids from 500 g of tomatoes (June picking) with system I gave good separation of the carotenoid diol and polyol fraction, but the hydrocarbon and monol fractions were not separated. When the aliquot of the sample solution (in benzene) was evaporated and the residue treated as usual with 50 ml of the upper layer of system I, considerable insoluble material (ap-

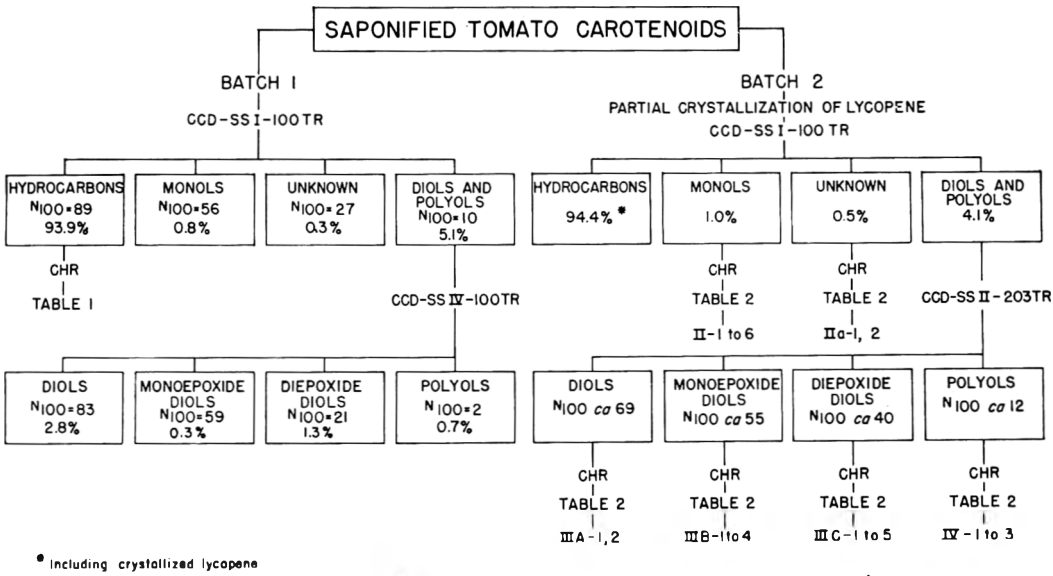

Fig. 1. Fractionation of saponified tomato carotenoids by countercurrent distribution.

parently lycopene) remained, mostly floating. This dissolved slowly during distribution, so that a minor part of the lycopene moved more slowly than that originally in solution.

On repeating this separation using the carotenoids from 43 g of tomatoes, separation was as shown in Fig. 1 (Batch 1). The diol-polyol fraction was then further fractionated by countercurrent distribution with system IV. The percentage of the various carotenoid fractions in the total carotenoids is given in Fig. 1.

Thus, because of the great preponderance of lycopene and its low solubility in either petroleum ether or methanol, only relatively small amounts of the saponified tomato carotenoids could be used directly in a countercurrent distribution run with system I. Under these conditions the amounts of the various xanthophyll fractions obtained were much smaller than the optimum amount for chromatography.

Partial crystallization of lycopene. In the following experiments the carotenoid mixture from the October tomatoes was used.

The benzene solution of the saponified carotenoid fraction was divided into 2 equal parts, and evaporated in vacuo, and the residues were dissolved in several small aliquots of benzene (total about 11–12 ml) and transferred to 1-oz screw-cap bottles. The bottles were filled by carefully layering methanol and petroleum ether, respectively, on top of the benzene solution. In both cases, on standing in the refrigerator, a considerable amount of deep-red crystalline material separated. The solutions were filtered on sintered-glass funnels precoated with filter aid, and the filter cake was washed a few times with petroleum ether and methanol, respectively. Countercurrent distribution runs with the carotenoid mixtures in the filtrates were then carried out with system I. The results are summarized in Fig. 1 (Batch 2).

In both cases, well over half of the total carotenoids were separated out, respectively 73 and 59%, with benzene-methanol and benzene-petroleum ether. The crystals were rather pure lycopene, as judged by the melting point (171–172°, unc. in vacuo) and the spectral absorption curve. The percentages of the xanthophyll fractions agreed closely in amount in the two experiments, and are in good agreement with results obtained on the other batch of fruit (Fig. 1). The benzene-methanol procedure is preferable since more lycopene was removed.

The two monol fractions (II) were combined for chromatography. The fractions III (diols and polyols) were combined and further fractionated by a 203-transfer countercurrent distribution run with solvent system II (Fig. 1).

Chromatography of fraction I. The hydrocarbon fraction obtained from the first batch of fruit was chromatographed. The fractions obtained are listed in Table 1 in order of elution, beginning with phytoene. All of the fractions obtained were among the tomato carotenes listed by Trombly and Porter (1953). Those workers used a much larger quantity of tomatoes, including some specially selected for their content of delta-carotene, zeta-carotene, etc. The great preponderance of lycopene in the hydrocarbon fraction is shown in Table 1.

A countercurrent distribution run with system I was made with lycopene; the N_{100} value obtained was 89, whereas the value for β-carotene from cling peaches (Curl, 1959) was 91.

Chromatography of fraction II. The N_{100} value in system I of the monol fraction was 56, the same as for cryptoxanthin (from cling peaches), indicating that this fraction

Table 1. Hydrocarbon carotenoids and polyenes obtained from tomatoes (in order of elution).

Constituent	Spectral absorption maxima in petroleum ether (mμ)	Percentage of hydrocarbon fraction [a]
Phytoene	(297), 286, (275)	5.6
Phytofluene	366 , 348, 331	3.0
α-carotene	472 , 443, 319	0.03
β-carotene	477 , 450, (427)	3.9
Phytofluene-like	367 , 347, 331	0.29
zeta-carotene	425 , 400, 378	1.0
γ-carotene	490 , 461, 432	1.3
Neurosporene	468 , 438, 415	0.9
Lycopene	519 , 485, 457 [b]	83.8

[a] Based on total absorbance of each constituent at the principal spectral absorption maximum.
[b] In benzene.

consisted mainly or entirely of 3-hydroxy carotenes. Chromatography showed the presence of at least 9 bands, several of which appeared to be mixtures but were present in too small quantity to rechromatograph; six of these bands are listed in Table 2. No cryptoxanthin or hydroxy-α-carotene was found. The strongest band had spectral absorption maxima in petroleum ether at 487, 455 and 428 mμ, in good agreement with those of delta-carotene (Porter and Murphey, 1951); the shape of the spectral absorption curve was also similar, and included low cis-peaks at 346 and 333 mμ, which are several mμ lower in wavelength than those of γ-carotene (Zechmeister *et al.*, 1943). The spectral absorption curve indicates the presence of 10 conjugated double bonds, none of which are in a β-ionone ring. On treatment with hydrochloric acid in methanol-ether (1 plus 4.5 plus 4.5, respectively), the principal maximum was at 457 mμ, only a slight change, showing that this substance was not a 5,6-epoxide. [This test is a modification of one previously described (Curl and Bailey, 1954) in which 5,6-epoxides were treated with 1 ml of hydrochloric acid in 9 ml of methanol. The ether was added to increase the solubility of the monol carotenoid.] It may be 3-hydroxy-delta-carotene [delta-carotene has recently (Kargl and Quackenbush, 1960) been shown to have an α-ionone ring on one end of the molecule and an open chain on the other.] It is referred to here as "monol 487."

Above this band on the column was another with spectral absorption maxima at 453 and 431 mμ in petroleum ether; the shape of the spectral absorption curve resembled that of pro-γ carotene except that the maxima were several mμ shorter in wavelength. The two *cis*-peaks were similar to those of "monol 487." This band was tentatively identified as a poly*cis* monol 487.

The next hand above on the column had spectral absorption maxima at 458 and 437,

Table 2. Xanthophylls obtained from tomatoes.

	Identity	Spectral absorption maxima [a] (mμ)	Percentage of total xanthophylls [b]	
II-1	Monol 487	487, 455, 428	6.8	9.8
II-2	Polycis monol 487 [c]	453, 431	3.0	
II-3	Polycis rubixanthin [c]	(490), 458, 437	4.4	
II-4	Polycis lycoxanthin a [c]	(497), 468, (447)	2.1	4.7
II-5	Polycis lycoxanthin b [c]	(496), 469, (448)	1.6	
II-6	Lycoxanthin	504, 473, 445	1.0	
IIa-1	398 substance	398, 376, 357	0.8	
IIa-2	378 substance	378, 357, 344	2.0	
IIIA-1	Lutein	487, 455, 431	33.5	
IIIA-2	Zeaxanthin	490, 461, (435)	1.3	
IIIB-1	Lutein 5,6-epoxide	483, 452, 425	2.0	
IIIB-2	cis-antheraxanthin [c]	481, 453, 429	1.7	
IIIB-3	Flavoxanthin	459, 430, 406	0.8	
IIIB-4	cis-mutatoxanthin [c]	461, 434, 411	0.9	
IIIC-1	Violaxanthin	484, 453, 426	12.5	
IIIC-2	Luteoxanthin a	461, 432, 408	0.6	2.1
IIIC-3	Luteoxanthin b	460, 431, 407	1.0	
IIIC-4	cis-luteoxanthin a	456, 427, 405	0.2	
IIIC-5	cis-luteoxanthin b	456, 427, 404	0.3	
IV-1	Neoxanthin	479, 447, 423	11.7	
IV-2	Trolliflor [c]	482, 449, 423	0.4	
IV-3	Neochromes [c]	457, 431, 408	0.9	

[a] In petroleum ether for II and IIa; benzene for remainder.

[b] Based on total absorbance of each constituent at the principal spectral absorption maximum.

[c] Tentative identification.

with an inflection at ca 490 mμ; the spectral absorption curve resembled fairly closely that of pro-γ carotene, a naturally occurring polycis isomer. Zechmeister and Schroeder (Zechmeister and Schroeder, 1942) obtained a substance from *Pyracantha angustifolia* fruit that appeared to be a pro-rubixanthin (3-hydroxy-γ-carotene), which may be the same substance as that obtained from tomatoes.

The last band eluted had spectral absorption maxima and a spectral absorption curve quite like those of lycopene. On countercurrent distribution of this substance in system I, the N_{100} value was found to be 54 (cryptoxanthin was 56). The difference in the N_{100} values of these two substances was the same as between lycopene and β-carotene. Hence, this band was identified as lycoxanthin (3-hydroxylycopene), previously found in tomatoes by Zechmeister and Cholnoky (1936). The above data show that the N_{100} values are only slightly changed on changing from two β-ionone rings to open chains, as in β-carotene and lycopene.

Just below lycoxanthin on the column were two distinct violet bands with almost identical spectral absorption curves, with maxima at ca 469, inflections at ca 497 and 447, and cis-peaks at ca 362 and 347 mμ (the last are quite close to those of lycoxanthin). These bands appeared to be poly-*cis* isomers of lycoxanthin.

The monol fraction thus consists mainly of several poly*cis* carotenoids, accompanied by at least two of the corresponding all-trans isomers. Poly*cis* isomers are rather rare naturally occurring substances; two were found in the hydrocarbon fraction of tomatoes by Trombly and Porter (1953).

Chromatography of fraction IIa. When a carotenoid mixture is subjected to countercurrent distribution with system I, with 100 transfers there is often a fairly wide gap between the monol and the combined diol-polyol fractions. Sometimes a maximum occurs in this gap that is attributable to monol epoxides. With tomato carotenoids there was a weak maximum in this area with N_{100} value of 27. Hence, the intermediate tubes (nos. 23–39) were combined and the product chromatographed. Small amounts of lutein and "monol 487" from the adjoining fractions were present, but no epoxides were found. Several of the fractions had pronounced maxima in the ultraviolet region, which indicated the presence of unusual carotenoids (or polyenes). Two of these with respective spectral absorption maxima at 398, 376, and 357 mμ, and 378, 357, and 344 mμ, are referred to here as the "398 substance" and "378 substance" (Table 2). The 398 substance, on treatment with hydrochloric acid in ether-methanol, showed very little change in the wavelength of the spectral absorption maxima, indicating that it was not a 5,6-epoxide. Under similar treatment the 378 substance was converted to a substance with spectral absorption maxima at 402, 378, and 358 mμ (in hydrochloric acid–ether–methanol). The 378 substance also was not a 5,6-epoxide. The wavelength shift indicates the addition of another double bond to the conjugated system, instead of the loss that occurs when a 5,6-epoxide is treated with acid. The additional double bond is probably due to the presence of a hydroxyl group allylic to the conjugated double-bond system, which loses a molecule of water under the hydrochloric acid treatment. A similar substance was found in the carotenoids from Meyer lemon peel (unpublished work). The 398 and 378 substances apparently contain 6 and 5 conjugated double bonds, respectively.

Chromatography of fraction IIIA. The diol fraction, which amounted to about half of the total xanthophylls, was found to consist of lutein, together with a much smaller amount of zeaxanthin (about 25:1). This situation is similar to that in leaves (Strain, 1938), whereas in other fruits examined by the author, such as oranges (Curl, 1953), tangerines (Curl and Bailey, 1957a), cling peaches (Curl, 1959), and Japanese persimmons (Curl, 1960b), zeaxanthin was present in greater quantity that lutein. However, the lutein-zeaxanthin ratio was 3:1 in apricots (Curl, 1960). Lycophyll, which was previously found in very small amounts in tomatoes (Zechmeister and Cholnoky, 1936), would be expected to occur in this fraction, but it was not found in the present work.

Chromatography of fraction IIIB. The largest band obtained from the monoepoxide diol fractions was identified as lutein 5,6-

epoxide. Its spectral absorption curve was very much like that of violaxanthin. In the hydrochloric acid–ether test (Curl and Bailey, 1954) this fraction gave a light-blue color characteristic of a monoepoxide (Curl, 1959). The next band, which gave a similar test, was tentatively identified as a cis-antheraxanthin. Other very minor bands were apparently the corresponding 5,8-epoxides, flavoxanthin and a *cis*-mutatoxanthin.

Chromatography of fraction IIIC. The major band of this fraction was identified as violaxanthin by the spectral absorption curve, and of course by the countercurrent distribution fraction (N_{100} in System II was 41; violaxanthin from leaves was 40) in which it occurred. It was accompanied by much smaller amounts of the corresponding 5,6,5′,8′-diepoxide isomers (luteoxanthins), but the 5,8,5′,8′-diepoxide isomers (auroxanthins) were not found.

Chromatography of fraction IV. The main band of the polyol fraction was identified as neoxanthin by the spectral absorption curve and behavior on countercurrent distribution in system IV, in which it had an N_{100} value of 62; the same value was obtained with neoxanthin from leaves. A very minor band above neoxanthin had a spectral absorption curve similar to that of violaxanthin, and an N_{100} value in system IV of 30. Similar fractions have previously been obtained from oranges and cling peaches (Curl, 1960 c). This fraction was tentatively identified as trolliflor (Lippert and Karrer, 1956), a monoepoxide $C_{40}H_{56}O_5$ which is apparently a tetraol.

The xanthophyll constituents, except for the monols, were qualitatively and quantitatively quite similar to those of green leaves (Strain, 1938; Curl and Bailey, 1957 b), and are those which would be expected to accompany the chlorophylls in the green fruit. On ripening there is a tremendous increase in the carotenes, especially lycopene, but apparently little change in the diol and polyol xanthophylls.

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The Chemical Composition of Beef Protein Fractions Before and After Irradiation [a]

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SUMMARY

A protein from beef prepared by ammonium sulfate fractionation of a hot-water extract, gave a "wet dog" odor when irradiated. This protein has an amino acid constitution similar to that of gelatin, is associated with a nitrogen-containing polysaccharide, and exhibits the solubility properties of a glucoprotein. When irradiated, 15% of the nitrogen becomes dialyzable and 13% of the amino acids are destroyed. The sulfhydryl content decreases markedly, whereas the ammonia, as determined after acid hydrolysis, and the ultraviolet absorption increase. Similar radiation-induced changes can be observed with proteins that do not produce an odor. Consequently, the odor discussed in this report does not appear to be produced from simple amino acids *per se*.

INTRODUCTION

Considerable evidence indicates that proteins are the major food component in which undesirable irradiated flavors and odors originate (Batzer and Doty, 1955; Bellamy and Lawton, 1954; Hedin *et al.*, 1960). This is not completely unexpected, because the underlying lability of proteins to irradiation is well documented. As early as 1913, Bovie (1913) reported on the coagulation of egg albumin by ultraviolet irradiation. Barron and Finkelstein (1952) observed the precipitation of bovine serum albumin solutions upon X-irradiation. Also of interest is the observation that the ultraviolet absorption between 250 and 300 mμ can either increase or decrease when proteins are irradiated (Arnow, 1935; Barron and Finkelstein, 1952; Carroll *et al.*, 1954).

Both serum albumin and hemoglobin, when irradiated at room temperature and examined with the ultracentrifuge, produced a continuous series of lower-, as well as higher-, molecular-weight substances (Svedberg and Brohult, 1939). Another observation that may involve molecular-weight changes was that of Jayko and Garrison (1958), who reported that radiation-induced cleavage of the peptide bond yields high-molecular-weight carbonyls and amides.

Although there have been many reports on changes caused by the irradiation of individual amino acids, the fate of the constituent amino acids after irradiation of a protein is not well understood. Barron *et al.* (1955), subjecting serum albumin solutions to X-irradiation, found decreases in glycine, alanine, glutamic acid, lysine, threonine, and tyrosine. Proctor and Bhatia (1950), on irradiation of fish muscle with high-voltage electrons, found no significant losses of any of the essential amino acids by microbiological methods. Drake *et al.* (1957), on the other hand, found a general loss of amino acids from aqueous insulin solutions.

In a previous study conducted in this laboratory (Hedin *et al.*, 1960), a protein fraction was obtained by refluxing in water that gave a "wet dog" odor when irradiated. This fraction was shown to be a mixture of at least two electrophoretically separable proteins and to contain small amounts of iron and phosphorus. Upon irradiation there was a considerable destruction of histidine, methionine, proline, glycine, and cystine, with a number of other amino acids being labile to a lesser degree.

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This study is chiefly concerned with the chemical composition of an electrophoretically homogeneous beef protein fraction isolated from the mixture (Hedin *et al.*, 1960), that gave the "wet dog" odor when irradiated. The composition of some other protein fractions was also determined to serve as a comparison.

EXPERIMENTAL

A. *Preparation of protein fractions from beef.*

The samples were prepared as described in a companion paper (Hedin *et al.*, 1961). As an aid in determining the procedure for sample preparation, the following code is used: HW denotes hot-water extraction, and CW denotes cold-water extraction. The numbers 25, 50, 75, and 100 denote the per cent saturation of ammonium sulfate required to precipitate the fraction. "I" represents a fraction irradiated previous to investigation, and "C" represents the unirradiated control.

B. *Methods of analyses.*

1. *Nitrogen.* Protein nitrogen was determined by the Kjeldahl procedure.

2. *Sulfur.* Total sulfur was determined as $BaSO_4$ according to the AOAC method. Sulfhydryl groups were determined amperometrically with mercuric chloride (Stricks *et al.*, 1954) and colorimetrically with bis(*p*-nitrophenyl) disulfide (PNPD) (Ellman, 1958).

3. *Ultraviolet absorptions.* Samples were dispersed in 0.1*M* phosphate buffer, pH 7.0, and the absorptions between 220 and 320 mμ determined with a model 14M Cary Recording Spectrophotometer.

4. *Quantitative amino acid analysis.* Fifty-milligram samples were dissolved in 2 ml of 6*N* HCl, dearated by bubbling with nitrogen, sealed in small tubes, and maintained at 105°C for 48 hours. The contents were washed with water through a filter and evaporated to dryness at reduced pressure. The hydrolysate was dissolved in water, adjusted to pH 7.2, and allowed to stand 4 hours at room temperature to ensure oxidation of any cysteine to cystine. The hydrolysate was then readjusted to pH 2.2 and diluted with buffer to a final concentration of 1 mg/ml protein hydrolysate. Two-milligram samples were then chromatographed and analyzed with a commercial amino acid analyzer constructed as described by Spackman *et al.*, (1958 a, b).

C. *Concentration and characterization of a polysaccharide moiety from HW-50-C and HW-75-C.*

Two per cent aqueous solutions of HW-50-C and HW-75-C were deproteinized by mixing with an equal volume of a chloroform-butanol mixture (3:1 V/V). After separation, two volumes of ethanol were added to the upper (aqueous) layer to precipitate the polysaccharide. The precipitate was then subjected to Partridge's procedure for removal of contaminating material (Partridge, 1948).

Aliquots were hydrolyzed for 24 hours in 1*N* HCl in sealed tubes at 105°C. They were chromatographed with an n-butanol–pyridine–water mixture (2:2:1 V/V/V). The uronic acids were detected with the naphthoresorcinol reagent, and the hexosamines were detected with the Elson-Morgan reagent.

D. *Irradiation techniques.*

The irradiation was performed in the gamma facility at Argonne National Laboratory, Lemont, Illinois. Dried samples were sealed in evacuated metal cans (300 × 200) and irradiated with 5 megarads.

RESULTS AND DISCUSSION

Effect of irradiation on structural integrity. Five-gram samples of HW-50-C and HW-50-I were dissolved in water and dialyzed against deionized water. The samples were then analyzed for nitrogen, sulfur, sulfhydryl, and ultraviolet absorbancy as described in the experimental section.

These results show that the loss of sulfur

Table 1. Irradiation-induced changes in composition.

Content of nondialyzables	HW-50-C		HW-50-I [a]	
	Before dialysis	After dialysis	Before dialysis	After dialysis
Sulfur (%)	0.306	0.304	0.304	0.303
RSH, moles × 10^{-6}/g, PNPD method	1.37	1.35	1.27	0.20
RSH, moles × 10^{-6}/g, amperometric method	1.20	1.10	0.40	0.30
Nitrogen (%)	16.77	16.65	16.85	14.35
Absorbancy 260 mμ 0.1% solution	0.210	0.204	0.315	0.208 [b]

[a] 5 megarads of gamma irradiation.
[b] Absorbancy 260 mμ of dialyzable fraction at equivalent concentration 0.093.

from the nondialyzables is so small that it cannot be measured gravimetrically. Amperometrically and colorimetrically, however, the loss of most of the sulfhydryl groups was demonstrated. Whether this represents a formation of low-molecular-weight compounds, which are dialyzable, or an oxidation of large-molecular-weight compounds, or both, is not known. It is of interest to point out that mercuric chloride does not react with the sulfhydryl groups in the irradiated protein before dialysis, whereas PNPD does. This difference in reactivity toward different sulfhydryl reagents, previously recognized (Guzman-Barron, 1951), provides additional evidence for irradiation-induced rearrangement of sulfhydryl groups in proteins.

Approximately 15% of the nitrogen became dialyzable when precipitate HW-50 was irradiated (Table 1). Since a full spectrum of amino acids was found when the hydrolyzed dialyzables were chromatographed two-dimensionally, the cleavage of the peptide bond at many locations was apparent.

Also observed was an irradiation-induced increase of absorption in the ultraviolet region (Table 1). Other workers have attributed this increase to hydroxylation of the aromatic rings (Arnow, 1935) and polymerization (Carroll *et al.*, 1954). The decrease of the absorbancy after dialysis to that of the control is not easily explained in terms of hydroxylation or polymerization. It is possible, however, that this increase in absorbance may be caused by the formation of radiation-induced cyclic structures. These structures could conceivably contain sulfur in view of the difference in reactivity of the sulfhydryl groups toward two different reagents after irradiation. The subsequent decrease in the absorbancy of the irradiated protein when dialyzed could be explained by some cleavage mechanism since the absorbancy of the dialyzables at 260 mμ was shown to account for the decreased absorbancy of the nondialyzables.

The absorbancies of the other ammonium sulfate fractions prepared from the hot-water extract were also found to increase after irradiation. Fractions HW-25, HW-75, and HW-100-S, respectively, increased by 42, 20, and 7%, and after dialysis fell to that of the control, as in the previous instance. These results suggest that the increased absorption is not necessarily related to the odor but is generally common to irradiated proteins.

Quantitative amino acid analysis of the protein fractions. Quantitative amino acid analysis studies were made on three protein fractions—one that produced considerable "wet dog" odor (HW 50), one that produced only a slight amount (HW 75), and one that produced no "wet dog" odor (CW 100). This was done in order to determine whether structural differences were large enough to explain differences in the magnitude of odor production. These results are summarized in Table 2.

Irradiation caused a decrease in the content of nearly every amino acid in all three protein fractions. This phenomenon was also observed by Drake *et al.* (1957), with insulin. The losses of glycine, proline, alanine, and glutamic acid were considerable in each fraction. In view of the small amounts of sulfur amino acids originally present, the losses were not quantitatively impressive. Nevertheless, this does not necessarily exclude the possibility that the "wet dog" odor compound contains sulfur, because the threshold for noting sulfurous odors such as hydrogen sulfide and mercaptans is so low that even microquantities can be detected.

There was also a marked increase in the ammonia content of all of the irradiated fractions after acid hydrolysis. With two proteins (HW-50 and CW-100), the molar increase in ammonia could account for essentially all of the amino acid losses.

The only new peaks produced by irradiation were methionine sulfoxide and an unknown, which emerged before arginine in HW-50. The methionine sulfoxide recovered could account for only ⅓ of the lost methionine. Since cysteic acid was found in two of the fractions, both before and after irradiation, its presence may in part he due to oxidation.

It can also be seen from Table 2 that the HW-50 fraction contains 60% more glycine,

Table 2. The amino acid recovery from three protein hydrolysates [a] (μM/mg).

	HW-50		HW-75		CW-100	
Amino acid	Control	Irradiated	Control	Irradiated	Control	Irradiated
Lysine	0.492	0.466	0.784	0.714	0.685	0.645
Histidine	0.083	0.075	0.160	0.092	0.322	0.195
Ammonia	1.255	2.157	1.400	1.860	2.320	3.480
Arginine	0.436	0.366	0.356	0.271	0.207	0.215
Glucosamine [b]	0.043	0.034	0.059	0.023	0	0
Cysteic acid	0.002	0.002	0	0	0.026	0.003
Aspartic acid	0.586	0.523	0.785	0.734	0.843	0.690
Methionine sulfoxide [b]	0	0.002	0	0.007	0	0.015
Hydroxy proline	0.387	0.314	0.247	0.203	0	0
Threonine [c]	0.172	0.169	0.238	0.207	0.308	0.260
Serine [c]	0.145	0.148	0.172	0.189	0.315	0.270
Glutamic acid	1.228	1.095	1.492	1.290	0.873	0.700
Proline	0.885	0.690	0.515	0.478	0.379	0.285
Glycine	2.782	2.265	1.520	1.460	0.685	0.535
Alanine	1.280	0.906	0.989	0.905	0.751	0.640
Half cystine [c]	0.005	0	0.006	0	0.005	0.002
Valine	0.369	0.318	0.343	0.280	0.655	0.565
Methionine [c]	0.073	0.059	0.085	0.067	0.122	0.086
Isoleucine [d]	0.198	0.171	0.172	0.138	0.301	0.250
Leucine	0.423	0.372	0.448	0.398	0.779	0.660
Tyrosine	0.016	0.015	0.040	0.038	0.207	0.140
Phenylalanine	0.128	0.125	0.098	0.076	0.344	0.255
Before arginine	0	0.175	0	0	0	0

[a] Irradiated with 5 megarads of gamma irradiation.
[b] Tentative identification based on peak eluant volume.
[c] No correction made for recognized losses.
[d] L-isoleucine plus D-alloisoleucine.

proline, and hydroxyproline, and 30% more alanine than the samples that produced little or no odor when irradiated. These amino acids are known to be relatively radiolabile (Barron *et al.*, 1955). The sulfur amino acids, also known to be radiolabile (Barron and Flood, 1950; Batzer and Doty, 1955), are present in essentially the same concentration in all three proteins.

The molar values for HW-50 were converted to mole fractions based on approximate bimolar values for threonine, and compared with literature values for calfskin gelatin (Neumann, 1949), beef myosin (Kominz *et al.*, 1954), and beef whole muscle (Greenwood *et al.*, 1951). These data demonstrated that there was a marked similarity between HW-50 and gelatin. The minor differences may be attributed to sample origin.

Calculations for total amino acid recovery were made for each of the proteins by converting the molar values to micrograms (Table 3). When a correction for added weight due to water of hydrolysis was made (approximately 15%), it could be seen that not all of the original material was protein. The remainder could be accounted for at least in part by nucleic acids, because the ash content of HW-75 and CW-100 was moderately high (0.18–0.34%), and by polysaccharide, because these fractions contained glucosamine and, though precipitable by am-

Table 3. Combined amino acid losses caused by gamma irradiation.

	HW-50	HW-75	CW-100
Nitrogen (%)	16.77	15.05	15.62
Ash (%)	0.02	0.34	0.18
Amino acid recovery [a] before irradiation (%)	113.50	104.50	110.20
Amino acid recovery after irradiation (%)	99.00	92.10	92.45
Irradiation destruction (%) [b]	12.75	11.85	16.15

[a] Per cent of original protein weight.
[b] Percentage loss based on amino acid recovery before irradiation.

monium sulfate, were soluble in boiling water, as are glucoproteins.

When the samples were irradiated there was no marked difference in the degree of

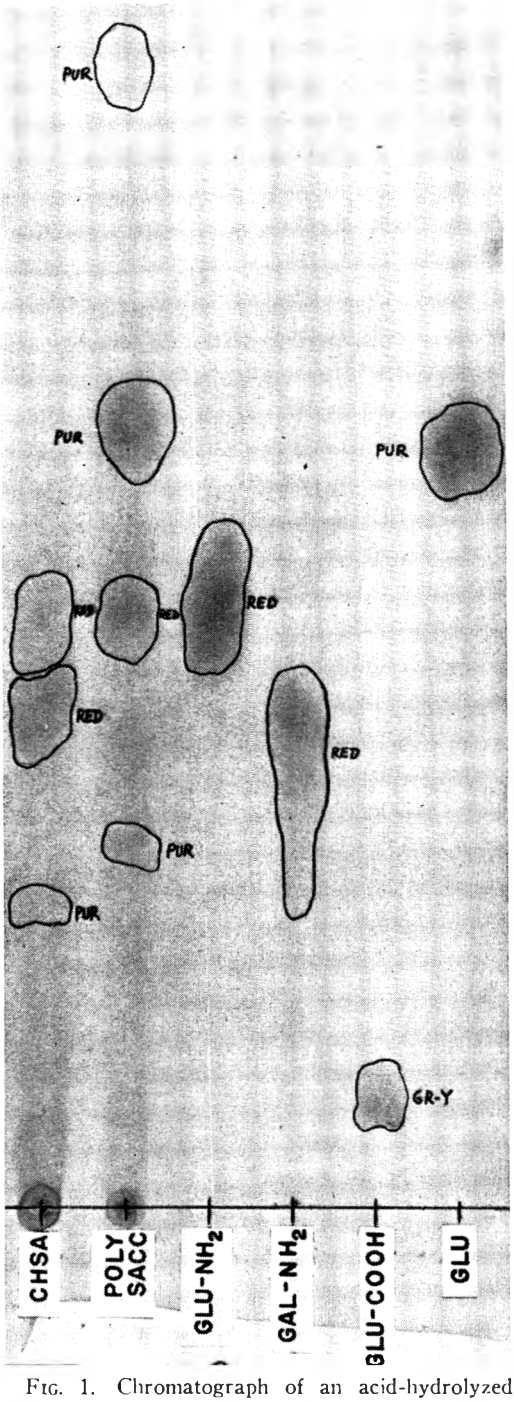

FIG. 1. Chromatograph of an acid-hydrolyzed polysaccharide isolated from HW-50 and HW-75. Hydrolysates were chromatographed with an n-butanol–pyridine–water mixture (2:2:1 V/V/V) and developed with the Elson-Morgan reagent. CHSA = chondroitin sulfate; GLU-NH_2 = glucosamine; GAL-NH_2 = galactosamine; GLU-COOH = glucuronic acid; and GLU = glucose.

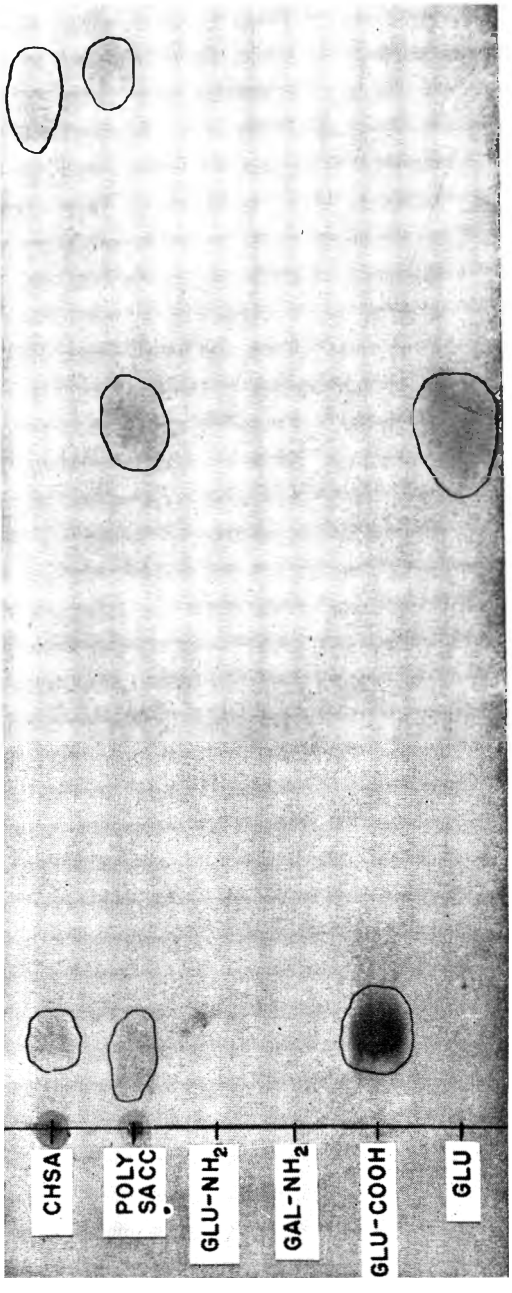

FIG. 2. Chromatograph of an acid-hydrolyzed polysaccharide isolated from HW-50 and HW-75. Hydrolysates were chromatographed with an n-butanol–pyridine–water mixture (2:2:1 V/V/V) and developed with the naphthoresorcinol reagent.

amino acid destruction (Table 3). This observation might be expected if equal amounts of similar materials had equal capacity to absorb energy. However, if the magnitude of destructon is not related to odor production, the relative amino acid concentrations, sequence, and presence of nonprotein material should be alternately considered.

Evidence for the association of the protein with carbohydrates. Since glucosamine was found (Table 2) in hydrolysates of HW-50 and HW-75, but not of CW-100, studies were instigated to determine the carbohydrate content of these two fractions. A Molisch-positive precipitate was isolated, hydrolyzed, and chromatographed as described (see experimental). It was found to contain glucuronic acid, glucosamine, glucose, and glucuronolactone (Fig. 1, 2). Since glycogen is known to accompany mucopolysaccharides obtained from animal sources (Partridge, 1948), glucose would be an expected contaminant. The glucuronolactone would also be expected because of the tendency of the glucuronic acid to cyclize when heated. Analysis for sulfate was negative. Comparing the two most likely nitrogen-containing polysaccharides, the evidence favors hyaluronic acid, which contains D-glucuronic acid and *N*-acetyl-D-glucosamine rather than chondroitin sulfate, which contains D-glucuronic acid, *N*-acetyl-D-galactosamine, and sulfuric acid.

Carbonyl-containing compounds such as sugars are known to react with hydrogen sulfide to produce mercaptals, many of which posess disagreeable odors. Although evidence has been obtained for the presence of polysaccharide in the odor-producing protein, further work must be done to establish a significant production of mercaptals.

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Modification of the Pad-plate Method of Determining Chlortetracycline in Egg White

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SUMMARY

The sensitivity of the pad-plate method for detecting chlortetracycline in egg white is increased by using pH 4.5 citrate buffer rather than pH 4.5 phosphate buffer as a diluent. It is also increased by allowing about four hours for diffusion of antibiotic prior to incubation of the assay plates. Under these conditions of assay, protein binding of the antibiotic was not an important factor.

Feeding or injection of high-level chlortetracycline (CTC) and oxytetracycline (OTC) to control poultry diseases results in measurable quantities of these antibiotics in whole egg and yolk (Broquist and Kohler, 1954; Durbin *et al.*, 1954; Elliott and Romoser, 1957; Frye *et al.*, 1958; Raica *et al.*, 1956). Difficulty has been encountered in detecting CTC in egg white even when added shortly before analysis (Elliott and Romoser, 1957; Frye *et al.*, 1958; Raica *et al.*, 1956). This has been attributed to the instability of CTC in alkaline media (Baron, 1950; Karel and Roach, 1951), such as egg white, and to a possible CTC-protein binding effect (Elliott and Romoser, 1957).

OBJECTIVE

This study was made to modify the pad-plate method in an attempt to improve the sensitivity for determining CTC in egg white. This involved: a) an investigation of buffers that lower the pH of egg white, and b) adjusting the assay technique to avoid appreciable dilution of sample.

METHODS

Chlortetracycline was determined by a modification of the pad-plate method (Grove and Randall, 1955). The test organism was spores of *Bacillus cereus* var. *Mycoides* ATCC 9634. Pyrex baking dishes, #232CBB, 1½-qt size, were poured with 100 ml of seeded agar (Difco Penassay Seed Agar) and allowed to harden before triplicate pads (Schleicher and Scheuell, ½-inch, No. 740E) containing 0.1 ml of sample were placed on them. The dishes were placed in a refrigerator for 1 hr or longer to allow the antibiotic to diffuse from the pads into the medium. The plates were then incubated 18–20 hours at 27°C, and the diameters of the zones of inhibition were measured with vernier calipers. A standard curve was prepared for each set of antibiotic analyses. The readings were made as directed for the pad-plate and method.

RESULTS AND DISCUSSION

Influence of buffers. The pad-plate method makes use of 0.1*M* KH_2PO_4 solution of pH

Table 1. The effect of dilution buffer and pH on the zone of inhibition of chlortetracycline in egg white.

Trial	Diluent [a]	pH	Zone of inhibition (mm)
1	Egg white + water (1:1)	8.6	0
	Egg white + 0.1*M* phosphate solution pH 4.5 (1:1)	6.7	18
	Egg white + 0.2*M* phosphate solution pH 4.5 (1:1)	6.3	21
2	Egg white + 0.1*M* phosphate solution pH 4.5 (1:1)	6.5	27
	Egg white + 0.1*M* citrate buffer (b) pH 4.5 (1:1)	5.4	35

[a] The final solution in all cases contained 0.333 μg/ml chlortetracycline.

4.5 as an extractive or diluting solution. It was used for diluting egg white 1 : 1. This lowered pH only from 8.6 to 6.7 (Table 1), which is still in the pH zone where CTC is unstable (Baron, 1950; Karel and Roach, 1951). A citrate buffer of pH 4.5 proved satisfactory. It was made by combining 0.1*M* citric acid and 0.2*M* disodium phosphate (buffer a) or citric acid and sodium citrate (buffer b). When this was used for diluting egg white 1 : 1, pH was reduced to 5.4 and the zone of inhibition (antibiotic activity) was much greater (Table 1).

Influence of pH. Citrate buffer solutions ranging from pH 2.8 to 5.8 were prepared (Table 2). Egg white was diluted with 0.2*M* citric acid to produce solutions of like pH. Chlortetracycline at a level of 0.125 gamma per ml was added to each buffer and egg-white solution. After mixing and standing for a few minutes at room temperature, the samples were analyzed for antibiotic content. The results are summarized in Table 2. A pH below 6 appears to be essential for detecting a small amount of CTC in egg white buffered with a citrate buffer. There is a slight binding or inhibitory effect of egg white on CTC. This is shown by the greater zones of inhibition of CTC in buffer at different pH than in egg white.

Table 2. The bacteriostatic effect of CTC in buffer and egg white.[a]

pH	Zone of inhibition (mm diam)	
	In buffer (a)	In egg white
2.8	22.6	22.4
3.8	22.1	21.0
4.8	21.3	19.2
5.8	20.9	18.5
6.8	17.1	0.0

[a] 0.125 gamma of CTC per ml of buffer and egg white solutions.

Loss of antibiotic effect. Two sets of tubes were prepared. One contained equal amounts of 0.2*M* solutions of buffer of different pH and egg white, giving a final dilution of 0.1*M*. The other set of tubes contained 0.1*M* buffer solutions of the same pH. Antibiotic (CTC) was added to all tubes at a level of 1.5 mg CTC per ml. Periodically, samples were removed, adjusted to pH 4.5 with 4*N* HCl, brought to constant volume with 0.1*M* citrate buffer, pH 4.5, and assayed for antibiotic content. The results are summarized in Table 3. Increasing the acidity of the antibiotic to pH 4.5 resulted in preservation of most of the antibiotic in solutions held one day at 45°F. There was some decline in antibiotic effect during a five-day holding period, especially at pH above 4.5.

Table 3. The effect of pH using citrate buffer diluting solution for egg white on the chlortetracycline zone of inhibition.

Set I composition	Buffer added	pH of contents	μg of CTC assayable after	
			1 day	5 days
Distilled water + CTC [a,b]	none	.....	0.11	0.05
pH 8 buffer + CTC	0.1*M* K phosphate	8.0	0.00[c]	0.00
pH 6.5 buffer + CTC	0.1*M* K phosphate	6.5	0.11	0.00
pH 4.5 buffer + CTC	0.1*M* Na citrate	4.5	0.14	0.15
Set II Composition				
Distilled water + albumin (1 + 1)	none	8.4	0.04	0.00
pH 8 buffer + albumin (1 + 1)	0.2*M* K phosphate	8.05	0.00	0.00
pH 6.5 buffer + albumin (1 + 1)	0.2*M* K phosphate	6.6	0.04	0.00
pH 4.5 buffer + albumin (1 + 1)	0.2*M* Na citrate	4.9	0.14	0.10

[a] Contents of tubes diluted 1:10 prior to assay so that theoretical CTC is equal to 0.15 μg/ml. All assays tested at pH 4.5.
[b] CTC in concentration of 1.5 μg/ml in each tube.
[c] Less than the sensitivity of assays that varied from 0.01 to 0.03 μg/ml.

Binding of antibiotic. Freshly prepared assay plates were placed in a household refrigerator for 1–5 hours before incubation, to see if high concentrations of albumen slow up the rate of diffusion of CTC. The data are summarized in Table 4. The combination of an acidified albumen sample along with an extended diffusion time of 4 hours or more resulted in nearly identical zones of inhibition by the antibiotic in either buffer or egg white. No binding effect of CTC by the egg white was indicated, as had been suggested by earlier workers (Elliott and Romoser, 1957).

Table 4. The effect of the duration of refrigeration on the size of the zone of inhibition produced by chlortetracycline–egg white mixtures.

Duration of refrigeration (hr)	Zone of inhibition (mm diam) produced by 0.125 μg/ml CTC in:	
	Buffer [a] (pH 3.8)	Egg white concentrated (pH 3.8)
1	20	18.9
2	20	18.9
3	21.2	20.5
4	21.5	21.4
5	21.7	21.7

[a] The buffer used is citric acid phosphate.

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Sensory Evaluation of Accessory Foods With and Without Carriers [a]

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SUMMARY

Accessory foods like jelly or catsup can be evaluated by consumers for preference without an appropriate carrier, such as bread or hamburger, at least as effectively as with the carrier. Time and money are saved, and accuracy is increased.

One problem that often arises in sensory evaluation work is whether accessory foods or condiments such as mayonnaise, jelly, or soy sauce should be served alone or in combination, i.e., mayonnaise on a salad, bread with jelly, soy sauce with chow mein, etc. There are many advantages in eliminating the carrier when evaluating accessory foods. Much time may be spent in selecting a proper item as a carrier, and in this selection many problems arise. Is there a dilution effect of the carrier on the item to be tested? Do we know that catsup served on frankfurters will give as good discrimination as when served on, say, hamburgers or eggs? If the experiment will take considerable time, can the quality of the carrier be maintained throughout many months of storage? Purchasing an adequate amount of carrier food at the initiation of the study and storing at freezing temperatures is not always satisfactory. This is so particularly when the accessory food is to be studied for storage stability over 6–12 months. A carrier would be suitable only if one had assurance that its quality would not change.

It has been independently established that the simpler preparation methods give better discrimination in testing for differences per se or differences in preference. This, in itself, would contraindicate using a carrier; furthermore, its preparation adds more chance for error on the part of the experimenter. The cost of the evaluation is increased by the cost of the carrier and, possibly, the additional time required to prepare it.

METHOD

Three variants of each of several accessory food items were prepared by one of two procedures: 1) A chemical was added to the item in increasing amounts to alter preference; 2) two different commercial products plus an experimental sample, prepared by adding a chemical to a blend of the commercial products, were used. The three samples of each set were tested in a separate run, being served both plain and with a carrier, using the single-stimulus method with the hedonic scale (Peryam and Pilgrim, 1957). Only three samples were tested by each subject, and the presence or absence of the carrier was confounded with judge group, i.e., each subject tested under one condition or the other, but not under both. Each test group consisted of 18 consumers chosen randomly from a population of 700 male and female employees at the Chicago Administration Center. The experiment was replicated on another day, giving a total N of 36 consumers rating each set of three samples. Analyses of variance were performed, and where the main effect of treatment, i.e., the difference between the food samples, or the treatment-carrier interaction was significant, special contrasts were applied.

RESULTS

Table 1 gives the details for each of the experiments and presents the results. For the analysis of variance, the particular contrast, i.e., comparison, is identified, followed by three columns indicating the significance of: a) treatment effect, i.e., whether the

[a] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 2030 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

Table 1. Mean preference ratings for experimental treatments of accessory foods with and without carriers, and significance of analysis of variance effects for each test.

Accessory food and experimental treatment	Carrier food	Preference: Without carrier	Preference: With carrier	Analysis of variance: Contrast	Significance of effects (%): Treatment	Significance of effects (%): Carrier	Significance of effects (%): Interaction
Cheese spread (β-phenylethyl alcohol added)	Crackers						
0.0%		6.1	6.5	Linear	<0.1	5.	5.
0.02%		4.8	6.0	Quadratic	Not		Not
0.04%		4.2	5.6				
Total		5.0	6.0				
Cake icing (saturated salt solution added)	White cake						
0.0%		7.0	6.9	Linear	<0.1	Not	1.
1.7%		6.2	7.1	Quadratic	Not		Not
3.4%		5.5	6.4				
Total		6.2	6.8				
Catsup	Frankfurters						
Brand A		7.6	7.3	A vs B	Not	Not	Not
Brand B		7.4	6.9	A + B vs			
Experimental [a]		5.1	4.4	exptl.	<0.1		Not
Total		6.7	6.2				
Worcestershire sauce	Hamburgers						
Brand A		5.1	6.2	A vs B	Not	Not	Not
Brand B		5.5	5.9	A + B vs			
Experimental [b]		4.8	5.6	exptl.	5.		Not
Total		5.1	5.9				
Soy sauce	Egg foo yong						
Brand A		4.1	6.4	A vs B	Not	<0.1	Not
Brand B		3.2	6.4	A + B vs			
Experimental [c]		3.7	6.2	exptl.	5.		Not
Total		3.7	6.3				
Cheese sauce (sugar added)	Macaroni						
0 tbsp/12 oz		6.6	6.2	Linear	Not	Not	Not
1 tbsp/12 oz		7.1	6.3	Quadratic	Not		Not
2 tbsp/12 oz		6.4	6.5				
Total		6.7	6.3				
Whipped topping [d]	White cake						
Fresh cream		7.3	7.5 [e]	7.4 [f]	A vs B	Not	0.1	Not
Topping A		6.7	7.0	7.3	A + B vs			
Topping B		6.6	7.2	6.5	fresh	1.		Not
Total		6.4	7.2	7.1				

[a] 1 tsp peppermint oil per 16 oz brands A&B combined.
[b] 12 ml smoke flavor per 16 oz brands A&B combined.
[c] 30 ml vanilla & 0.2 ml maple concentrate to 100 ml of brands A&B combined.
[d] N = 12 for each mean; means in all other experiments based on N of 36.
[e] Instruction I: Rate whipped cream and cake.
[f] Instruction II: Rate whipped cream and cake, but pay special attention to whipped cream.

contrast indicated was significant; b) the main effect of carrier, i.e., whether the samples as a group rate the same with as without the carrier; and c) the treatment-carrier interaction, which shows whether the differences between treatments were the same with and without the carrier.

The cheese spread and cake icing both showed a decline in preference with addition of the chemical additive. This marked decrease was significant at the 0.1% level. The interaction of treatment-carrier was slightly significant (at the 5% level). The direction of the decrease in preference was the same for both items without and with a carrier. The difference was greater, however, when the items were served without a carrier. This fact accounts for the treatment-carrier interaction. The special contrasts showed that in each case the effects were linear—there was no curvature in the relationship between preference and amount of additive.

The catsup, Worcestershire sauce, and soy sauce also showed a decline in preference upon the addition of chemicals—respectively significant at the 0.1%, 5%, and 5% levels. The difference between the two commercial samples in each case was not significant. The contrast that was significant was the experimental treatment vs the average of the commercial products. The interaction of treatment-carrier was not significant, indicating that the difference was the same without or with a carrier.

The cheese-sauce evaluation is shown in the table, though there were no significant main effects or interactions. Thus, this item failed to contribute to the experiment.

The whipped-topping experiment showed a significant difference for the treatments; however, the treatment-carrier and treatment-instruction interactions were not significant, which indicates that discrimination was equally good for the item without or with a carrier.

DISCUSSION

The six experiments in which there were significant treatment effects all support the hypothesis that accessory foods can be evaluated in the absence of their normal carriers. Furthermore, two experiments—icing and cheese spread—showed that discrimination was greater without the carrier. Thus, in some cases one can use a smaller number of subjects, or else be more certain of his results, when the accessory food is tested alone.

In three experiments the average level of preference between the with and without conditions differed significantly, but this fact is of no importance when one is attempting to assess differences between products.

There is also evidence that the converse of these studies yields the same kind of result. Two crackers were tested and found of different quality or level of preference. It was thought that with a spread on them, the difference might not be noticed. Therefore, the crackers were served plain to one group, and with a cheese spread to another group. In both cases a marked difference in preference was demonstrated.

Although the number of products tested is limited, the results suggest that one can generally dispense with the added work and cost of using carriers for accessory foods. One also avoids the possibility of introducing experimental errors arising from nonuniformity of the carrier.

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Pectic Substances of Valencia Oranges at Different Stages of Maturity [a]

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(Manuscript received May 9, 1960)

SUMMARY

Seasonal changes in concentrations of pectic substances of the peel and pulp were followed by determining the total- and water-soluble pectic substances as anhydrogalacturonic acid during growth and development under normal conditions. With the rapid development of fruit growth in the early part of the season, a tremendous initial increase occurred in the total- and water-soluble pectic materials followed by a gradual decrease through the remainder of the season. The percentage methylation of the pectic substances of Valencia orange peel rises rapidly to approximately an 80 per cent level and remains relatively constant during the rest of the season.

INTRODUCTION

The stage of development at which an orange is harvested has an effect on many factors relating to eating quality, shipping, storing, marketing, processing qualities, and especially yields and profits. Some oranges are harvested when still green and color development accelerated by special storage conditions in order to take advantage of a favorable market. Oranges, unlike pears, apples, bananas, etc., do not continue to mature when detached from the tree and placed in storage, because they have no carbohydrate reserve. In other instances oranges are held on the tree past peak maturity because of a poor market. It is important, therefore, to know something of the fruits' chemical constitution at many stages of maturity. The pectic substances are one of the constituents of primary importance in citrus, whether sold as fresh fruits or processed.

Some chemical changes occurring in the pectic substances of Valencia oranges in late-immature through the yellow-mature stages have been reported (Sinclair and Jolliffe, 1958). This period of near-maturity and maturity has received most of the attention by research workers for many fruits (Gaddum, 1934; Sinclair and Crandall, 1953). A continuation of the study on Valencia oranges has been made during the time when the fruit is extremely small and immature, when the build-up of pectic substances is taking place along with development and differentiation of the various fruit tissues; a period when there is much change and synthesis as compared with the rather static period already studied. The changes of the pectic substances during the entire growth cycle of the Valencia orange are given in this paper with considerable emphasis on the early immature stages, which have been neglected in past work.

METHODS

In this study the Valencia orange was sampled at two-week intervals from fruit about the size of a green pea until full-size, mature fruit. Measurements of the fruit were determined with calipers at each sampling, and the average area and volume found. The study was started on May 20, but it was not until July 15 that the pulp was sufficiently developed that an adequate and reliable sample could be obtained for analysis. No juice analysis was made on the early fruit samples, because not enough juice was present.

The data were obtained with the procedures used in previous work (Sinclair and Jolliffe, 1960). Alcohol-insoluble solids were determined on each fruit sample using 80% ethyl alcohol and grinding in a Waring blender (Sinclair and Crandall, 1951; Sinclair and Jolliffe, 1960). Two such extracts were made of the peel and pulp samples, the first extraction period being for about two hours and the next one overnight. About 20 g of peel and 60 g of pulp were used, duplicate samples being

[a] Paper No. 1263, University of California Citrus Experiment Station, Riverside, California.

collected on the more ample peel. The alcohol-insoluble solids were recovered using Whatman #50 paper and a Buchner funnel, and washed with 95% ethyl alcohol, and finally with petroleum ether. This material was dried in a 65°C oven and weighed. The percentage of moisture was determined also at 65°C on the respective samples, and the data calculated on a dry-weight basis.

The total pectic substances were calculated by making CO_2 analysis on the alcohol-insoluble solids and multiplying this percentage by four to obtain the percentage of anhydrogalacturonic acid (McCready *et al.*, 1946). Extracts were made of the alcohol-insoluble solids using hot water and hot 0.1*N* HCl, three ten-minute extractions for each. The respective extracts were analyzed for the percentage of anhydrogalacturonic acid using the carbazole colorimetric method (Dische, 1947; McComb and McCready, 1952). Methoxyl determinations on the peel and pulp alcohol-insoluble solids were made by the saponification method (Romeo, 1933). It is known that calcium chloride precipitates many biological constituents other than the pectic substances, thus giving falsely high calcium pectate values. No determinations were made by the traditional calcium pectate method of Carré and Haynes (1922), for values found by this procedure indicate only the proportion of polygalacturonic acid that is precipitated as neutral calcium salts, and not the complete range of molecular sizes.

The figures presented represent the seasonal trends for data collected during a three-year period. The importance of absolute values is not emphasized, because factors such as climate, irrigation and soil, age of trees, and disease will certainly affect these, but normal growth and maturation will lead to curves for the development of pectic substances not significantly different from those presented.

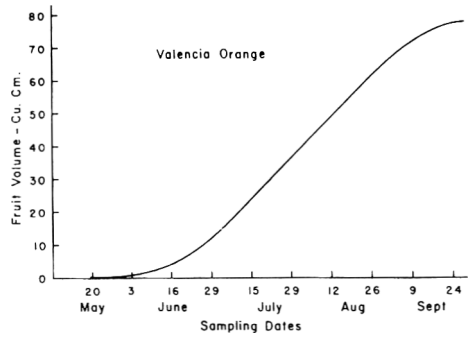

FIG. 1. Change in fruit volume during the first five months after fruit set.

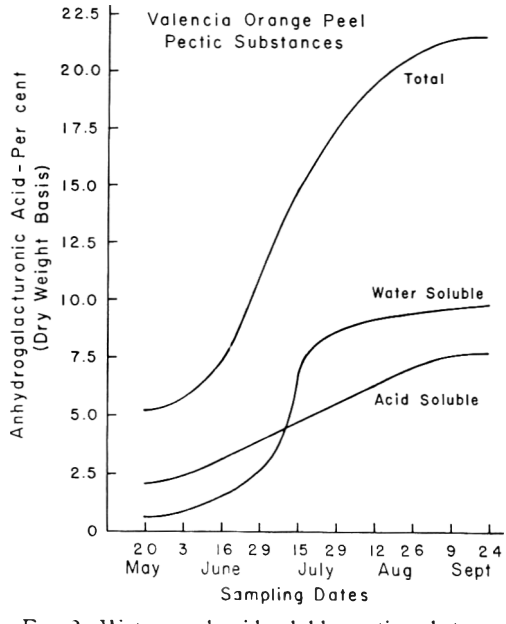

FIG. 2. Water- and acid-soluble pectic substances in the peel of early immature Valencia orange peel.

RESULTS AND DISCUSSION

The extremely small fruit in the early samples were very hard and consisted of over 90% peel, and the rate of fruit growth is shown in the change in fruit volume during the first five months after fruit set (Fig. 1). The fruit sample picked on September 24 had a fully developed pulp and had peel thickness only slightly greater than that of normal mature fruit. The greatest period of fruit growth occurred between June and September, inclusively, and from then on the fruit gradually increased in size with the advance of the season.

Pectic substances in orange peel. Fig. 2 shows the relationship of the increase of the total pectic substances in the peel and its component water- and acid-soluble fractions with growth and development of the fruit. A rapid increase in the total pectic substances occurred during the first 17-week period of fruit growth, beginning when the fruit was approximately 5 mm in diameter. There was better than a fourfold increase in the percentage (dry-weight basis) of total anhydrogalacturonic acid during these early stages.

The water- and acid-soluble fractions of the total pectic substances increased during the early period. For the first eight weeks

of this study, when the fruit volume was between 0.2 and 13.4 cc, the acid-soluble fraction was 2–3 times as large as the water-soluble fraction. During a two-week period from June 29 to July 15 there was about a threefold increase in the concentration of the water-soluble fraction, and it then became the major pectic component (Fig. 2).

The data in Fig. 3 show the changes in

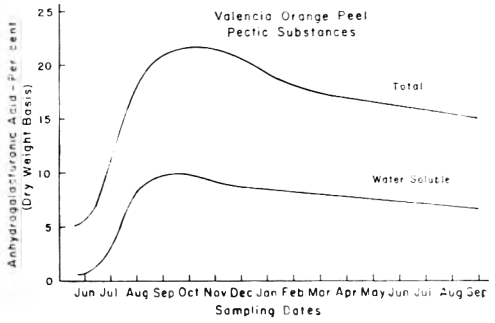

FIG. 3. Changes in the total- and water-soluble anhydrogalacturonic acid of Valencia orange peel (dry-weight basis).

total- and water-soluble pectic substances of the peel for the complete growth cycle of the fruit. The water-soluble fraction can be seen to have an S-shaped curve during this study, and during the remainder of the season there was a gradual decrease in the percentage of anhydrogalacturonic acid on a dry-weight basis. A 25–30% reduction of this concentration took place during the rest of the fruit's development and maturation on the tree. This reduction, due to a decrease in the concentration of alcohol-insoluble solids calculated on a dry-weight basis, must have been caused by an increase in a dry-weight factor in the alcohol-soluble fraction, probably carbohydrates.

Pectic substances in orange pulp. In these experiments, orange pulp is defined as the edible portion of the fruit, consisting primarily of the segments (carpels) and juice vesicles (juice sacs) and contents. An observation of Fig. 2 and 4 shows that the total anhydrogalacturonic acid (dry-weight basis) was significantly lower in the pulp tissue than in the peel. The amount of water-soluble pectic material increased gradually, but did not manage to obtain the level of the acid-soluble fraction at any time during this study (Fig. 4). There was a much more limited increase in the total pectic substances in the pulp, expressed as percentage of anhydrogalacturonic acid (dry-weight

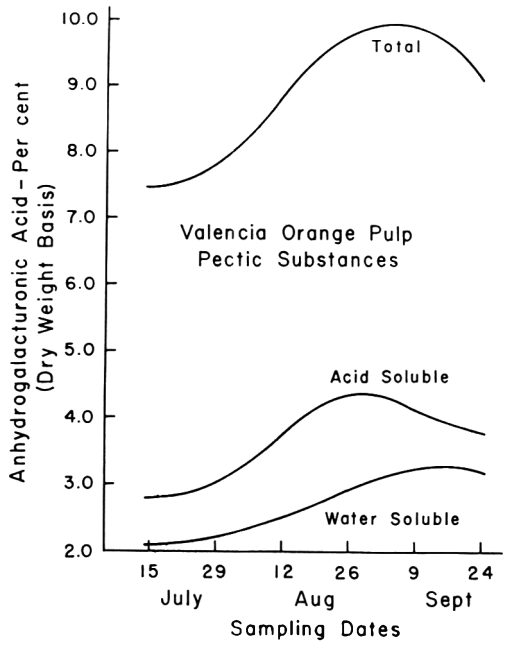

FIG. 4. Water- and acid-soluble pectic substances of the pulp of early immature Valencia orange pulp.

basis), than that of the peel (Fig. 4), whereas the reduction during the remainder of the season was comparable to that of the peel (Fig. 5).

It can be noted from Fig. 2 and 4 that the sum of the water- and acid-soluble fractions accounts for about 70–80% of the total pectic

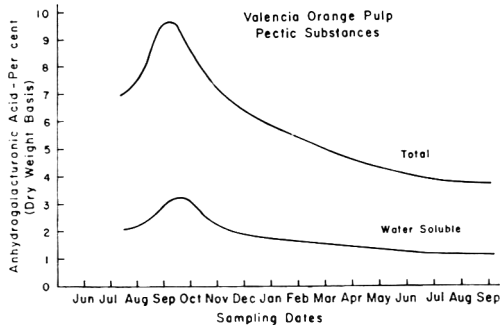

FIG. 5. Changes in the total- and water-soluble anhydrogalacturonic acid of Valencia orange pulp (dry-weight basis).

substances on an anhydrogalacturonic acid basis. An explanation is possible. The hot-water extraction mainly removes pectic materials and colloidal pectinic acid of sufficiently high methoxyl content to be soluble. The mild acid extraction removes the pectates, pectinic acids, and hydrolyzed protopectin. These extractions are not sufficient to release all the protopectin and place it in solution, because some fractions are interwoven and bound or combined with different intensities with various polysaccharides, cellulose, and hemi-cellulose, and likely some protein as well. Gaddum reported yields of water-soluble pectic material for albedo and pulp, respectively, calculated as 75 and 92% of the total pectic substances. These values, which are based on his gravimetric determination of precipitated alcoholic coagulum, are much higher than any based on the anhydrogalacturonic acid content. Gaddum was working with albedo instead of with whole peel (both albedo and flavedo), and this might make some difference. Further, his water-soluble pectic material was obtained by five extractions with boiling water, a treatment drastic enough to solubilize many insoluble materials by degradation.

To account for the un-extracted pectic materials, the residue was recovered as well as possible and CO_2 determinations made on it. This, expressed as anhydrogalacturonic acid, accounted for 5–15% of the total pectic substances. The results show that the higher percentages are found in the early stages and diminish as the fruit matures, which is consistent with the known behavior of protopectin. Then, another 10% loss of pectic material can be expected in such an extract analysis, due to degradation during extraction, etc.

Relation of the anhydrogalacturonic acid to the alcohol-insoluble solids of peel and pulp. It has been shown in other papers (Sinclair and Crandall, 1951, 1953; Sinclair and Jolliffe, 1958, 1960) that the uronide fraction of citrus fruits occurs in the alcohol-insoluble solids portion, a fraction that consists of cell wall constituents of such material as cellulose, hemicelluloses, pectic substances, and proteins. The percentage of anhydrogalacturonic acid found in the alcohol-insoluble solids of the peel rises to a

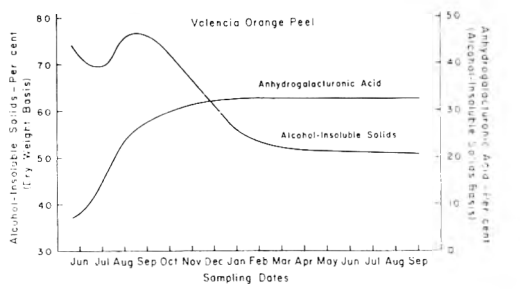

FIG. 6. Relation of the anhydrogalacturonic acid to the alcohol-insoluble solids of Valencia orange peel.

point and remains fairly constant throughout the rest of the season, which demonstrates that there is no real diminution of the pectic substances acting as structural components (Fig. 6). The data also show that in very small immature fruits picked early in June the peel contained about 75% of the dry matter as alcohol-insoluble solids. With an increase in seasonal fruit growth, the alcohol-insoluble solids are rapidly reduced, chiefly as a result of the rapid influx into the fruit of soluble carbohydrates, thus increasing the dry weight of the fruit.

In the pulp tissue the percentage of alcohol-insoluble solids on a dry-weight basis diminishes while the percentage of anhydrogalacturonic acid on an alcohol-insoluble solid reaches a constant early in the season. This is the same behavior that is seen in the peel, and the percentage of anhydrogalacturonic acid constant is about the same, showing that the composition of the structural material is similar in both peel and pulp (Fig. 7). A notable difference between peel and pulp is that the peel contains a

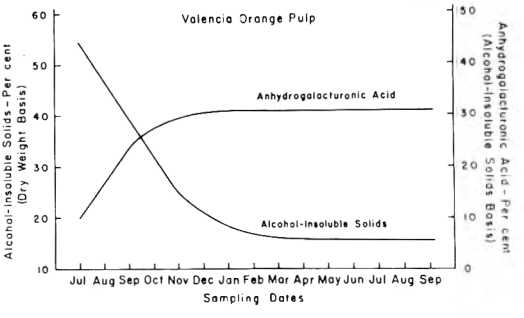

FIG. 7. Relation of the anhydrogalacturonic acid to the alcohol-insoluble solids of Valencia orange pulp.

greater amount of alcohol-insoluble solids as a percentage of the dry weight.

Water-soluble pectic substances of the alcohol-insoluble solids. The water-soluble substances of the peel and pulp are extracted from the alcohol-insoluble fraction, which contains the total pectic substances. In the absence of an appreciable quantity of starch, procedures used for isolation of the various fractions in the alcohol-insoluble solids are considerably simplified and shortened. The chief difficulty in these procedures is the isolation of the pectic materials from the cellulose-hemicellulose complex. Since the molecular composition of the extracted pectic material varies from one sample to another, the uronide represented by the last traces of CO_2 in the residue of the alcohol-insoluble solids yields a complex of variable composition. At present there are no practical chemical procedures for separating the various polygalacturonic acid components from extractives of pectic material.

The percentage of anhydrogalacturonic acid in the water extract on an alcohol-insoluble solids basis is shown to increase to a maximum and then become relatively constant for the remainder of the season (Fig. 8). This constancy of the water-soluble fraction is somewhat surprising since in many other fruits a rapid increase generally occurs in water-soluble pectic substances at peak maturity and thereafter. Comment should be made, however, that the concentration of water-soluble pectic substances in the fruits depends significantly on the basis on which the calculations are made. For example, in Fig. 3 and 5 the water-soluble

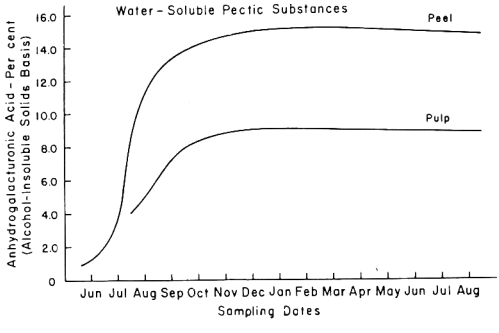

FIG 8. Changes of the water-soluble pectic substances of the peel and pulp of Valencia orange fruit (alcohol-insoluble solids basis).

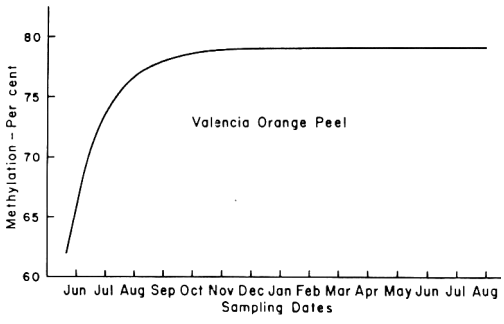

FIG. 9. Seasonal trends in methylation of the pectic substances of Valencia orange peel.

pectic substances, calculated on a dry-weight basis, show an increase to a maximum early in the season, followed by a slow decrease for the remainder of the growing period. There is no real decrease in the water-soluble fraction since this decrease is based on the percentage of total pectic substances and the latter has been shown to be affected by the per cent alcohol-insoluble solids, which is decreasing percentage-wise because of an increasing dry weight.

Degree of methylation of pectic substances of Valencia orange peel. The degree of esterification of the carboxyl groups is shown by the amounts of methoxyl determined on the alcohol-insoluble solids and reported as a percentage of the anhydrogalacturonic acid (Fig. 9). The anhydrogalacturonic acid being calculated from the total CO_2 is equal to the sum of the esterified and non-esterified carboxyl groups. The percentage of esterification is determined by the amount of carboxyl groups liberated upon saponification and compared with the total carboxyl units present.

The percentage of methylation of the pectic substances of Valencia orange peel rises rapidly to about 80%, and remains relatively constant during the rest of the season. Some fluctuation was observed, but this is attributed to the inherent error in the determination of methoxyl content on alcohol-insoluble solids and biological variation. The high percentage of methylation of anhydrogalacturonic acid of the alcohol-insoluble solids is no indication that a pectic substance could be isolated from this material that would be methylated to this extent.

The percentage of methylation of anhydro-

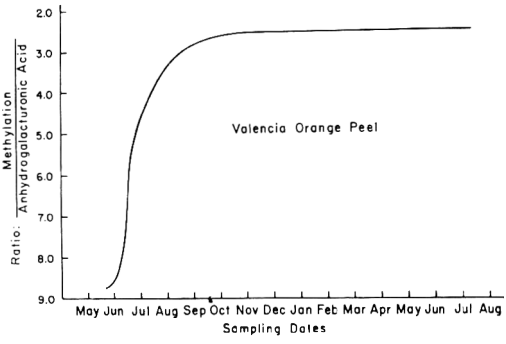

FIG. 10. Ratio of the change in percentage of methylation of the anhydrogalacturonic acid to the change in percentage of anhydrogalacturonic acid of the alcohol-insoluble solids of Valencia orange peel during the season.

galacturonic acid increases to a maximum with fruit growth; still, the percentage of anhydrogalacturonic of an alcohol-insoluble solids basis increases at a greater rate than the percentages of methylation. If the ratio of the change in percentage of methylation to the change in percentage of total anhydrogalacturonic acid on an alcohol-insoluble solids basis is graphed against time it can be seen that a ratio is reached early in the season that remains relatively constant (Fig. 10). This ratio was found to be much more consistent than the percentage of methylation as shown in Fig. 9, and there appears to be an equilibrium relationship between the percentage of methylation and the percentage of total anhydrogalacturonic acid. No trend was observed in the methoxyl content of the pulp alcohol-insoluble solids. Perhaps a period of rapid change took place during the time that insufficient pulp could be obtained for reliable analysis, and the fairly constant methylation values obtained are due to having already reached a plateau.

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Carbonyls in Oxidizing Fat. IV. The Role of Various Fatty Acid Components in Carbonyl Generation

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SUMMARY

Volatile monocarbonyls produced by mildly oxidized esters of oleic, linoleic, linolenic acids and fats have been characterized by paper chromatography of 2,4-dinitrophenylhydrazone derivatives. The unsaturated fatty acid esters yielded a total of 7 *n*-alkanals, 8 *n*-alk-2-enals, and 4 alk-2,4-dienals; and similar results were obtained with the fats. Each unsaturated acid produced three major characteristic aldehydes. Comparisons of esters and glycerides showed the presence of keto or aldehyde ester scission products.

Fats become rancid at widely varying peroxide levels (Dugan, 1959; Lea, 1953; Lips, 1952; Nikkila and Linko, 1955). This is true, to a smaller degree, even in fats of the same kind (Chipault *et al.*, 1945; Gaddis *et al.*, 1959; Naumann *et al.*, 1951). Consequently, peroxide numbers are of little specific value as indices of oxidative rancidity. Similarly total carbonyl values appear to be of limited use (Gaddis *et al.*, 1959). Although other factors (Chipault *et al.*, 1945; Dugan, 1959; Watts and Wong, 1951) are unquestionably involved, a primary factor would presumably be due to differences in fatty acid composition, with resulting variation in the kind and amount of carbonyls produced.

A considerable amount of work (Gaddis *et al.*, 1959; Lea *et al.*, 1953; Morris, 1954) has been done over the years on the volatile carbonyls formed by autoxidized unsaturated fatty acid esters and various animal and vegetable fats. For the most part, oxidation has been considerably beyond the point of rancidity, and, because of inadequate methods, analyses have been incomplete.

This work applied newly developed micromethods (Ellis and Gaddis, 1959; Ellis *et al.*, 1958; Gaddis and Ellis, 1957, 1959 a, b; Gaddis *et al.*, 1959, 1960) to qualitative determination of the volatile monocarbonyls produced from mildly oxidized esters of oleic, linoleic, and linolenic acids, and animal and vegetable fats.

MATERIALS AND METHODS

Methyl stearate, methyl palmitate, methyl oleate, and ethyl linoleate were furnished by Dr. H. B. Knight, of our Eastern Regional Laboratory. Edible-grade refined palm oil (fruitcoat) was furnished by Dr. V. K. Babayan, of E. F. Drew and Company, Inc. Highly purified oleate triglyceride and methyl linolenate were obtained from The Hormel Foundation, Austin, Minnesota. Pork, beef, and lamb fats were steam-rendered, and stored in vacuum-packed cans at −30°F until required. Cocoa butter and linseed oil were purchased from a chemical company. Soybean and peanut oils were obtained from reliable commercial sources.

Twenty-five grams of esters (8 g methyl linolenate), 25 g of mixtures of esters, and 25 g of the fats were oxidized in thin films in 11-cm-diameter containers by ultraviolet light at room temperature (28–30°C). Peroxide values were followed on 0.200 g samples by the method of Kenaston *et al.* (1955). Steam-volatile carbonyl 2,4-dinitrophenylhydrazones (2,4-DNPH) were isolated from 2–10 g unheated and heated (165°C for 15 minutes) samples by methods described by Gaddis *et al.* (1959). Volatile carbonyl 2,4-DNPH's were separated into mono- and dicarbonyl derivatives by methods described earlier (Gaddis and Ellis, 1959 b; Gaddis *et al.*, 1959, 1960). Nonvolatile and bound carbonyls remaining in the steam distillation residues were isolated by reaction with Girard T reagent and Dowex 50 cation resin at room temperature (Gaddis *et al.*, 1960). Car-

bonyl 2,4-DNPH's isolated by the Girard T reagent were fractionated into volatile and nonvolatile mono- and dicarbonyl groups (Gaddis *et al.*, 1960). Steam-volatile monocarbonyls were separated into classes, and the classes resolved into individual compounds that were identified by previously reported methods and applications (Ellis and Gaddis, 1959; Ellis *et al.*, 1958; Gaddis and Ellis, 1957, 1959a, b; Gaddis *et al.*, 1959). Total determinable carbonyls were measured by the method of Henick *et al.* (1954), Gaddis *et al.*, (1960). Iodine values were measured by the Wijs method (Official and Tentative Methods of the Am. Oil Chemists' Soc., 1954).

Methyl oleate, trioleate glyceride, and ethyl linoleate were oxidized separately and simultaneously. Amounts of oxidation permitted were such as to give a combined peroxide value of about 30 for mixtures of the two unsaturated acids commonly found in pork, beef, and lamb fat. Similarly, mixtures of methyl palmitate, stearate, oleate, and ethyl linoleate corresponding to the three animal fats were oxidized to peroxide values of about 30. Methyl linolenate was oxidized to a peroxide level of 93. No attempt was made to synchronize oxidation of this ester with that of the others, since it is ordinarily present in most animal fats in very small quantities. Finally, eight animal and vegetable fats of widely varying fatty acid composition were oxidized to peroxide values in the region of 30.

RESULTS AND DISCUSSION

The primary purpose of this paper was to report the effect of fatty acid composition on the kind of volatile monocarbonyls generated by oxidation. However, considerable quantitative data have been accumulated in the process, and it seems appropriate to give a brief account of some of the more significant findings.

Unheated and heated steam-volatile carbonyls from the mixtures of fatty acid esters, based on total determinable carbonyl content (Gaddis *et al.*, 1959; Henick *et al.*, 1954), were considerably higher than those obtained from oxidized pork fat (4% and 19% greater, respectively) (Gaddis *et al.*, 1960). Similarly, the trioleate glyceride yielded a much lower amount of volatile carbonyl than the simultaneously and equally oxidized methyl oleate. The methyl oleate contained 0.8% polyunsaturate impurities that may have influenced the course of oxidation. However, it would be expected that some scission fragments, such as keto or aldehyde acid, would be volatile in the case of the esters and should show up mostly in the "dicarbonyl" fraction. This was clearly indicated in comparison of the volatile "dicarbonyl" fractions from methyl oleate, trioleate glyceride, and mixtures of fatty acid esters. An interesting observation was that the volatile "dicarbonyl" fractions of the fatty acid esters, mixtures thereof, and also the trioleate glyceride increased greatly on heating. This was quite different from the behavior of the "dicarbonyl" fraction of beef, lamb, and pork tissue and rendered fat (Gaddis and Ellis, 1959b; Gaddis *et al.*, 1959, 1960), which showed little quantitative change. The presence of volatile carbonyl-acid fragments may not account for all of the differences since the percentage of monocarbonyls was also much higher. The differences are evidently related to retention of carboxyl carbonyls, and possibly the presence of some other kind of binding in the glyceride structure. Some of these bound and nonvolatile forms are doubtless isolated by the Girard T reagent (Gaddis *et al.*, 1960). The nonvolatile carbonyls are the major fraction, and are of considerable interest for a number of reasons (Berry and McKerrigan, 1958; Gaddis *et al.*, 1960). Evidence was unmistakable that much of the total determinable carbonyls are present in bound forms (Gaddis *et al.*, 1960). The distribution of volatile and nonvolatile mono- and dicarbonyl fractions isolated by the Girard T reagent from methyl oleate and ethyl linoleate was similar (Gaddis *et al.*, 1960). The distribution of the Girard T fractions from linolenate, however, was quite different. Linolenate volatile carbonyls isolated by the Girard T reagent were 65% of the total, and high-molecular-weight polycarbonyls were 90% of the nonvolatile fraction. Fugger *et al.* (1951) found that there was marked difference in the course of linolenate oxidation compared to that of oleate and linoleate. Scission and polymerization occurred immediately upon oxidation of linolenate.

Table 1 shows determinations of micromoles of volatile monocarbonyls per 10 g material for methyl oleate, trioleate glyceride, ethyl linoleate, and methyl linolenate.

Table 1. Total monocarbonyl values of oxidized esters.[a]

		Monocarbonyl 2,4-dinitrophenylhydrazones			
		Micromoles per 10 g			
			Per cent		
	Peroxide	Total	Alkanal	Enal	Dienal
Methyl oleate	40.8	7.63	58.2	41.8	
		35.72	29.9	70.1	
Methyl oleate	52.0	7.12	61.8	38.2	
		49.63	28.1	71.9	
Methyl oleate	71.0	15.79	60.9	39.1	
		54.89	32.7	67.3	
Trioleate	43.0	4.86	60.1	39.9	
		30.12	27.4	72.6	
Trioleate	52.0	4.94	59.7	40.3	
		26.85	32.3	67.7	
Trioleate	80.0	6.93	54.4	45.3	
		.....	.....	.....	
Ethyl linoleate	200.0	16.87	52.3	23.5	24.2
		109.56	11.5	16.3	72.2
Ethyl linoleate	336.0	29.04	52.7	19.6	27.7
		125.15	14.5	19.4	66.1
Ethyl linoleate	536.00	53.43	57.9	19.1	23.0
		172.01	24.3	12.2	63.5
Methyl linoleate	93.3	8.35	43.4	24.0	32.6
		27.77	12.4	15.4	72.2

[a] The first of each pair of lines is for unheated; the second is for heated.

In the methyl oleate and trioleate, the proportions of alkanal were greater than those of alk-2-enal, though the latter increased considerably upon heating. In the ethyl linoleate and methyl linolenate, the alk-2,4-dienal class increased greatly when exposed to heat. Since both of these polyene acids, as will be shown later, generate low-molecular-weight alkanals and alk-2-enals, an appreciable loss of these compounds probably took place when heated (Gaddis *et al.*, 1959). Also, the alk-2-enal class is in error to the extent of the amount of alkanal C_2 present, since that compound separates with that class (Gaddis and Ellis, 1959a, b).

Table 2 compares quantitative monocarbonyl class data for oxidized pork, lamb, and beef rendered fat, mixtures of palmitic, stearic, oleic, and linoleic ester, and calculated combinations of data from separately oxidized oleic and linoleic esters. The mixtures were made up as follows: pork—methyl palmitate 30.1%, methyl stearate 16.2%, methyl oleate 46.6%, and ethyl linoleate 7.1%; lamb—24.4%, 34.5%, 39.1%, and 2.0%, respectively; and beef—32.0%, 21.0%, 45.9%, and 1.1%, respectively. These are about average compositions for the three fats, but not necessarily the composition of the fats examined. Unheated proportions of classes were similar. In the heated samples, the proportions of class were about the same for the mixtures and fats. However, calculated data gave much lower proportions of alkanals and higher proportions of alk-2-enals. The total amount of monocarbonyls also was much higher in the calculated samples. Apparently the release of volatile monocarbonyls was greater in separately oxidized unsaturated acids than when they were mixed. Possibly, more polymerization took place in the mixtures.

Table 3 shows quantitative class data for animal fats and vegetable oils oxidized to approximately the same peroxide values. The data are arranged in the order of increasing linoleic and linolenic acid content. Except for linseed oil, which was initially

Table 2. Total monocarbonyl values of ester mixtures and fats.[a]

		Monocarbonyl 2,4-dinitrophenylhydrazones			
		Micromoles per 10 g			
			Per cent		
	Peroxide	Total	Alkanal	Enal	Dienal
Pork fat mixture	28.0	4.34	61.8	28.8	9.4
		10.63	39.0	36.6	24.4
Pork fat	31.0	3.50	63.4	24.0	12.6
Pork fat comb. (calculated)	33.2	9.64	35.4	27.8	36.8
		4.76	56.7	37.2	6.1
		24.42	24.1	52.9	23.0
Lamb fat mixture	31.0	5.87	59.3	33.4	7.3
		12.36	51.5	38.3	10.2
Lamb fat	30.0	4.76	53.8	41.4	4.8
		10.31	51.5	39.7	8.8
Lamb fat comb. (calculated)	27.1	3.94	59.1	32.7	8.2
		21.90	36.5	65.9	7.6
Beef fat mixture	33.0	3.93	62.3	30.3	7.4
		15.49	43.6	47.6	8.8
Beef fat	31.5	4.73	62.2	30.0	7.8
		10.31	49.2	41.6	8.9
Beef fat comb. (calculated)	27.6	3.91	60.4	35.0	4.6
		24.16	27.3	68.9	3.8

[a] The first of each pair of lines is for unheated; the second is for heated.

Table 3. Carbonyl values of oxidized fats and oils.[a]

				Carbonyl 2,4-dinitrophenylhydrazones				
				Volatile monocarbonyl				
				Micromoles per 10 g				
						Per cent		
	Iodine value	Peroxide	Total determinable absorbance λ max	Total	Dienal	Alkanal	Enal	Dienal
Beef	38.7	31.5	48.6	4.73	0.29	62.2	30.0	7.8
				10.31	1.35	49.5	41.6	8.9
Lamb	36.5	30.0	51.5	4.76	0.23	53.8	41.4	4.8
				10.31	0.91	51.5	39.7	8.8
Cocoa butter	38.3	33.0	47.3	3.00	0.35	64.0	24.3	11.7
				5.52	0.60	56.1	33.0	10.9
Pork	58.8	30.8	40.3	3.50	0.44	63.4	24.0	12.6
				9.64	3.55	35.4	27.8	36.8
Palm oil	55.9	27.0	26.5	2.61	0.29	69.7	19.2	11.1
				5.86	0.82	58.4	27.6	14.0
Peanut oil	104.9	26.0	29.8	2.20	0.41	55.5	23.6	20.9
				5.85	1.79	46.0	30.6	23.4
Soybean oil	136.1	29.3	19.9	1.43	0.18	63.6	23.7	12.7
				4.28	1.77	30.8	27.8	41.4
Linseed oil	181.0	33.0	50.4	10.45	3.33	44.0	23.6	32.4
				16.13	7.55	30.5	22.7	46.8

[a] The first of each pair of lines is for unheated; the second is for heated.

in poor condition, the total determinable carbonyl value (Gaddis *et al.*, 1960; Henick *et al.*, 1954) and monocarbonyls in general tended to decrease with increase in iodine values and polyene acids. However, the vegetable oils, two of which (cocoa butter and palm oil) are similar in composition to lamb and pork fat, were invariably lower in most of these values than the animal fats, and appeared to be in a separate group. It is therefore difficult to say at this time whether this is due to the presence of antioxidants in the vegetable oils or the higher content of polyene acids. In most cases, the percentage of alk-2,4-dienal class increased with total unsaturation and polyene acid content. Perhaps the most striking difference was in the pork fat. This showed a much greater increase in alk-2,4-dienals upon heating than other fats of the same or much higher polyene fatty acid content. Palm oil has a composition very similar to that of pork fat, but showed little change in proportion of dienals. The proportion of dienal obtained from pork fat was higher than that obtained from peanut oil, and almost as high as that from soybean oil. Peanut oil has four times as much linoleic acid, and soybean oil has seven times as much linoleic and 30 times as much linolenic acid. The extreme vulnerability of pork fat to rancidity is difficult to explain on the basis of its polyunsaturated acid content. While the above differences may be due entirely to the action of antioxidants, the possible effect of differences in glyceride structure seems worth considering. Mattson and Lutton (1958) have observed that pork fat has a unique triglyceride structure. The unsaturated acids are predominantly in the 1 and 3 positions. Beef, lamb, and vegetable oils have most of the saturated acids positioned in the 1 and 3 positions. It would seem that unsaturated acids would be more accessible to oxidation in the 1 and 3 positions than in the 2 position. This might explain the relatively much higher yield of alk-2,4-dienals from pork fat. The possibility seems worthy of further investigation.

Volatile monocarbonyls identified from methyl oleate, ethyl linoleate, and methyl linolenate are shown in Table 4. Trace and

Table 4. Volatile monocarbonyls identified in oxidized unsaturated esters.[a]

	n-Alkanals	Enals	Dienals
Methyl oleate	C_6*		
	C_8		
	$C_9\Delta$	C_8*	Trace
		$C_{10}\Delta$	
	C_{11}	$C_{11}\Delta$	
Ethyl linoleate	C_2		
	C_3	C_6*	
	$C_6\Delta$	$C_7\Delta$	
		C_8	
	C_9*	C_9	C_9*
		C_{10}*	$C_{10}\Delta$
		C_{11}*	
Methyl linolenate	C_2		
	$C_3\Delta$		
	C_4	C_5	
		$C_6\Delta$	
		C_8	$C_7\Delta$
		C_7	C_9*
		C_9*	

[a] *trace; Δ major.

major compounds are indicated. Some of the trace compounds probably result from impurities. The methyl oleate sample contained 0.8% polyunsaturates. The trace compounds tended to increase with degree of oxidation. In the most highly oxidized oleate sample, a trace of alk-2,4-dienal class was observed. Trace *n*-alkanal C_6 probably comes from linoleate. It might at first appear that trace *n*-alk-2-enal C_9 would come from linoleate, but if that were the case, the major alk-2-enal C_7 should also be present.

Little has been reported concerning the identity of the volatile monocarbonyls produced by oxidized oleate. Swift *et al.* (1948) isolated alk-2-enal C_{11} from heated oleate hydroperoxide. Bickford *et al.* (1948) found C_8, C_9, C_{10}, and C_{11} isomeric hydroperoxides formed in oxidized oleic acid. These would yield *n*-alk-2-enals C_{11} and C_{10} and *n*-alkanals C_9 and C_8.

The ethyl linoleate used was 97–98% pure and had an iodine value of 164.9. Trace compounds alkanal C_9 and alk-2-enals C_{10} and C_{11} probably come from oleate impurity. Trace alk-2-enal C_6 could come from linolenate, but may not have, because the major linolenate alk-2-enal C_5 should also be present. Chang and Kummerow (1953) found *n*-alkanals C_3, C_5, and C_6 among the volatile decomposition products of linoleate oxidative polymers. The compound *n*-alkanal C_5 was not detected in this study. Recently, Patton *et al.* (1959) isolated alkanal C_6, alk-2-enal C_7, C_9, and C_{10}, and alk-2,4-dienal C_{10} from heated methyl linoleate. Badings (1959) found alkanal C_2, alk-2-enal C_8, and alk-2,4-dienal C_{10} in oxidized ammonium linoleate.

The highly purified methyl linolenate yielded no trace compounds that could be logically traced to the other two unsaturated fatty esters. Kawahara *et al.* (1952) found alkanal C_2 and C_3 and alk-2-enal C_5 among the volatile cleavage products of oxidized linolenate. Johnson *et al.* (1953) found methyl ethyl ketone, alkanal C_2 and C_3, and alk-2-enal C_5 in the products of decomposed oxidative polymers of linolenate.

Table 5. Volatile monocarbonyls identified in oxidized mixtures of fatty acid esters.[a]

	Alkanal	Alkenal	Dienal
Pork mixture fatty acid esters	C_2		
	$C_6\Delta$		
		$C_7\Delta$	
	C_9	C_9	
	$C_9\Delta$	C_9	C_9
		$C_{10}\Delta$	$C_{10}\Delta$
	C_{11}	$C_{11}\Delta$	
			C_{12}*
Beef mixture fatty acid esters	C_2		
	C_6		
		C_7	
	C_8	C_9	
	$C_9\Delta$	C_9	C_9
		$C_{10}\Delta$	$C_{10}\Delta$
	C_{11}	$C_{11}\Delta$	
			C_{12}*
Lamb mixture fatty acid esters	C_2		
	C_6		
		C_7	
	C_8	C_9	
	$C_9\Delta$	C_9	C_9
		$C_{10}\Delta$	$C_{10}\Delta$
	$C_{11}\Delta$	$C_{11}\Delta$	
			C_{12}*

[a] *trace; Δ major.

A compound of an apparently new class was detected in small amounts in the oxidized linolenate. This separated in paper chromatography (Gaddis and Ellis, 1959a, b) slightly below the alk-2,4-dienal class, had a maximum of 390 mμ in CCl_4, and a probable carbon chain length of C_9 to C_{11}. This compound might be an alk-2,4,6-trienal (Braude and Jones, 1945; Nazarov *et al.*, 1957). A deca-2,4,6-trienal could be formed by scission of a 9-hydroperoxide of linolenate. This fraction requires further study.

Based on the above findings, an oxidized mixture of the three unsaturated fatty acids would be expected to produce the following volatile monocarbonyls: *n*-alkanals C_2, C_3, C_4, C_6, C_8, C_9, and C_{11}; *n*-alk-2-enals C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, and C_{11}; and *n*-alk-2,4-dienals C_7, C_9, and C_{10}. Volatile monocarbonyls found in oxidized mixtures of stearic, palmitic, oleic, and linoleate are shown in Table 5. The expected compounds were found, with minor exceptions: alkanals C_3 was not found. A tentative alk-2,4-dienal trace C_{12} was detected repeatedly.

Compounds found in the 8 fats and oils are shown in Table 6. Qualitative differences were as might be predicted from the composition. Over-all, there was a remarkable qualitative similarity. As indicated by variations in major compounds, and the previously discussed differences in class proportions, there were obviously great quantitative differences between fats. Tentative

Table 6. Volatile monocarbonyls identified in oxidized fats and oils.[a]

	n-Alkanals	Enals	Dienals
Pork fat	C_2*		
	C_3		
	$C_4\Delta$		
		C_7	C_7
	C_8*	C_8	
	$C_9\Delta$	C_9	C_9
		C_{10}	$C_{10}\Delta$
	C_{11}*	C_{11}	
			C_{12}*
Beef fat	C_2*		
	C_3*		
	C_3		
	C_7	C_7	C_7
	C_8	C_8	
	C_9	$C_9\Delta$	C_9*
	C_{10}	$C_{10}\Delta$	C_{10}
	C_{11}*	$C_{11}\Delta$	C_{11}
			C_{12}*
Lamb fat	C_6		
	C_7	C_7*	C_7
	C_8*	C_8*	
	$C_9\Delta$	$C_9\Delta$	C_9*
	C_{10}	$C_{10}\Delta$	C_{10}
	C_{11}	$C_{11}\Delta$	
			C_{12}*
Peanut oil	$C_6\Delta$		
		$C_7\Delta$	C_7
	C_8	$C_8\Delta$	
	C_9	$C_9\Delta$	C_9
		C_{10}*	$C_{10}\Delta$
	C_{11}	C_{11}*	
			C_{12}*

	n-Alkanals	Enals	Dienals
Palm oil	$C_6\Delta$		
		C_7	C_7
	C_8	C_8	
	C_9	C_9	C_9
		C_{10}	$C_{10}\Delta$
	C_{11}	C_{11}	
	C_{12}*		C_{12}*
Linseed oil	C_3		
	$C_3\Delta$		
	C_4	C_4	
		$C_6\Delta$	
	$C_6\Delta$	$C_6\Delta$	
		$C_7\Delta$	$C_7\Delta$
	C_9	C_9	C_9
			C_{10}
Cocoa butter	$C_6\Delta$		
		C_7	C_7
	C_8	C_8	
	$C_9\Delta$	C_9	C_9
		$C_{10}\Delta$	$C_{10}\Delta$
	C_{12}*	C_{12}	
			C_{12}*
Soybean oil	C_2*		
	C_3		
		C_5	
	$C_6\Delta$	C_6	
		$C_7\Delta$	$C_7\Delta$
		C_9	
	C_9	$C_9\Delta$	C_9
		C_{10}	$C_{10}\Delta$
		C_{11}	
			C_{12}*

[a] * trace; Δ major.

alk-2,4-dienal trace C_{12}, found in the fatty acid ester mixtures, also appears here. Alkanal C_7 and C_{10} were detected in beef and lamb fat. It will be noted that alk-2,4-dienal C_7, which is characteristic of linolenate, appears in beef, pork, and lamb, although that acid is almost a trace component in those fats.

Gaddis and Ellis (1959b) found that hexanal was the major volatile monocarbonyl in pork tissue fat oxidized at low temperature. However, the above data on rendered pork fat indicate that alkanal C_9 was equally abundant. Alkanal C_9 comes from oleate, and C_6 from linoleate. This is in line with the observation of Watts and Wong (1951) that hemoglobin oxidizes linoleic and linolenic acids selectively. The action of catalysts, enzymes (Koch *et al.*, 1959), and pro- and antioxidants, and the conditions of oxidation may have considerable influence on the qualitative and quantitative nature of the volatile monocarbonyls. Slover and Dugan (1957) found that gamma irradiation of oleate caused no significant departure from unirradiated autoxidation. However, Witting and Schweigert (1958) found alk-2,4-dienal C_{11} to be the predominant carbonyl from lard oxidized by gamma irradiation. This compound was observed by Gaddis and Ellis (1959b) as a minor component in oxidized pork tissue fat. However, it was not observed in the present work except in beef tallow. The major linoleate dienal was C_{10}. Witting and Schweigert (1958) comment on the variation of the principal dienal linoleate degradation products in different fats. They also found alkanals C_3, C_4, C_5, C_6, C_9, and C_{10} and alk-2-enals C_3 and C_4. Pippen *et al.* (1958) found acetone and methyl ethyl ketone (Gaddis and Ellis, 1957); alkanals C_2, C_3, C_4, C_5, C_6, C_8, and C_9; alk-2-enals C_5, C_6, C_7, C_{10}, and C_{11}; and alk-2,4-dienal C_7. We have not detected alkanal C_5 or alk-2-enal C_3. Traces of the latter might be missed in our systems, since it forms mixtures with alkanal C_2. The new class of monocarbonyl (a possible trienal), found in linolenate, was also detected in linseed oil.

Heating the esters or fats at 165° did not affect the qualitative composition of the volatile monocarbonyls, although in some instances small amounts of low-molecular-weight products were lost through volatilization (Gaddis *et al.*, 1959). There were large increases in volatile monocarbonyls, and doubtless large changes in relative amounts of the individual compounds similar to those shown for pork tissue fat by Gaddis and Ellis (1959b).

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The Effect of Methylation Upon the Antioxidant and Chelation Capacity of Quercetin and Dihydroquercetin in a Lard Substrate [a, b, c]

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SUMMARY

The relative importance of the hydroxyl group positions of quercetin and the metal-complexing sites of the molecule to antioxidant capacity in lard was investigated. The antioxidant action of quercetin seems to be a combination of reaction with free radicals and metal-ion complexing; with the former being the more important. Selective methylation of the hydroxyl groups of the quercetin molecule generally decreased the antioxidant activity. Ultraviolet absorption spectra of quercetin and copper–quercetin complexes suggest that quercetin will complex two moles of copper ion by intramolecular complexing and a third by intermolecular complexing. Copper–quercetin complexes retain some antioxidant ability although it is much less than that of uncomplexed quercetin.

The flavone quercetin, and the flavanone dihydroquercetin, are distributed widely in nature, where they may exist in the free form or as glucosides (Bate-Smith, 1954; Hergert and Goldschmid, 1958; Paech and Tracy, 1955). Dihydroquercetin is found in the bark of Douglas fir trees to the extent of 5%. The annual supply of this bark exceeds two million tons (Kurth and Chan, 1951).

Quercetin and dihydroquercetin as stabilizers for fats and oils are of great potential importance. Their known antioxidant ability in a wide variety of substances (Heimann *et al*, 1953; Kurth and Chan, 1951; Mehta and Seshadri, 1958; Richardson *et al*, 1947; Simpson and Uri, 1956; Weyerhaeuser Timber Co., 1957), their wide natural occurrence (Bate-Smith, 1954; Kurth and Chan, 1951; Paech and Tracy, 1955), and their apparent nontoxicity (Bate-Smith, 1954; Booth and DeEds, 1958; Griffith *et al*, 1955) suggest their possible use as food antioxidants.

The mechanism of antioxidant action by the flavonoids has not been clearly elucidated, although Lea (1958) and Kelley and Watts (1957) recently discussed two possible modes of action. Heimann *et al* (1953), Pazina (1957), Simpson and Uri (1956), and Mehta and Seshadri (1958) suggested that the antioxidant activity of the polyhydroxyflavones is primarily a function of their ability to act as free-radical acceptors. The latter two groups of workers mention that the metal-chelating property of these molecules may make some contribution to their activity.

Recent studies utilizing flavones as antioxidants have resulted in definite conclusions as to the relation of certain molecular moieties to antioxidant action. Richardson *et al* (1947) suggested that the $-C-C=C-$ (with O above the double bond) group in the pyrone ring or in the open chalcone is responsible for antioxidant activity. Heimann and Reiff (1953) investigated the antioxidant properties of certain flavone and flavone derivatives in ethyl lino-

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[b] Presented at the 20th Annual Meeting of the Institute of Food Technologists, San Francisco, May 17, 1960.

[c] From an M.S. thesis by David Crawford.

leate and concluded that: (a) the α-β-unsaturated ketone structure of the pyrone ring or in the corresponding chalcones was of decisive importance, (b) the free-hydroxyl group on C3 in the chromone ring was very important, (c) the antioxidant effect of the chromone ring system was decreased by meta hydroxyls, and (d) ortho hydroxyl groups on the phenyl side ring (pyrocatechol residue) increase the antioxidant effect of the flavones considerably.

Simpson and Uri (1956) confirmed the observations of Heimann and Reiff and added that the *p*-quinol structure in the 2-phenyl ring appears to impart considerably greater activity than does the *o*-quinol structure.

Mehta and Seshadri (1958) evaluated a large number of flavones as antioxidants for lard. Their results indicated that meta hydroxyl groups on the chromone ring do not necessarily lower the antioxidant capacity of the molecule. The position and orientation of the hydroxyl groups on the chromone ring was found to be of great importance. They suggested that the keto-enol tautomerism of the hydroxyl group on C3 and the carbonyl in the four position were major factors in the antioxidant action of the molecule.

The metal-complexing ability of quercetin and other flavonoids has been studied by many workers (Clark and Geissman, 1949; Kelley and Watts, 1957; Lewis and Watts, 1958; Pazina, 1957). Detty *et al* (1955) demonstrated the pH dependency of the metal-complexing ability of the flavones. Shifts in ultraviolet absorption maxima in various chelating solvent systems have been used in structure determinations of flavones (Jurd, 1956; Jurd and Horowitz, 1957). The complexing properties of flavones have also been used in the preparation of selectively alkylated flavones (Jain *et al*, 1953).

Theoretically, there are possibly three intramolecular chelation systems present on the quercetin molecule: (a) between the C5 hydroxyl and the carbonyl, (b) between the C3 hydroxyl and the carbonyl, and (c) between the ortho hydroxyl groups on the pyrocatechol moiety (Detty *et al*, 1955; Kelley and Watts, 1957; Paech and Tracy, 1955).

The purpose of this investigation was to evaluate the relative importance of the hydroxyl-group position on the flavone nucleus and the metal-complexing sites of the molecule to antioxidant capacity in lard.

EXPERIMENTAL

Apparatus

Active oxygen method apparatus (Am. Oil Chemists' Soc., 1959) and Beckman DK-1 Recording Spectrophotometer.

Flavonoids and derivatives

Obtained from the Oregon Forest Research Center, Oregon State College, Corvallis.

Lard

Steam-extracted lard, a sample from the middle portion of one large vat, was obtained from a local commercial source. The lard was packed at 70°C in 307 × 409 "C" enamel cans and held at −18°F until used. The lard had a free fatty acid content of 0.55% (oleic acid) (Am. Oil Chemists' Soc., 1959). No peroxides were found (Am. Oil Chemists' Soc., 1959). The iodine number (Hanus method) of the lard was 57.53 (Assoc. Offic. Agr. Chemists', 1955). After ignition, the residue from 25 g of lard gave a negative copper-ion spot test with phosphomolybdic acid (Feigl, 1958).

Antioxidant activity of the flavonoids and derivatives

Melted lard at 70°C was added to known quantities of the flavonoids in absolute ethanol (10 ml) solution to adjust their molarity to 5×10^{-4} moles per kg of lard. The ethanol was removed by holding the mixture at 70°C under reduced pressure and flushing with nitrogen for 30 minutes. The stability of the treated lard was then determined via the Active Oxygen Method (Am. Oil Chemists' Soc., 1959), using 125 meq of peroxide per kg lard as the endpoint of AOM stability.

Antioxidant activity of copper–quercetin complex

The copper–quercetin complex with the desired Cu^{++}–quercetin ratio was obtained by addition of 0.04*M* absolute ethanolic $CuCl_2$ to a known quantity of quercetin in an absolute ethanol solution. The absolute ethanol solution containing the copper–quercetin complex was adjusted to 20 ml. The concentration of the copper–quercetin complex was adjusted with lard at 70°C to 5×10^{-4} moles per kg on the basis of quercetin present. The complex was then mixed into the lard by agitation for 5 minutes and its stability determined via the Active Oxygen Method (Am. Oil Chemists' Soc., 1959), using 125 meq of peroxide per kg lard as the AOM stability endpoint.

Ultraviolet absorption spectra of quercetin and the copper–quercetin complexes

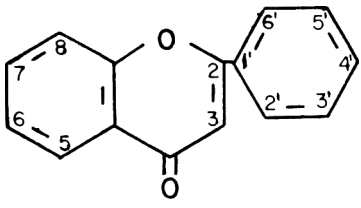
Stock solutions of quercetin and $CuSO_4$ were prepared according to the following procedure. Quercetin (5.380 mg) was weighed on a microbalance and diluted to 100 ml with redistilled 95% ethanol to give a $1.780 \times 10^{-4} M$ stock solution. From a $0.1 M$ $CuSO_4$ solution a $8.900 \times 10^{-4} M$ aqueous $CuSO_4$ stock solution was prepared. A 5-ml portion of the quercetin stock solution plus 6.0 ml of distilled H_2O was diluted to 50 ml with redistilled 95% ethanol. The copper–quercetin complexes were prepared by adding the $CuSO_4$ solution in quantities necessary to attain the desired Cu^{++}–quercetin molar ratio with 5.0 ml of the quercetin stock solution. The amount of water as added via the $CuSO_4$ solution was adjusted to 6.0 ml, and the complex diluted to 50 ml with redistilled 95% ethanol. The ultraviolet absorption spectra of quercetin and copper–quercetin complexes were determined from 220 to 500 mμ.

RESULTS

Antioxidant activity of flavones and derivatives in lard

Flavones and derivatives, with their respective AOM stability times, are listed in Table 1. The AOM oxidation curves for the determination of stability of lard containing the flavones and derivatives listed in Table 1 are shown in Fig. 1 and 2. All lard oxidation curves presented are from uninterrupted AOM analysis.

Flavone Molecule



Ultraviolet absorption spectra of quercetin and copper–quercetin complexes

The ultraviolet absorption spectra of quercetin and its copper complexes are presented in Fig. 3. The spectra of Cu^{++}–quercetin complex ratios from 1.0 to 5.0 were determined. No shift in absorption maxima was observed beyond a ratio of 3.0.

Table 1. AOM stability of lard containing flavonoids.

Compound at $5 \times 10^{-4} M$/kg of lard	AOM stability (hr)
3, 5, 7, 3′, 4′–pentahydroxyflavone (quercetin)	66.1
3, 5, 7, 3′, 4′–pentahydroxyflavanone (dihydroquercetin)	47.0
3, 5, 7, 2′, 4′–pentahydroxyflavone (morin)	27.0
3, 5, 3′, 4′–tetrahydroxy–7–methoxy-flavone (rhamnetin)	48.6
3, 7, 3′, 4′–tetrahydroxy–5-methoxyflavone	59.7
3, 3′, 4′–trihydroxy–5, 7–dimethoxyflavone	37.7
5, 3′, 4′–trihydroxy–3, 7–dimethoxyflavone	45.8
5–hydroxy–3, 7, 3′, 4′–tetramethoxyflavone	8.9
3,5,3′,4′–tetrahydroxy–7–benzyl-oxyflavone	71.0
3,5,3′–trihydroxy–4′,7–dibenzyloxy-flavone	10.8
Lard	8.8

Antioxidant activity of the copper–quercetin complexes

The Cu^{++}–quercetin ratios and the relationship of the complex substituents to the lard are illustrated in Table 2. The AOM stability time is also given for the substrate and the substrate plus quercetin, copper ion, and copper–quercetin complexes. The endpoints for the substrate plus copper ion and for the copper–quercetin complexes are not exact AOM procedure endpoints. Only single determinations from one tube below and above 125 meq were obtained. Peroxide formation, once initiated, was very rapid.

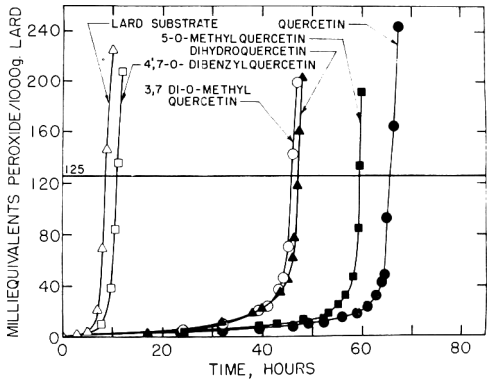


FIG. 1. AOM oxidation curves of lard containing flavonoids.

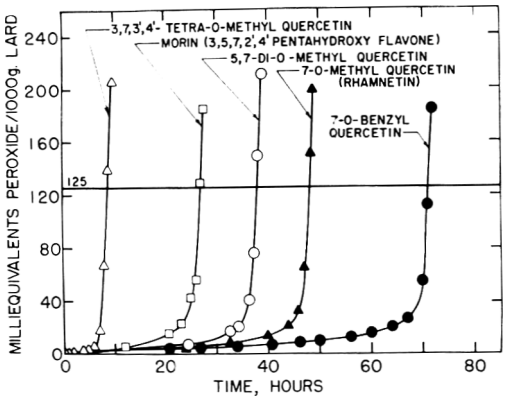


FIG. 2. AOM oxidation curves of lard containing flavonoids.

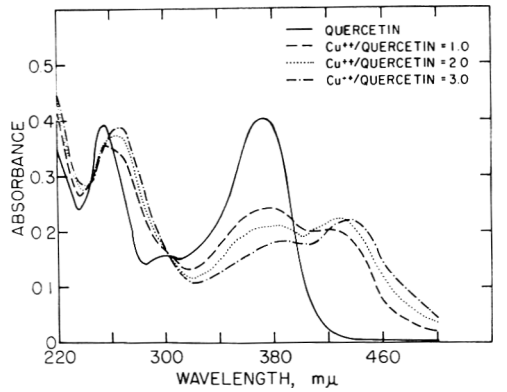


FIG. 3. Ultraviolet absorption spectra of quercetin and its copper complexes.

This is illustrated by the complete oxidation curves of the substrate plus copper ion and copper–quercetin complexes in Fig. 4. The relationship between the ratio of Cu++–quercetin and AOM endpoint time as listed in Table 2 is graphically presented in Fig. 5.

DISCUSSION

Polyhydroxyflavones possess molecular structural features that are required of antioxidants to function as free-radical acceptors and as metal-complexing agents. It is difficult to evaluate the exact contribution of each mode of action to the total antioxidant capacity. Changes in the number and position of the hydroxyl groups on the flavone nucleus probably influence both modes of action.

In this study the number and positions involved in metal complexing were altered

Table 2. Relationship of copper–quercetin complex substituents to the AOM stability of lard.

Concentration of complexing substituent		Cu++, quercetin	AOM stability
Moles quercetin, kg lard	Moles Cu++/kg lard		125 meq
			8.8
5×10^{-4}			66.1
5×10^{-4}	1.25×10^{-4}	0.25	10.6
5×10^{-4}	2.5×10^{-4}	0.50	9.1
5×10^{-4}	5.0×10^{-4}	1.0	6.7
5×10^{-4}	10.0×10^{-4}	2.0	6.8
5×10^{-4}	15.0×10^{-4}	3.0	5.4
	5.0×10^{-4}		2.1

by selective alkylation of the hydroxyl groups on the quercetin molecule. It was anticipated that the differences in antioxidant capacity of these derivatives would indicate the relation of the various complexing

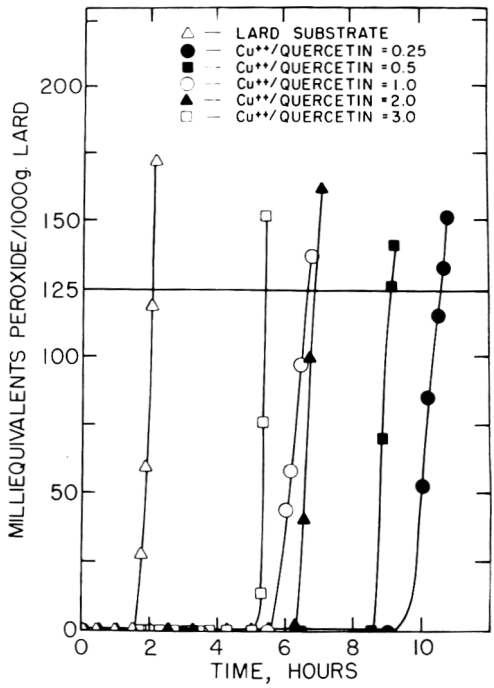


FIG. 4. AOM oxidation curves of lard containing copper–quercetin complexes.

sites to the antioxidant capacity of the molecule. At the same time, the importance of the metal-complexing mechanism could be evaluated.

The results indicate that both mechanisms operate. The exact contribution of each

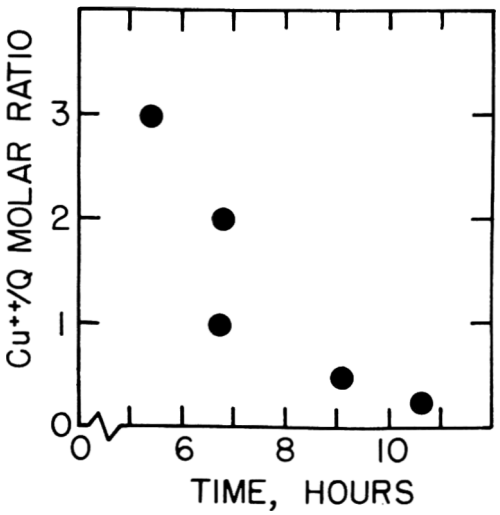


FIG. 5. Relationship of the complexed Cu^{++}–quercetin molar ratios to AOM stability.

mechanism to total antioxidant ability was not readily apparent from the experimental results.

Certain conclusions can be drawn regarding the relation of the hydroxyl groups to the antioxidant capacity of quercetin. Methylation of the hydroxyl groups of quercetin results in a decrease in antioxidant capacity. This finding is in partial agreement with experimental results of Simpson and Uri (1956), Uri (1958), and Heimann and Reiff (1953).

Benzylation of the seven hydroxyl positions on the chromone ring of quercetin increases the antioxidant capacity of the molecule. The reason is not readily apparent. The resonance contribution of the benzyl substituent may be a possible explanation of the increased antioxidant effect. Further investigation of C7 hydroxyl substitution is indicated.

The free ortho hydroxyl groups in the 3′, 4′ positions on the 2-phenyl ring of quercetin are essential to antioxidant capacity. The much greater antioxidant ability of 7-benzylquercetin over the 4′, 7-dibenzylquercetin in lard is evidence of the importance of the 4′ position. Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone), with free meta hydroxyl groups (2′, 4′), is a markedly poorer antioxidant than quercetin; this further verifies the importance of ortho hydroxyl groups on the quercetin molecule.

Methylation affects the antioxidant contribution of the C3 hydroxyl position only slightly. The difference in stability between lards treated with 7-methylquercetin and 3, 7-dimethylquercetin was only slight. No compounds were tested without the hydroxyl group in the C3 position, hence no conclusion can be drawn from this work as to the importance of its presence. However, in consideration of the importance attached to the C3 hydroxyl group by other workers (Heimann and Reiff, 1953; Mehta and Seshadri, 1958; Simpson and Uri, 1956), the result obtained is unexpected.

The C5 hydroxyl group is of minor importance to the antioxidant capacity of quercetin. This fact is illustrated by the absence of antioxidant protection for lard afforded by the 3, 7, 3′, 4′-tetramethylquercetin. Methylation of only the C5 hydroxyl group slightly decreases the antioxidant capacity of quercetin.

Considerable decrease in antioxidant action is observed when the C7 hydroxyl group is methylated. When both the C5 and C7 hydroxyl groups are methylated, even further decrease results. This decrease is even greater than that obtained by methylation of the C3 and C7 hydroxyl groups. Therefore, the C5 and C7 hydroxyl groups together must influence the antioxidant capacity of the molecule.

The reduction of the double bond between C2 and C3 of the chromone ring of quercetin results in an appreciable loss in antioxidant capacity. This is illustrated by the greater stability exhibited by lard with additions of quercetin than with dihydroquercetin. This result is in agreement with other workers (Clark and Geissman, 1949; Heimann and Reiff, 1953; Kurth and Chan, 1951).

Ultraviolet absorption maxima shifts of quercetin when molar ratios of copper ion are added demonstrate that complexes of quercetin and copper ion are being formed. The absorption maxima did not change beyond a Cu^{++}–quercetin molar ratio of 3.0. Apparently, the metal-complexing capacity of quercetin is saturated with three moles of copper ion in this solvent system. The large maxima shift on the addition of one and two moles of copper ion per mole of

quercetin suggests intramolecular complexing. The small maxima shift exhibited when a third mole of copper is added probably indicates intermolecular complexing.

The retention of some antioxidant capacity by copper–quercetin complexes is illustrated by the results listed in Table 2. The copper-quercetin complexes of the molar ratios indicated were prepared and then added to the lard. Lard treated with these complexes was more resistant to oxidation than lard to which copper ion alone was added. The degree of protection afforded the lard by the complexes was much less than that given by uncomplexed quercetin. It should be noted that, once a peroxide value was obtained (Fig. 4), the reaction proceeded at almost an instantaneous rate. This is in contrast to the much slower accumulation of peroxides observed with quercetin and its derivatives. It is evident also from Table 2 and Fig. 5 that the oxidation rate is dependent on the copper concentration in the complexes. This dependency exists up to a molar ratio of Cu^{++}–quercetin of one and beyond the ratio of two.

These results suggest certain conclusions. Copper-complexed flavonoids cannot act in their full capacity as free-radical acceptors. This is shown by the AOM stability of lard treated with complexed and uncomplexed quercetin. Since molar ratios of Cu^{++}–quercetin of one and two exhibit the same protection for lard, an equilibrium between the complex and the uncomplexed substituents is suggested. The rapid change in the oxidation pattern of the lard treated with the complexes may be interpreted as additional evidence of this equilibrium. Further investigation is necessary for confirmation.

ACKNOWLEDGMENT

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The Carbohydrates in the Peel of Oranges and Grapefruit [a]

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(Manuscript received May 31, 1960)

SUMMARY

The carbohydrates of the citrus peel were divided according to their solubility in 80% ethanol. The alcohol-soluble solids were composed of about 80% total sugars. Free xylose was found in trace amounts. Glucose, fructose, and sucrose were the main sugars present. Sucrose content of the peel was in general lower than the total reducing sugars. The alcohol-insoluble solids were separated into "pectic substance," "hemicellulose," and "cellulose" fractions by extracting with different solvents. The "pectic substance" fraction upon hydrolysis yielded arabinose, galactose, and galacturonic acid. "Hemicellulose" fraction contained xylose, arabinose, galactose, glucose, and some uronic acids in its hydrolysate. In the "cellulose" fraction, glucose was dominant with some xylose and arabinose being also present. Traces of galactose and some uronic acids and in some cases mannose were found. On the average only between 53 and 70 per cent of the alcohol-insoluble solids were recovered as carbohydrates in the peel of various citrus varieties.

The peel of citrus fruit is becoming increasingly important to the Florida citrus industry, not only because of its relationship to the storage quality and external appearance of the fruit but also because of its actual value in the production of dry animal feed and molasses. In addition, various by-products of citrus peel are being developed from time to time. The composition of citrus peel has not been studied as extensively as that of the edible portion of the fruit, although its study may prove helpful in better understanding the condition of the fruit during maturation.

With few exceptions, the same components that occur in the juice are usually found in the peel, although in different proportions. For example, pulp-free juice contains no cellulose. The dominant components of the peel alcohol-insoluble solids are cellulose, hemicellulose, and pectic substances, with proteins occurring only in small amounts. In the juice, proteins may be as high as 25% of the alcohol-insoluble solids (Ting, 1958). The alcohol-soluble fraction of the peel contains various components such as sugars, amino acids, organic acids and their salts, flavonoids, vitamins, and volatile component.

Carbohydrates of the peel are by far the most abundant of all the constituents. Economically these are probably also more valuable than any other group of substances found in the peel. Sinclair and co-workers (Sinclair and Crandall, 1949; Sinclair and Jolliffe, 1960) found that over two-thirds of the dry weight of mature grapefruit peel was alcohol-soluble. In the orange this value was over 50% (Sinclair and Crandall, 1953). The soluble sugars are the major components of the alcohol-soluble solids. Poore (1934) reported 8.68% sugar on fresh-weight basis in California grapefruit peel, and 6.35% in Florida grapefruit peel. On a dry basis Harvey and Rygg showed that the total sugars varied between 29 and 44% in orange peel (Harvey and Rygg, 1936a) and between 21 and 35% in grapefruit peel (Harvey and Rygg, 1936b). Swift and Veldhuis (1957) determined the soluble solids of the peel juice refractometrically, and found the range between 11 and 15% for Florida oranges while the total sugars ranged between 8 and 12%. Although little is known about the sugar composition of the alcohol-soluble carbohydrates, it is generally believed that the three main

[a] Florida Agricultural Experiment Stations Journal Series, No. 1093. In cooperation with Florida Citrus Commission.

sugars found in citrus juices (McCready *et al.*, 1950) are also present in the peel.

The alcohol-insoluble solids components of the citrus peel have been studied by Sinclair and Crandall (1949, 1953) as to the total pentosan and polyuronide content. With the aid of chromatography the identification and estimation of the carbohydrates in both the alcohol-soluble and -insoluble fractions have been facilitated. By extraction with different solvents, the alcohol-insoluble solids may be divided into several major groups such as pectic substances, hemicellulose, and cellulose. Each of these groups contains a mixture of polysaccharides that are difficult to separate but on hydrolysis yield a number of carbohydrate monomers. Although the polysaccharides cannot be easily separated, the sugars from each group can be readily identified and estimated on paper chromatograms.

MATERIALS AND METHODS

Sampling. Samples of 40 fruits, obtained at monthly intervals from four labeled trees at Lake Alfred during the 1957–58 season, included three varieties of oranges (Hamlin, Pineapple, and Valencia) and one of grapefruit (Marsh). Sampling of the Marsh grapefruit began in August, when the fruit was quite immature. Sampling of Hamlin and Pineapple oranges started in October. The December samplings of all varieties were made the day after the severe freeze of that year. After the freeze, only two samples of Hamlin orange and one each of the Marsh grapefruit and Pineapple orange were made. Sampling of Valencia orange was continued through April. None of the trees from which the samples were taken was damaged by the freeze, though all trees dropped some fruit.

The fruit was brought to the laboratory, weighed, and peeled. The peel included both the albedo and flavedo. Longitudinal slices of the peel from each fruit were removed, and between 100 and 200 g were collected. The samples were preserved in 80% alcohol in a manner similar to that of Sinclair and Crandall (1949) and stored in sealed jars at 0°C until analyzed.

Preparation of alcohol-insoluble solids. Alcohol-insoluble solids were prepared according to Sinclair and Crandall except that a Waring blender Model CB-2 was used at top speed (16000 rpm) to grind the sample. Three extractions were made, and the final alcohol extraction showed only a trace of soluble carbohydrate when tested with anthrone. The alcohol-insoluble solids were air dried, and finally dried at 60°C and weighed. The dried solid was ground in a Wiley mill through a 40-mesh screen and stored in tightly stoppered bottles. Moisture content of the sample was determined by drying 12 hours at 100°C.

The three alcohol extracts were combined and concentrated to 250 ml in a flash evaporator under reduced pressure. Aliquots were dried 24 hours at 75°C, and finally 6 hours at 100°C. The total weight of the alcohol-soluble solids and the moisture-free alcohol-insoluble solids was considered the dry weight of the sample. Inevitably all the volatile substances were lost on drying. During storage and evaporation of the alcohol-soluble extractives, some precipitate was formed. It was collected and incorporated with the alcohol-insoluble solids. Tests indicated it to be mainly flavanones (Davis, 1947).

Chromatographic technique. The alcohol-soluble carbohydrates were spotted directly on Whatman No. 1 filter paper. The alcohol-insoluble solids were chromatographed after fractionation and hydrolysis. Two irrigating solvents used were: (I) butanol–pyridine–benzene–water (50:30:4.5:30, v/v), and (II) butanol–acetic acid–water (4:1:5, v/v). In solvent (I), 16–24 hours were sufficient to separate all the sugars. With solvent (II), 24–32 hours were required. Sugar spots were revealed by spraying the developed and dried chromatograms with 10% ammoniacal silver nitrate, para-anisidin phosphoric acid, or aniline phthalate (Block *et al.*, 1958). Known quantities of various sugars were spotted as standards on each chromatogram. Triplicate chromatograms of each sample were made and compared visually with the standards on each chromatogram. The results thus obtained were only semiquantitative but did give approximate amounts.

Sugar analysis. Total reducing sugars, fructose, and total sugars were determined on separate aliquots of the alcoholic extract by a colorimetric method (Ting, 1956). Treatment of the extract with neutral lead acetate did not significantly change the amount of sugars found in the peel extract. Glucose and sucrose were calculated.

Fractionation of the alcohol-insoluble solids. Two and a half grams of the alcohol-insoluble solids were shaken with 400 ml of 0.05*N* sodium hydroxide solution and allowed to stand for 30 minutes. The mixture was adjusted to pH 5.5 with glacial acetic acid. Five ml of filtered aqueous extract of Pectinol 100-D (10 g/100 ml) were added and allowed to stand for 4 hours at room temperature before filtering. The filtrate was covered with toluene and let stand overnight at room temperature. The residue was washed with alcohol, dried, and weighed. Hemicellulose was extracted from the residue in a 50-ml Erlenmeyer

flask according to Jermyn and Isherwood (1956) with only one extraction with 50 ml of 4*N* KOH. The acidified extract was precipitated with 80% ethanol, washed with acetone, dried, and weighed.

Hydrolysis of different fractions. The remaining crude cellulose was hydrolyzed by dissolving 0.5 g in 4 ml of 72% sulfuric acid and letting stand at room temperature for 2 hours. The mixture was then diluted with 360 ml of water, refluxed for 6 hours, and the sulfuric acid removed by precipitation with barium carbonate. After filtering, the solution was concentrated and chromatographed. The Pectinol-hydrolyzed fraction was concentrated to 125 ml and chromatographed directly. Two-tenths of a gram of the crude hemicellulose was hydrolyzed in a sealed tube with 1*N* HCl and chromatographed.

RESULTS

The alcohol-soluble and total solids in the orange and grapefruit peel. Except in Valencia, the dry weight (total solids) of the peel did not show any consistent trends (Table 1). The alcohol-soluble solids, however, increased with season in the varieties examined. About one-third of the dry weight of the immature fruit was soluble in 80% alcohol. As the fruit ripened, the soluble solids in some instances exceeded the insoluble solids.

Free sugars in the alcohol extract. Besides the presence of glucose, fructose, and sucrose, paper chromatograms also showed traces of free xylose and rhamnose (Fig. 1). The quantities of xylose and rhamnose were about 1–2 mg per 100 g fresh peel. Concentrated extract was used to show the presence of these two sugars on the chromatogram.

The monthly values for fructose, glucose, and sucrose as determined colorimetrically appear in Table 1. In Marsh grapefruit and Valencia orange, fructose and glucose occurred in about equal amounts. Fructose

Table 1. Seasonal differences of the sugar composition of orange and grapefruit peel (1957–58 season).

Time of sampling	Dry weight %	Alcohol-soluble solids	% dry wt			Total sugars
			Glucose	Fructose	Sucrose	
Hamlin orange						
Oct	25.39	39.2	7.6	9.6	13.9	31.1
Nov	24.79	48.0	10.1	14.2	14.4	38.8
Dec	26.43	49.7	10.3	16.8	11.8	38.8
Jan	27.54	44.4	12.6	14.9	8.6	36.2
Feb	27.50	52.3	14.0	17.5	10.2	41.7
Pineapple orange						
Oct	25.56	36.8	6.3	10.4	14.3	31.0
Nov	26.79	44.2	5.2	15.0	13.8	34.0
Dec	28.31	44.5	8.3	15.8	10.4	34.5
Jan	21.02	54.8	10.8	21.2	13.9	45.8
Valencia orange						
Dec	26.11	29.5	7.8	12.1	5.1	25.1
Jan	24.45	37.2	10.6	11.8	8.0	30.4
Feb	21.21	38.4	12.6	11.5	6.0	30.0
Mar	18.87	41.3	15.2	13.2	5.3	33.7
Apr	19.52	42.3	10.9	10.0	16.5	37.4
Marsh grapefruit						
Aug	17.30	31.4	11.7	10.4	4.0	26.2
Sep	16.74	33.4	8.2	8.3	9.0	25.5
Oct	18.36	43.1	7.2	6.8	16.2	30.3
Nov	19.31	45.1	8.5	7.2	20.9	36.7
Dec	19.71	48.9	11.6	12.8	14.3	38.7
Jan	17.91	50.2	13.5	13.5	11.9	39.0

FIG. 1. Free sugars in the alcohol extracts of orange and grapefruit peel. Chromatogram irrigated 20 hr with butanol, pyridine, benzene, and water (5:3:0.45:3 v/v), and sprayed with para-anisidin phosphate. K) known sugars; 1) sucrose; 2) glucose; 3 fructose and arabinose; 4) xylose; A) orange peel; B) grapefruit peel. 1) sucrose; 2) glucose; 3) fructose; 4) xylose; 5) rhamnose.

was higher than glucose in the Hamlin and Pineapple varieties. This was particularly evident in the latter, since the fructose content was nearly twice that of the glucose. Total reducing sugars (glucose and fructose) are higher than sucrose except for the October and November sampling of Marsh grapefruit. The concentration of the total sugars, like that of the alcohol-soluble solids, increased with season. Between 70 and 85% of the alcohol-soluble solids appeared to be sugars.

Sugars in the hydrolysates of various fractions of the alcohol-insoluble solids. The alcohol-insoluble solids ranged from about 70% of the total solids in the immature fruit to about 50% of those in mature fruit. The "pectic substance" fraction removed by the 0.05*N* NaOH and Pectinol solution amounted to about 50% of the alcohol-insoluble solids (Table 2), and the crude cellulose after the extraction of hemicellulose by 4*N* potassium hydroxide solution ranged between 30 and 40%. Except in Valencia, the crude cellulose fraction seemed to be lower in more mature fruit. The hemicellulose fraction obtained was about 10% for all samples.

The monosaccharides in the hydrolysates of various fractions of the Pineapple orange peel are shown in Table 3. The other varieties had similar monosaccharide composition. Almost all of the galacturonic acid was found in the "pectic substance" fraction, together with arabinose and galactose. The hemicellulose fraction contained xylose, arabinose, glucose, galactose, and traces of uronic acids and rhamnose. Glucose was by far the most abundant monosaccharide in the hydrolysate of the crude cellulose fraction. Xylose, arabinose, and another uronic acid were also present. Traces of galactose were also found in this fraction, and mannose was observed in some samples.

Since no clear separation of each polysaccharide was obtained, the assumption was adopted that each sugar was originally derived from a single polysaccharide (Jermyn and Isherwood, 1956). Thus, arabinose from all three fractions was considered as derived from the polysaccharide araban. Since there were no marked monthly trends, averages for the season were used. Table 4 shows the amounts of various polysaccharides in the alcohol-insoluble solids.

Table 2. Percentage distribution of various fractions in the alcohol-insoluble solids of orange and grapefruit peel.

Date of sampling	Alcohol-insoluble solids (% DW)	Fractions (% alcohol-insoluble solids)			Not accounted for
		Pectic substance	Hemicellulose	Cellulose	
Hamlin orange					
Oct	60.8	46.2	10.2	38.7	6.9
Nov	52.0	50.1	10.3	35.3	4.3
Dec	50.3	55.4	8.5	31.2	4.9
Jan	55.6	50.4	8.9	33.9	6.8
Feb	47.7	54.5	9.9	31.1	4.5
Pineapple orange					
Oct	63.2	53.4	9.9	30.2	6.5
Nov	55.8	55.1	10.3	29.1	5.5
Dec	55.5	58.0	10.8	24.2	7.0
Jan	45.2	56.5	9.9	26.6	7.0
Valencia orange					
Dec	70.5	59.0	11.7	24.8	4.5
Jan	62.8	56.7	10.8	26.5	6.0
Feb	61.6	59.2	12.6	21.2	7.0
Mar	58.7	50.8	12.2	31.0	6.0
Apr	57.7	51.1	11.3	30.4	7.2
Marsh grapefruit					
Aug	68.6	44.0	9.4	39.5	7.1
Sep	66.6	42.5	12.1	40.4	5.2
Oct	56.9	42.1	12.4	40.3	5.2
Nov	54.9	48.3	11.4	35.2	5.1
Dec	51.1	46.1	10.2	37.7	6.0
Jan	49.8	52.4	11.7	30.8	5.1

DISCUSSION

The free sugars of the peel of oranges and grapefruit amounted to as much as 50% of the total solids or dry weight. As the fruit ripened, the sugar concentration in the peel increased in all varieties. Similar trends for the increase of sugar concentration in the peel with ripening were shown by Swift and Veldhuis (1957). They found a greater percentage of the peel juice to be of a non-sugar nature than that of the segment juice. Our results showed that about 80–90% of the alcohol-soluble solids were accounted for by the sugars. Undoubtedly, the salts of organic acids, nitrogenous materials, and some flavonoids are the major constituents of the non-sugar fraction of the alcohol-soluble material of the peel. Swift and Veldhuis also found that the peel juice had higher ash content than the segment juice, but concluded that the difference was not enough to account for the higher non-sugar solids unless the

Table 3. Monosaccharides in the hydrolysate of various fractions of Pineapple orange peel alcohol-insoluble solid.[a]

Fraction	Arabinose	Xylose	Galactose	Glucose	Galactose uronic acid	Other uronic acids	Mannose	Rhamnose
Pectic substance	+	trace	+	—	+++	++	—	—
Hemicellulose	++	+++	+	+	trace	trace	—	trace
Cellulose	++	+	trace	+++	—	++	trace[b]	—

[a] (+) signs indicate relative amounts of each monosaccharide in each fraction.
[b] Found only in some samples.

Table 4. Polysaccharides[a] in the alcohol-insoluble solids of citrus peel.[b]

Variety	Araban	Galactan	Xylan	Glucosan	Polygalacturonic acid	Other uronic acids	Total
			(Per cent of alcohol-insoluble solids)				
Hamlin orange	9.3	6.1	2.6	20.8	23.2	0.7	62.7
Pineapple orange	7.0	4.8	2.2	15.5	23.5	0.4	53.0
Valencia orange	9.7	5.3	2.1	14.8	23.6	0.5	56.0
Marsh grapefruit	9.9	4.9	2.7	27.9	23.4	1.2	70.0

[a] Factors used to convert monosaccharides to polysaccharides were respectively 0.88, 0.90, and 0.92 for pentoses, hexoses, and hexuronic acids.
[b] Mean values for entire season.

salts involved acids of very high molecular weight. The major organic acid in the peel is L-quinic (Ting and Deszyck, 1959), which has an equivalent weight nearly three times that of malic or citric acids. Sinclair and Crandall (1949) and Sinclair and Jolliffe (1960) found that total sugar accounted for about 60% of the total alcohol-soluble solids in grapefruit peel, and for 55.4 and 72.7% in the peel of Valencia and navel oranges, respectively. These values are lower than those found in the present experiment. This discrepancy could be due to the difference in methods by which the alcohol-soluble solid values were obtained. During the storage and evaporation of the alcohol extractive, the flavanone, glycosides of the peel, amounting to about 1% of the fresh weight (Hendrickson and Kesterson, 1954; Kesterson and Hendrickson, 1953), were precipitated, dried, and incorporated into the alcohol-insoluble solids.

The presence of free xylose is interesting. Since the amount is very small in comparison with other reducing sugars, its detection and estimation would have been almost impossible without paper chromatography. Axelrod and Seegmiller (1954) found traces of xylose in apple tissue.

It is generally believed that the citrus peel has sugar composition similar to that of the juice. From August to November, the total sugar content of the peel of grapefruit increased only slightly. There was an accumulation of sucrose and a simultaneous decrease in total reducing sugars. This trend then reversed, showing a decrease of sucrose with an increase in reducing sugars from November through January (Table 1). Since no early samplings were made on very immature fruit of the two early varieties of oranges, the period between October and January was comparable to the later period of the grapefruit. During this period, the reducing sugars of both of these varieties of oranges increased, and sucrose decreased in the Hamlin variety. Valencia orange in Florida matures during March and April, when the new growth takes place, and reaches its peak maturity about May. There was a slight increase in total reducing sugar from December through March. The last sample showed a significant increase in sucrose. This may be due to the decrease in rate of new growth.

The fractionation of the alcohol-insoluble carbohydrates into individual polysaccharide was not accomplished. However, the polysaccharides in each fraction showed their characteristic pattern of monoses upon hydrolysis. Polygalacturonic acids and cellulose glucosan were by far the predominant polysaccharides of the peel. The araban and galactan occurring in various fractions amounted to as much as 50–60% of the polygalacturonic acids. The amount of xylan was quite small and showed little difference among varieties. The cellulose glucosan was higher in grapefruit and Hamlin orange than in the other two varieties of orange. The glucose found in the hemicellulose fraction may or may not have its origin in cellulose. No test was made for starch, which has not been reported in citrus fruit.

The polysaccharide determination by the comparison of the monosaccharides in the hydrolysate with known sugar standards is at best a rough estimate. Even so, a general evaluation of the amounts of simple sugars in the various components was obtained.

The extraction with 0.05*N* sodium hydroxide and Pectinol solution removed 40–60% of the total alcohol-insoluble material of the various samples. The carbohydrate component of this fraction consisted mainly of pectin and its associated polysaccharides, araban and galactan, which were all hydrolyzed by the enzymes in Pectinol. Mineral acid hydrolysis of this fraction did not further increase its sugar content. Only about 60% of the extracted portion was accounted for by sugars and uronic acids. Protein, salts, and flavanone glycosides were probably the other major constituents extracted.

When the hemicellulose extracted by caustic was precipitated by alcohol in an acidified solution, 5–7% of the total alcohol-insoluble solids remained soluble in the alcohol. This portion was not recovered. Anthrone test indicated that some carbohydrate material was present, but the amount could not account for the total weight loss. Hydrolysis of hemicellulose gave only 55-70% yield of sugars as estimated by paper chromatography. The crude cellulose fraction also yielded only between 65–85% of its weight in sugars. Incomplete hydrolysis and decomposition of the monosaccharides during hydrolysis may be two of the main causes of the low yield. No attempt was made to account for the discrepancies.

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Destruction of *alpha*-Tocopherol by *gamma*-Irradiation[a]

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SUMMARY

Irradiation (Co^{60} source) of solutions of DL-α-tocopherol caused extensive destruction of this vitamin and gave rise to products similar to those obtained by autoxidation. Irradiation in saturated solvents (mineral oil, methyl myristate) destroyed more tocopherol than did irradiation in unsaturated solvents (methyl oleate, methyl linoleate). Destruction by 2.0 Mrad of irradiation increased with increasing tocopherol concentration, but the increase became negligible above 0.5% tocopherol in the saturated and 0.1% tocopherol in the unsaturated solvents.

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INTRODUCTION

Most vitamins are partially destroyed by irradiation, and it has been suggested (Becker *et al.*, 1956) that destruction of vitamin E was responsible for a lowered reproduction rate in rats fed irradiated foods. Vitamin E (α-tocopherol), a naturally occurring antioxidant, reduced the oxidation of methyl oleate and linoleate during irradiation (Pollister and Mead, 1954). The α-tocopherol was presumably destroyed during irradiation of these esters, but no data appear to be available on the extent of destruction under various conditions. A study of the destruction of α-tocopherol in mineral oil, and in methyl esters of saturated and unsaturated fatty acids, was therefore undertaken. The results are reported herewith.

MATERIALS AND METHODS

An NF grade of DL-α-tocopherol (Merck and Co.) was used throughout these studies. Pharmaceutical-grade mineral oil was purified by passage through a silica-gel column. Methyl oleate and linoleate of high purity (Hormel Foundation) were used without further purification. Methyl myristate was prepared from myristic acid, and purified by redistillation.

Tocopherol was determined by the iron-dipyridyl method after washing a petroleum-ether (boiling range 90-120°C) extract of the mixture with 80% sulfuric acid, followed by potassium hydroxide (Lips, 1956; Parker and McFarlane, 1940). Optimum concentrations of dipyridyl (0.2%) and ferric chloride (0.08%) were used. All solvents used in the determination were purified by redistillation and, where required, treated as directed. Light was excluded during the determination. Color density was determined by 515 mμ with an Evelyn colorimeter.

Accurately weighed portions (0.4985–0.5127 g) of the desired mixtures were irradiated in unsealed 13 × 100-mm glass tubes in a "Gamma-cell 220" containing approx 15,000 curie Cobalt-60. (Thanks are extended to the Commercial Products Division, Atomic Energy of Canada, Ltd., for making this apparatus available to us.) Under the conditions used the dose rate was 1.4 Mrad/hr. The temperature of the samples increased from ambient to 45°C during irradiation.

Control samples were treated in an identical manner except that they were placed in a cold oven and warmed to 45°C over a 20-minute period, then held at this temperature for the remainder of the period required for irradiation of the test samples. This procedure approximately duplicated the conditions measured in the "gamma-cell" when a thermocouple was inserted with the samples.

Analysis of both control and irradiated samples was begun as quickly as feasible after completion of the irradiation, usually within one hour. For simplicity, it was assumed that the amount of tocopherol found in the control samples represented the amount present when irradiation began, and that the amount found in the test samples was the amount present at the end of the irradiation period. The amount of added tocopherol recovered from control samples varied from about 99% in mineral oil to 50% in the unsaturated esters. However, all samples were prepared 18–24 hours in advance of irradiation, and, assuming a logarithmic decrease in tocopherol concentration with time, it can be calculated that autoxidation during and after irradiation would result in a maximum underestimation of 4% in the amount irradiated and of 2% in the amount destroyed.

[a] Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada. Issued as N.R.C. No. 6243.

Irradiation yields (G values) were calculated as:

$$\frac{\text{No. molecules tocopherol destroyed} \times 100}{\text{Irradiation dose (electron volts)} \times \text{weight of sample}}$$

Thus, the values obtained include surface effects, etc., but since these conditions were essentially constant the results are comparable between samples and between solvents.

No attempt was made to determine the extent of oxidation of the methyl esters during irradiation.

RESULTS

Undiluted DL-α-tocopherol was found to be very resistant to irradiation destruction, a dose of 25 Mrad having no detectable effect on the infrared or ultraviolet spectra, or on the reducing power. Spreading the tocopherol in a thin layer over glass beads during irradiation did not detectably increase its destruction. The ultraviolet spectrum of α-tocopherol irradiated in silicone (data not reported) showed the formation of quinones and conjugated material, as previously observed following autoxidation in this inert solvent (Lips, 1957). The infrared spectra of irradiated tocopherol–mineral oil mixtures was also similar to those of the same mixtures heated to high temperatures.

Irradiation (2-Mrad dose) of varying concentrations of tocopherol in mineral oil or in methyl myristate caused considerably more destruction of the vitamin than did irradiation in methyl oleate or methyl linoleate (Fig. 1). In all four solvents the amount destroyed increased with increasing tocopherol level, but the increase per unit increase in tocopherol became small at the higher tocopherol levels studied, and was less than the errors of measurement at levels above 500 μg tocopherol per 0.5-g sample in oleate and linoleate.

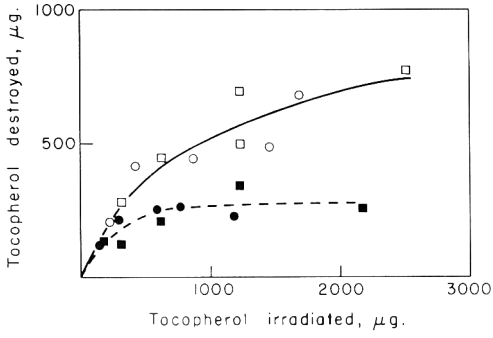

FIG. 1. Effect of increasing tocopherol concentration on the amount destroyed. Tocopherol dissolved in: ○, mineral oil; □, methyl myristate; ●, methyl oleate; ■, methyl linoleate.

Tests were attempted with still-higher concentrations of tocopherol in mineral oil, but the amount destroyed by a 2-Mrad dose became so small relative to the amount present that it could not be determined accurately. The average destruction of 7 tocopherol concentrations ranging from 3,000 to 400,000 μg per 0.5 g was 1,350 μg per sample (av G value approx 1.5). There was no evidence for an "optimum" concentration of tocopherol (where its destruction was minimal) in mineral oil or in any of the other solvents over the range tested.

Irradiation of one level of tocopherol at 0.5, 1.0, and 2.0 Mrad in each solvent also showed (Fig. 2-A) that more tocopherol was destroyed in mineral oil and in methyl myristate than in the unsaturated solvents. The amount irradiated in this test varied widely between solvents, but the close similarity of the amount destroyed when 770 μg was irradiated in oleate or 2175 μg in linoleate confirmed the conclusion, drawn from Fig. 1, that concentration in this range had little effect on the amount destroyed.

The yield (G value) for destruction of tocopherol by 2 Mrad of irradiation increased with increasing tocopherol, but appeared to approach a maximum at about 1.5 when mineral oil or myristate was used as solvent, and at about 0.75 when oleate or linoleate was used. At a fixed tocopherol level the yield decreased with increasing dose (Fig. 2-B).

DISCUSSION

Since the tocopherol concentration at the beginning of irradiation did not exceed 0.5% (w/w) except in a few tests with mineral oil, it is unlikely that direct "hits" of gamma rays on tocopherol molecules played a significant role in the destruction of tocopherol. The more probable mechanism involves the formation of active radicals from the solvents, followed by interaction of these radicals with (a) unchanged solvent molecules, (b) α-tocopherol, or (c) each other. The greater destruction of tocopherol

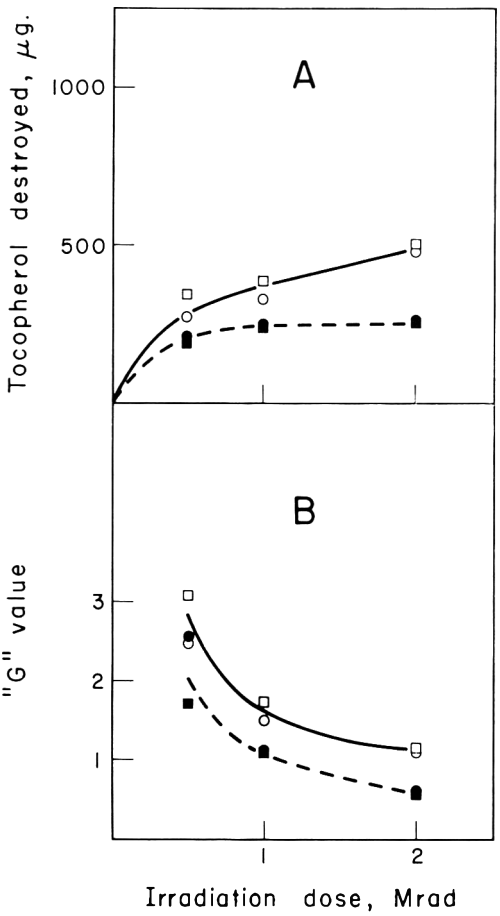

FIG. 2. A) Effect of increasing irradiation dose on amount of tocopherol destroyed. B) Irradiation "yield" for tocopherol destruction in various solvents. ○, 1457 μg tocopherol in mineral oil; □, 1235 μg tocopherol in methyl myristate; ●, 770 μg tocopherol in methyl oleate; ■, 2175 μg tocopherol in methyl linoleate.

in saturated than in unsaturated solvents indicates either that a greater number of active radicals was formed from the saturated solvents, or that more of those formed from unsaturated solvents were removed by reaction with unchanged solvent molecules (or with each other) before they came within reacting distance of a tocopherol molecule. The reactivity of unsaturated compounds suggests that the latter is the more probable explanation.

The absence of an "optimum" concentration of tocopherol in any of the solvents used contrasts sharply with the result of autoxidation studies (Lips, 1957). Presumably the pro-oxidant effect of higher concentrations of tocopherol (Lips, 1957; Quackenbush, 1949) was insignificant over the short time (85.6 min. irradiation at 45°C or below plus up to one hour storage at room temperature) involved in these tests, and tocopherol was destroyed only when it acted as an antioxidant. Higher concentrations would thus be increasingly effective in protecting the solvent ester, but increasing amounts (wt basis) of tocopherol should have been destroyed throughout the range studied.

Failure to detect significant increases in the destruction of tocopherol with increasing concentrations above about 0.5% in mineral oil and above 0.1% in unsaturated solvents (2-Mrad dose), may have resulted from the increasing errors involved as the percentage destruction decreased. However, the stability of pure tocopherol to irradiation also contrasts with its ease of autoxidation, and the constant destruction at higher concentrations of tocopherol may reflect the stability of the pure compound.

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Proteolytic Enzyme Activity During Storage of Radiation-Stabilized Raw Beef and Its Significance to Flavor[a]

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SUMMARY

This study has attempted to define the relationship of proteolysis to flavor during storage of irradiated raw beef. The data presented show that a decrease in consumer-type taste-panel preference during unrefrigerated storage of irradiated (4.5 megarad) raw ground beef correlates to some extent with the action of endocellular tissue proteolytic enzymes (cathepsins). The effective control of proteolytic activity using refrigerated storage is demonstrated. In the experiment cited, raw beef round steaks irradiated at a pasteurizing dose of 0.5 megarad were stable both microbiologically and enzymically, and the preference rating did not decline during six months at 3°C. The limited control of proteolysis during unrefrigerated storage using high-pH beef is shown. The use of high-pH beef for a limited extension of unrefrigerated storage life without significant decrease in preference rating is suggested. A need for further studies is indicated.

Traditionally, spoilage in meats has been due to microbiological activity. Successful control of this type of spoilage has been achieved by dehydration, brining, thermal processing, curing, freezing, and, in recent years, ionizing radiation. Concurrent with the use of ionizing radiation of raw meats has been noted a second type of spoilage, attributed to enzyme activity (Drake *et al.*, 1957). This spoilage has been characterized by a pronounced fluid exudate, production of amino acids and peptides, development of a "bitter" flavor, and marked decrease in taste-panel preference scores during storage at unrefrigerated temperatures (Cain *et al.*, 1958). Excellent data obtained by Zender *et al.* (1958a, b) on aseptic autolysis of meat indicate that endocellular proteolytic enzymes (cathepsins) are responsible for much of the exudation and increase in amino acids and peptides during storage. The significance of tissue proteolytic activity and flavor deterioration is not so well-defined, although the development of a "bitter" flavor during proteolytic enzyme hydrolysis of proteins has been reported by Murray and Baker (1952).

Practical control of enzymic spoilage in irradiation-stabilized products has been obtained by the use of heat (Chiambalero *et al.*, 1959). A second method of control has been reported in which an anti-autolytic effect was found in raw meat from animals given a pre-mortem epinephrine injection (Rosen, 1957). The effect of the epinephrine injection is to produce a high-pH meat through depletion of glycogen in the muscle and a consequent reduction in the production of lactic acid. Such high-pH meat has a characteristic dark red-purple, and animals producing such meat have been called dark cutters (Hedrick *et al.*, 1957). Methods of reproducibly producing dark cutters have been suggested in a series of studies by Howard and Lawrie (1956, 1957).

The present study started as an attempt to evaluate the use of high-pH (dark cutter) meat for radiation processing. As it progressed, the possibility of defining more fully the significance to flavor of cathepsin activity in raw beef during storage became apparent, and was followed.

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EXPERIMENTAL PROCEDURES

Processing

In Experiment 1 five ½-in.-thick steaks were packed in No. 2½ (401 × 411 interior-enameled cans, sealed under vacuum (28 in. Hg), and held at wet ice temperature until irradiated. Samples were gamma-irradiated (0.5 megarad) at Argonne National Laboratory within 24 hours of packing. In Experiment 2 meat from rounds was trimmed of excess fat and cut into strips of about 1 × 3 in. Three hundred grams of meat were vacuum packed (28 in. Hg) into 303 × 406 interior-enameled cans, and quick-frozen. Samples were thawed for 24 hours at 3°C just before being given a 4.5-megarad gamma-radiation dose at Materials Testing Reactor Facility, Idaho Falls, Idaho. Samples were refrozen after irradiation and maintained frozen during return shipment.

In Experiment 3 normal-pH ground beef was vacuum-sealed (25 in. Hg) in cans, frozen, and given a 4.5-megarad dose at Argonne National Laboratory. Storage samples were placed in a 38°C constant-temperature box, and the remainder maintained frozen until thawed (24 hours at refrigerated temperature before use). The dry amino acids were hand-kneaded into the ground beef for 10 minutes; all ground beef in the study was hand-kneaded similarly, whether amino acids were added or not. In Experiment 4 the ground beef was vacuum-sealed (25 in. Hg) in interior-enameled No. 2½ cans, frozen, and given a 4.5-megarad dose at Argonne National Laboratory.

Epinephrine Injection: Two Hereford steers, each weighing between 1200 and 1400 lb and with the same feed-lot history, were selected and held in a pen for 24 hours before slaughter. One steer was fed for this period according to standard hold-over-feeding practice. The other steer was starved and given epinephrine for 24 hours prior to slaughter. Epinephrine injections of 3 mg and 2 mg per 100 lb were administered 24 hours and 2 hours antemortem, respectively. The two steers were slaughtered, dressed, chilled, and cut into quarters according to recognized practice.

Meat: To obtain ground beef, trimmed beef round was ground through a ¾-in. plate, mixed, ground through a ¼-in. plate, and mixed again. Fat content of the ground beef was low, averaging 17%. Round steak was used in Experiment 1.

Analyses

Drip: In Experiment 1, drip is defined as the exudate remaining in the can when the steaks are removed. In Experiment 2, drip is defined as the total exudate expressed from 300 g of meat sample by a weight of 1 kg for 10 min.

pH: In the meat itself, the pH was determined by cutting thin ribbons from the meat, equilibrating them with an equal volume of distilled water for one minute, and reading on a Beckman Zeromatic instrument. For the drip measurements, the electrodes were immersed directly into a 1:2 solution of drip in distilled water.

Non-Protein Amino-Nitrogen: Values are expressed in terms of m*M* of tyrosine standard equivalent. In Experiments 1 and 2, a ml of drip was diluted with 6 ml of 5% trichloroacetic acid (TCA), stirred, held 30 minutes at 60°C, and filtered. One ml of the filtrate was diluted to an appropriate concentration for use in the Rosen (1957) ninhydrin method. In Experiments 3 and 4, the beef sample was diluted 1:1 with distilled water, comminuted in a Waring blender, and 10 g of this sample diluted with 10 ml of 10% trichloroacetic acid. The remainder of the procedure was similar to that above.

Total Amino-Nitrogen: In Experiment 2, the total nitrogen in the expressible drip is expressed as m*M* of tyrosine equivalent. Values were obtained on the hydrolyzed drip. For hydrolysis, one ml of drip was diluted with one ml 12*N* HCl, sealed in a glass tube, and heated 24 hours at 100°C.

Amino Acid Analysis: Calculations for amino acid content are based on protein as determined by Kjeldahl nitrogen assay. Two grams of ground beef were diluted to 10 ml with 5% TCA, mixed, held 30 minutes at 60°C, and filtered hot. One ml of the filtrate was diluted with 1 ml 12*N* hydrochloric acid, sealed in a glass tube, and hydrolyzed 24 hours at 100°C. One ml of the hydrolysate was diluted to 10 ml with pH 2.2 citrate buffer and analyzed in an automatic amino acid analyzer. Percentage error (standard error divided by average value determined) for each amino acid was no greater than ±5%.

Microbiological and Toxicity Testing: All irradiated samples except those maintained frozen after irradiation were subjected to microbiological examination before taste-panel analysis. This consisted of a standard plate count, a qualitative test for anaerobes, and a 72-hour mouse-injection toxin assay. After specimens were removed for microbiological analyses, samples were re-vacuum-packed in cans, and held 72 hours at 2–3°C before preparation for panel evaluation.

Taste Panel: Used for all evaluations was a consumer-type panel using the hedonic-scale method. In all cases, the subjects were told only that they were tasting either "steaks" or "ground beef." No seasoning was provided or used. For serving, the round steak was floured, browned, and sauteed for 30 minutes; the ground beef was broiled in an electric oven (preheated to 500°F)

Table 1. Mean preference ratings ($N = 36$) of irradiated (0.5 megarad) raw beef round steak during storage at 2–3°C.

	Storage (months)						
	1	2	3	4	5	6	Av
Treated[a] (pH 5.9)	6.1	6.5	7.2	5.8	6.2	6.6	6.4
Control (pH 5.5)	6.6	6.8	7.3	6.3	6.5	7.2	6.8

Analysis of variance

Source	DF	Sum of sq. dev.	F	Significance
Treatment	1	15.1875	11.84	Beyond 0.1%

[a] Meat from animal injected with epinephrine 24 hours and 2 hours pre-mortem.

for 7 min. on the first side and 5 min. on the second. After cooking, in all cases the samples were held in casseroles at 60°C (140°F) during serving.

RESULTS AND DISCUSSION

Experiment 1: The experiment was planned with the expectation that the high-pH beef would exhibit a greater stability than normal-pH beef during storage at 40°F. The meat from the epinephrine-injected (treated) animal was only pH 5.9, instead of the pH 6.2–6.7 desired. Howard and Lawrie (1957) also found that epinephrine injection is not always successful. The meat from the control animal was pH 5.4. The stability of the samples during storage at 3°C was unexpected, and the experiment was terminated only because sufficient samples had been provided for a six-month study.

As shown in Table 1, there was no decline in preference for either sample over a six-month period. Analyses of variance showed that the control samples were significantly (beyond 0.1% level) preferred to the treated samples. Although this difference in preference was accompanied by comments of toughness for the meat from the epinephrine-injected animal, no conclusions relating treatment and tenderness may be made, because only two animals were involved.

The highest microbiological count obtained during the experiment was 1700 organisms per gram in one four-month storage sample. In this experiment, therefore, adequate microbiological control was obtained from irradiation at a 0.5-megarad dose and refrigerated storage.

In the chemical evaluations, the pH's shown in the table were maintained throughout the storage period. The free drip increased from 0.25% to 1% in the treated samples, and from 1% to 3% in the control at one and at six months, respectively. In the same period the amino-nitrogen content increased from 43 μM to 340 μM (tyrosine equivalent) in the treated steaks, and from 240 μM to 1600 μM in the control steaks.

Thus, proteolysis was evident but not extensive. Of interest is the opinion by meat technologists that the typical "aged" meat flavor was not evident even at the six-months' evaluation. This suggests that much of the "aged" flavor in meats is due to bacterial action (or bacterial proteolytic enzyme action) rather than to muscle cathepsin action. The cathepsin inactivation due to irradiation would be small at this dose.

The taste panel evaluated only the cooked product presented. Quality-wise, the red color of the raw normal pH beef had faded considerably at the one-month evaluation, and was very faded at six months. The darker red color of the raw higher-pH meat faded to some extent, but at six months the meat still had an excellent appearance. In regard to odor, it was possible to detect a changed "irradiation" odor in the raw meat at the one- and two-month evaluations, but the irradiation odor was absent thereafter. During cooking, a slight "irradiation" odor change was noted at every evaluation.

Experiment 2: The use of a refrigerated temperature in Experiment 1 effectively controlled enzymic changes, and a comparison of normal and high-pH beef during storage at unrefrigerated temperatures was sought. Since animals with high-pH meat constitute about 1% of a normal packing-house operation, the meat used was selected in the chill room from beef carcasses slaughtered within

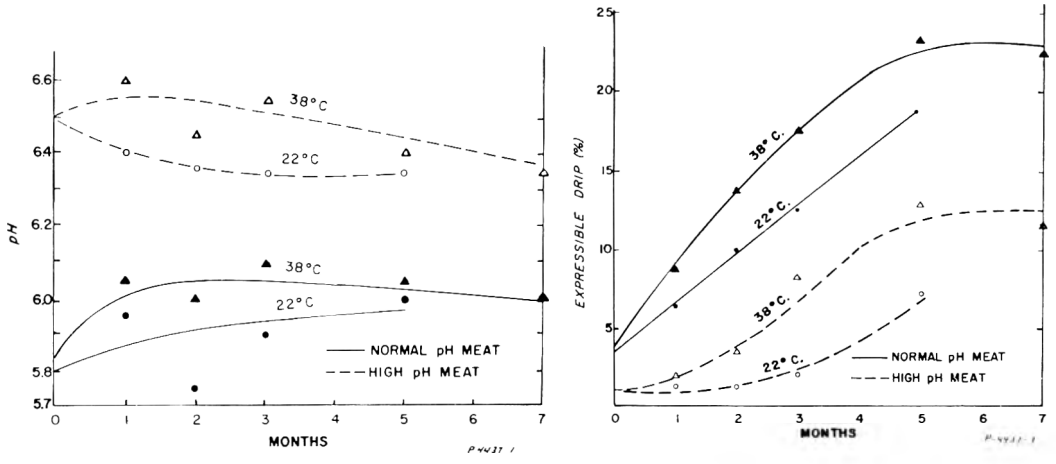

FIG. 1. Change in pH and in amount of expressible drip in normal and high-pH raw irradiated (4.5 megarad) beef during storage at 22° and 38°C (72° and 100°F).

the previous three days. The four high-pH meat rounds had pH's of 6.5, 6.5, 6.3, 6.1, and the four normal pH rounds had pH's of 5.4, 5.4, 5.3, and 5.3. Samples used were composites of the two pH 6.5 rounds, the pH 6.3 and 6.1 rounds, the pH 5.4 rounds, and the pH 5.3 rounds. Immediately after irradiation, the pH of the respective composites was 6.6, 6.4, 5.8, and 5.8. The increase in pH of the meat upon irradiation is quite marked, and may be related to the production of ammonia and amines (Burks *et al.*, 1959).

The results from analyses of the two samples for each treatment (normal- vs. high-pH meat) at each storage period–temperature were averaged to present the data in Fig. 1 and 2. In each figure, the averaged result is plotted, and a curve is drawn that appears to fit the data best.

The small change in pH with storage time is similar to that obtained in another study

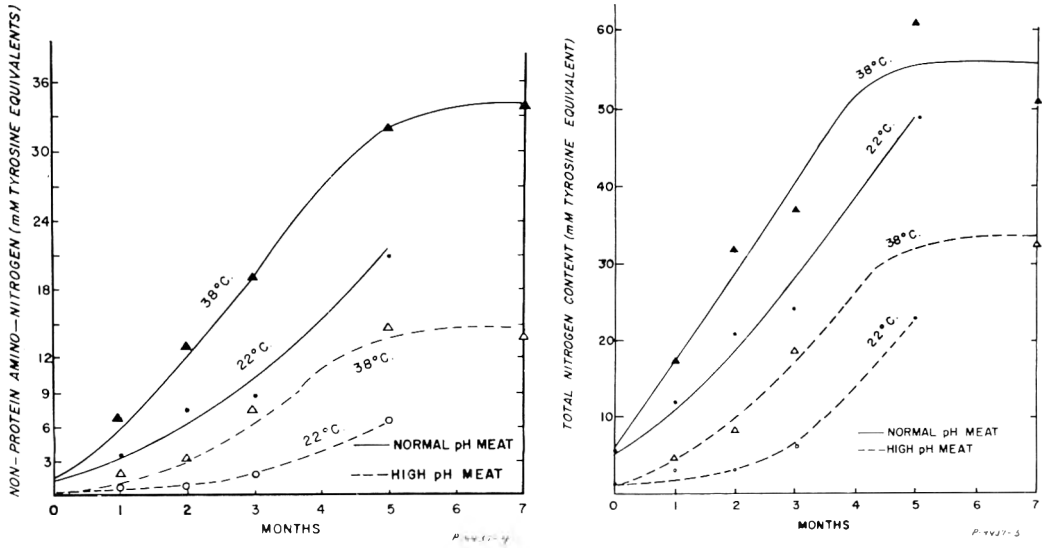

FIG. 2. Change in amount of non-protein amino-nitrogen and of total nitrogen present in expressible drip from normal and high-pH raw irradiated (4.5 megarad) beef during storage at 22° and 38°C (72° and 100°F).

(Zender *et al.*, 1958 a, b). If the ratio of non-protein nitrogen to total nitrogen is calculated, it can be seen that proteolysis is occurring during storage and that some control of proteolysis is obtained by using high-pH meat. The plateau at 5–7 months may be interpreted as a decrease in the rate of hydrolysis due to the accumulation of end-products and/or a denaturation of the cathepsins. No assay was made of the proteolytic enzyme activity *per se* of these samples.

Tyrosine crystals appeared in the normal-pH beef samples at 22°C storage for five months and at 38°C storage at two months. Tyrosine crystals were not evident in any of the high-pH beef samples, even though the non-protein nitrogen content of 38°C storage samples after 5 months of storage is equivalent to values where crystals appear in the normal-pH beef. This indicates that the course of proteolysis is somewhat different in the two sets of samples. This might be expected, however, since the changed pH would shift the rates of hydrolysis, and the high pH should favor the transamination reaction of cathepsins rather than the hydrolysis reaction (Fujii and Fruton, 1957).

Experiment 3: The use of high-pH meat in Experiment 2 showed that some control of proteolysis could be obtained, and it appeared desirable to attempt to determine the role of such activity in reducing preference ratings. The separation and evaluation of each enzyme system, however, would be a laborious undertaking. It was felt that a relatively simple check on the extent to which the cathepsin enzyme system contributes to lower ratings might be obtained by evaluating the effect of adding the end-products of proteolysis (in this case, commercially available amino acids). This assumes that the aggregate free amino acids would have the same effect upon flavor as when they are bound into peptides.

The amino acid content of the non-protein nitrogen moiety of the expressible drip from normal-pH beef stored at 38°C for one month was measured and was the basis for addition of amino acids (X) to irradiated beef for sensory evaluation. Table 2 shows the amino acid content of the non-protein moiety for the samples evaluated in Table 3. Statistical analyses of the sensory evaluation data indicate that for each addition of X amino acids there is a significant decrease (about 0.9 of a unit) in preference rating. At an amino acid addition that approximates the non-protein amino acid content of 38°C–one-month-storage irradiated beef, the preference ratings are the same.

Table 2. Amino acid content of non-protein moiety in irradiated (4.5 megarad) raw ground beef. Values are expressed as $\mu M/g$ protein.

	Frozen + X AA	Frozen + 2X AA	One month [a] 38°C	One month 38°C + X AA
Lys	66	110	150	210
His	110	160	130	180
NH_3	340	350	600	610
Arg	31	50	60	81
Asp	37	60	66	89
Thr	20	30	72	84
Ser	37	62	84	110
Glu	180	320	280	420
Pro	81	130	120	170
Gly	200	300	240	320
Ala	160	270	330	460
Val	43	82	120	170
Met	9	15	19	23
Ileu	17	30	66	81
Leu	28	50	110	140
Tyr	8	20	27	38
Phe	18	32	45	62
Total (w/o NH_3)	1045	1721	1919	2638

[a] Non-protein amino acid content in the expressible drip of this sample was basis (X) for adding amino acids to other samples.

Experiment 4: The addition of amino acids in Experiment 3 did correlate with decreased preference scores, and a limited control of proteolysis was obtained in Experiment 2 by use of high-pH meat. There-

Table 3. Mean hedonic ratings by a consumer-type panel (N = 20) on irradiated (4.5 megarad) raw ground beef.

Sample	Preference ratings: No storage	Preference ratings: 1 month storage
Frozen	5.5	
Frozen + X amino acids	4.2	5.7
Frozen + 2X amino acids	3.3	4.8
Stored [a] 38°C		4.8
Stored 38°C + X amino acids		4.0

[a] Non-protein amino acid content of expressible drip from this sample was basis (X) for adding amino acids to other samples.

fore a high-pH meat should have a longer unrefrigerated shelf-life than a normal-pH meat. To test this hypothesis, a pH 6.1 and a pH 5.5 beef loin were evaluated after storage. The evaluation times were based on the data from Fig. 3, which show that the non-protein amino-nitrogen content of the normal-pH beef at one-month 38°C storage was not attained in high-pH beef until the third month.

The results of the flavor evaluations are shown in Table 4. Statistical analysis of the data indicates that the control and high-pH ground beef stored one month are not significantly different, while all other samples are less preferred.

The non-protein amino acid content of the samples used in Trial 1 appears in Table 5. It is evident that the amount of proteolysis in the samples was greater than was found in Experiment 2, and shorter storage times for comparison of samples might have given more clear-cut results. It is of interest that the total amount of amino acids in the non-protein moiety is quite similar for each of the storage samples. The similarity here, and the apparent control of proteolysis exhibited by high-pH beef in Experiment 2, suggest that the non-protein moiety of high-pH beef contains peptides with a larger average number of residues than the normal-pH beef at equivalent storage intervals. Since specific amino acids do not appear to correlate with preference scores when both Experiments 3 and 4 are considered, these results would substantiate previous work (Murray and Baker, 1952) postulating that the types of peptides and/or the degree of protein hydrolysis influence taste.

Table 4. Mean hedonic ratings by a consumer-type panel (N = 20) during storage at 38°C of normal pH and high pH irradiated (4.5 megarad) raw ground beef.

Sample	Preference ratings	
	Trial 1	Trial 2
Normal pH (frozen control)	7.2	6.3
Normal pH (1-month storage)	5.5	4.9
High pH (1-month storage)	6.5	
High pH (2-month storage)	5.6	4.5
High pH (3-month storage)		4.4

Analysis of Variance

Source	DF	Sum of sq dev	F	Significance
Storage	3	38.8000	6.72	at 0.1%

Dunnett Test (1955): Compared to the normal pH frozen control, the high pH one month was not significantly different; the normal pH one month and the high pH two and three months were significantly different and less preferred.

Table 5. Amino acid content of non-protein moiety in irradiated (4.5 megarad) normal-pH and high-pH raw ground beef during storage. Values are expressed as $\mu M/g$ protein.

	Normal pH		High pH	
	Frozen	1 month 38°C	1 month 38°C	2 months 38°C
Lys	24	150	170	210
His	160	180	160	180
NH_3	340	720	700	730
Arg	20	67	47	44
Asp	30	76	94	73
Thr	13	84	71	82
Ser	20	91	86	100
Glu	95	320	330	370
Pro	54	110	140	140
Gly	200	260	440	420
Ala	86	370	380	460
½ CySH	tr.	7	6	6
Val	21	110	110	140
Met	2	25	22	24
Ileu	8	69	58	68
Leu	18	120	110	120
Tyr	4	30	22	28
Phe	8	51	44	48
Total (w/o NH_3)	763	2120	2290	2513

Further studies will be required to define the parameters of time/temperature proteolysis control in high-pH meats and to delineate more fully the relationship of proteolysis in meats to flavor preference. It is hoped that the work presented here points to the importance and likely success potential in such further work.

ACKNOWLEDGMENT

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The Volatile Sulfur Components of Cabbage [a]

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SUMMARY

Naturally occurring volatile sulfur compounds in cabbage have been identified by mass spectrometry and gas chromatography. Twenty sulfur compounds are reported of which five are isothiocyanates, five sulfides, nine disulfides and one trisulfide. Two additional isothiocyanates and one trisulfide are tentatively reported. The method of mass spectral analysis is described for representative members of these sulfur compounds. Gas chromatography of fresh, dehydrated and rehydrated cabbage clearly shows the presence of allyl isothiocyanate in fresh cabbage and its regeneration by enzymatic action in dehydrated cabbage. The identification of five isothiocyanates reveals the presence of corresponding thioglucosides from which the isothiocyanates are released by enzymatic hydrolysis. Similarly corresponding reactions leading to the formation of sulfides are discussed.

It has been proposed that enzymic action may be utilized to enhance the characteristic aroma of foods, especially of those that have undergone processing treatments such as dehydration (Hewitt *et al.*, 1956). Heat, with or without partial vacuum, removes volatile compounds, many of which are associated with the characteristic aroma and flavor of the food product. Identification of these components, and determination of the proportions of each required for reconstitution of flavor of a processed product, are problems of considerable magnitude. The utilization of biological processes in which the components associated with aroma are naturally produced offers a promising concept for the improvement of processed foods. Such a concept depends on the ability of the flavor precursors in fresh food to survive the processing treatment; that these do so has been suggested by sensory testing (Konigsbacher *et al.*, 1959).

Many compounds have been identified in cabbage although there have been relatively few studies involving the more volatile components. Konig and Kracht (1929) have reported the presence of methyl mercaptan and hydrogen sulfide. The isothiocyanates have been reported to occur in both the seeds and leaves of the vegetable (Bailey *et al.*, 1957; Clapp *et al.*, 1959; Jensen *et al.*, 1953). Dateo *et al.* (1957) reported disulfide as the volatile sulfur components of cooked cabbage. The thioglucosides, sulfoxides, and other non-volatile compounds found in cabbage are believed to be precursors of these different volatile components. Challenger (1953) reported that the characteristic odor and taste of cabbage and other members of the Cruciferae are due to complex thioglucosides that on hydrolysis yield glucose, potassium hydrogen sulfate, and the pungent isothiocyanate. The chemistry of these thioglucosides has been studied extensively (Ettlinger and Lundeen, 1956, 1957; Kajaer, 1959; Kajaer and Gmelin, 1956; Kajaer *et al.*, 1953, 1959).

The investigation described in this paper is part of a general program on the chemistry of natural products. Cabbage was selected for study as a representative member of the Cruciferae. The occurrence of compounds previously reported is confirmed, and additional compounds are identified. The enzymic release of isothiocyanates from dehydrated cabbage is demonstrated by mass spectrometry and gas chromatography.

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MATERIALS AND METHODS

Fresh cabbage. Experiments on fresh cabbage were carried out on samples secured from a single planting of the Harris Resistant variety of Danish white cabbage, supplied by Professor Robert E. Young, University of Massachusetts Experimental Station, Waltham. Several methods of reducing the cabbage were investigated, all of which gave substantially the same results. The different methods were shredding, chopping, or mincing the cabbage at room temperature, or freezing the whole cabbage head in liquid nitrogen followed by shattering. For the subsequent mass spectral analyses described in this paper, a 500-g sample of fresh cabbage was minced in a Waring blender. For the gas chromatographic studies of fresh cabbage, a 100-g sample was minced and transferred to a glass cell maintained at 60°C by a water jacket and sealed with a rubber serum cap. Atmospheric pressure was maintained by a slight flow of argon into the cell through a side arm fitted with an overflow outlet. This apparatus is shown in Fig. 1.

FIG. 1. Sample cell for gas chromatographic analysis.

Dehydrated cabbage. Dehydrated white cabbage was obtained from Dr. Fred Heiligman, QM Food and Container Institute, Chicago, Illinois. Samples of 20 g were reconstituted with 180 ml of distilled water at room temperature. Samples were allowed to stand for 15 minutes to ensure complete rehydration, and the volatiles were separated as described below. Enzyme treatment of dehydrated cabbage was accomplished by similar reconstitution of samples with water containing 0.6 g of myrosinase.

For control purposes, a sample of 0.6 g of myrosinase, inactivated by heating 20 minutes at 121°C, was added to a sample of reconstituted dehydrated cabbage.

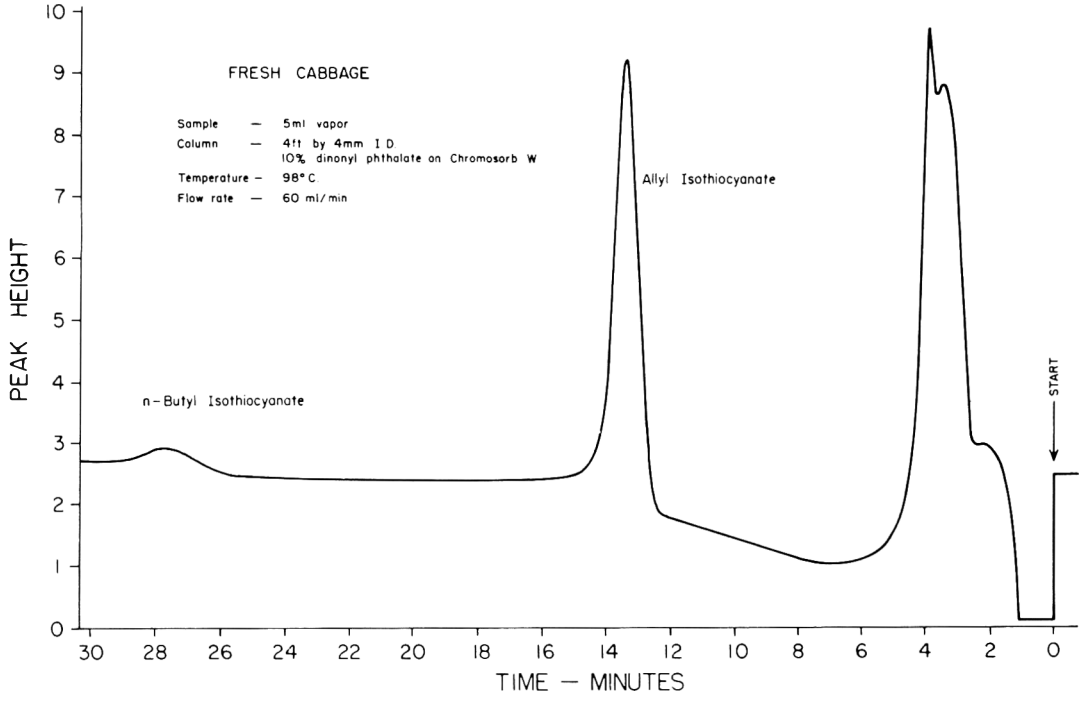
The enzyme preparations used were supplied by Dr. George W. Kurtz, QM Food and Container Institute, Chicago, Illinois, and Dr. Eric J. Hewitt, Evans Research and Development Corporation, New York.

Distillation of volatiles for mass spectral analysis. The distillation procedure followed was based on that of Merritt *et al.* (1959), employing low-temperature high-vacuum techniques. Prepared samples were transferred to a 1-L round-bottom flask and connected to a high-vacuum line previously evacuated to 1 μ of mercury. The sample flask was then opened to the evacuated system, and a portion of the total volatile constituents was condensed at −196°C in an adjacent gas bottle cooled with liquid nitrogen. Noncondensable air was then pumped out. This procedure was repeated several times, until the sample was free of air. Total condensables were then collected for 3 hours. A study of collecting periods ranging from 1 to 18 hours indicated that the 3-hour period was optimal since it sufficed for components of interest yet avoided the excessive amounts of water and carbon dioxide that accumulated over longer periods.

Further fractionation was required for separation of the volatile compounds from water and carbon dioxide. This was accomplished by using appropriate coolants and bulb-to-bulb distillation of the total condensate. Four fractions were obtained for mass spectral analysis: −60 to −80°C, −80 to −110°C, −110 to −140°C, and −140 to −196°C. Additionally, each of these fractions was further fractionated directly on the mass spectrometer by the method of Bazinet and Merritt (1959).

Mass spectral analysis. About 5×10^{-4} moles of each fraction was fractionated directly from the gas bottle into a Consolidated Engineering Corporation Model 21-103C analytical mass spectrometer. Components were identified by analysis of the fragmentation patterns in accordance with established direct-method techniques (Dunning, 1955; Rock, 1951).

Gas chromatographic analysis. Cabbage vapor samples were studied chromatographically in a Pye argon chromatograph employing 20 mc of strontium-90 in the ionization detector. For each analysis a 5-ml volume of the vapor above the sample was withdrawn and introduced by syringe into the chromatographic column. Operating conditions and resultant patterns are shown in Fig. 2.



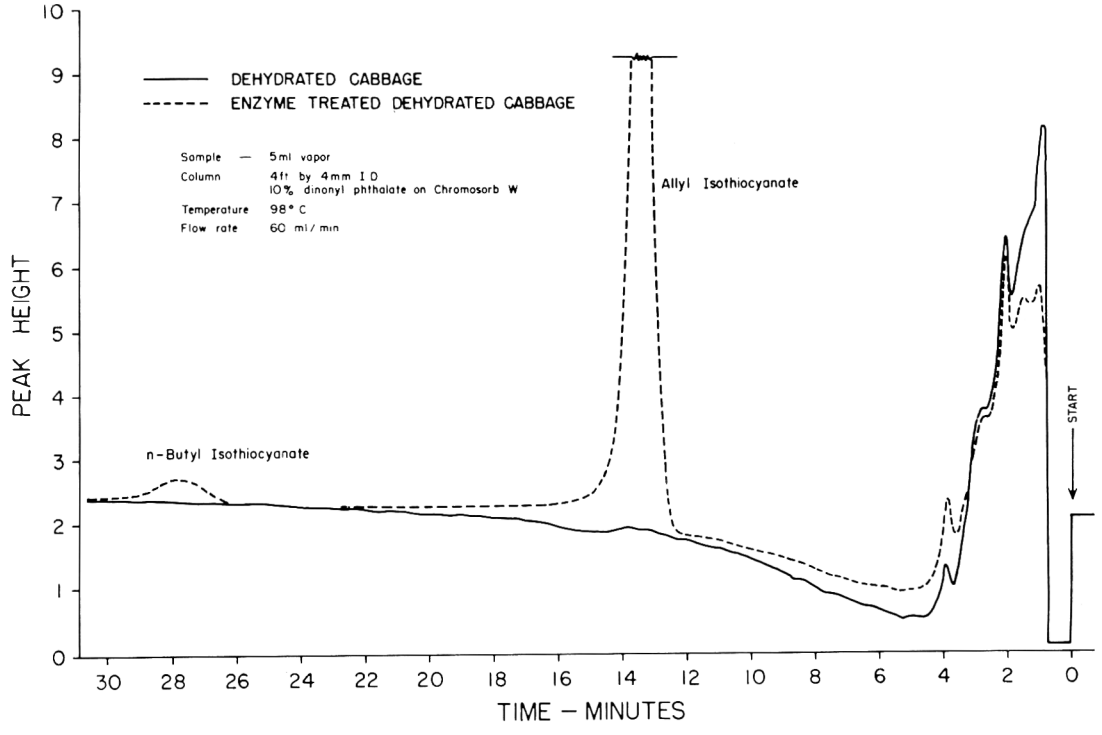


FIG. 2. Enzymic release of isothiocyanates.

RESULTS AND DISCUSSION

Results of mass spectral and gas chromatographic analyses of fresh and processed cabbage are summarized in Table 1. They are based on evidence exemplified by data shown in Fig. 3 and 4. Only qualitative data are presented, since the fractionation techniques used prevented accurate quantitative determinations. These results cover only sulfur components since, following the removal of water and carbon dioxide, sulfur compounds are found to represent the major volatile constituents. Of the non-sulfur components, 3-butene nitrile, a product of the hydrolysis of sinigrin, and various alcohols, aldehydes, and ketones were also observed.

Table 1. Volatile sulfur components of fresh and processed cabbage.

	Fresh cabbage	Dehydrated cabbage	Dehydrated cabbage + enzyme
Isothiocyanates			
Methyl	+	0	0
n-butyl	+	0	+
Butenyl	+	0	+
Allyl	+	0	+
Methylthiopropyl	+	0	0
Sulfides			
Hydrogen	+	0	0
Carbonyl	+	0	+
Dimethyl	+	+	+
Diethyl	+	0	0
Dibutyl	+	0	0
Disulfides			
Carbon	+	0	+
Dimethyl	+	0	+
Methyl ethyl	+	0	0
Diethyl	+	0	0
Ethyl propyl	+	0	0
Dipropyl	+	0	0
Propyl butyl	+	0	0
Propyl allyl	+	0	0
Diallyl	+	0	0
Trisulfides			
Dimethyl	+	0	0
TENTATIVE (because of lack of reference spectra)			
Isothiocyanates			
Methylthiomethyl	+	0	0
Methylthiobutyl	+	0	0
Trisulfides			
Diethyl	+	0	0

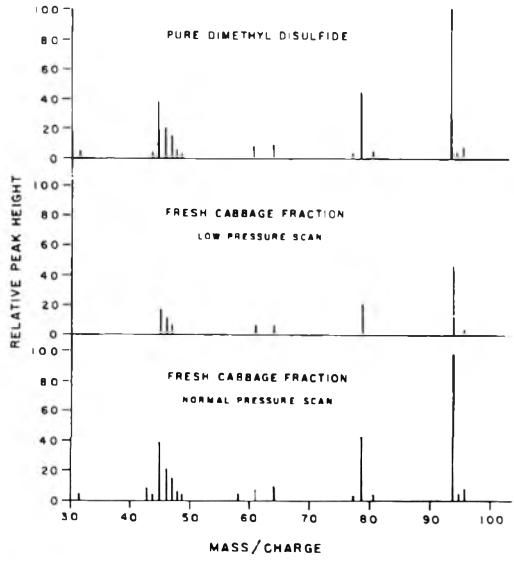


FIG. 3. Mass spectral identification of dimethyl disulfide.

Analysis of control samples for the presence of volatiles gave results identical to those observed in rehydrated cabbage without enzyme.

Identification of dimethyl disulfide in one of the fractions is depicted in Fig. 3. The upper spectrum is that of a pure sample of dimethyl disulfide. It is characterized by a high-intensity ion at the parent mass of 94

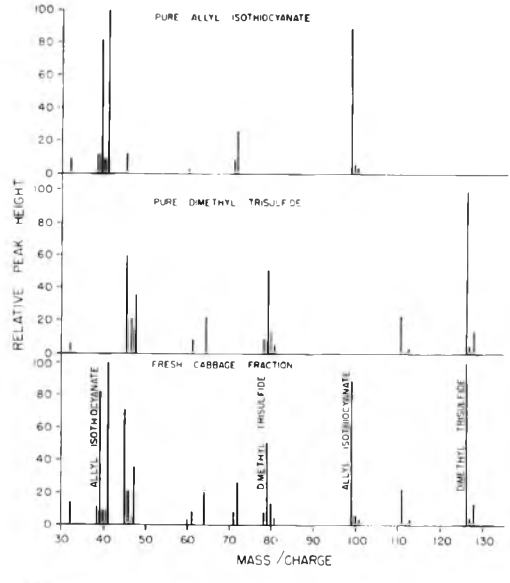


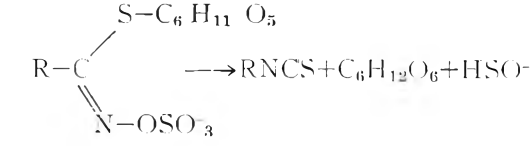
FIG. 4. Mass spectral identification of allyl isothiocyanate and dimethyl trisulfide.

and its associated typical isotopes at 95 and 96. Fragmentation peaks at mass numbers 79, 64, 61, 47, 46, and 45 and their isotopes complete the characteristic spectrum of this compound. The lower two spectra of Fig. 3, of a fresh-cabbage fraction, were obtained by direct low-temperature fractionation on the mass spectrometer (Bazinet and Merritt, 1959). The distillation of narrow temperature fractions is accomplished directly on the inlet line. As soon as part of the sample is vaporized, a mass spectrum of the fraction is obtained. The spectrum labeled "low-pressure scan" is an example of scanning begun when very little of the −80 to −110° sample is vaporized; thus, only the larger characteristic peaks of dimethyl disulfide, the major compound present in the fraction, are seen.

The spectrum below is that obtained as more of the sample is distilled and the pressure in the inlet system approaches the normal range. Here some of the less abundant ions of dimethyl disulfide can now be seen as well as two new fragments not found in the spectrum of the pure dimethyl disulfide. These two new ions, at mass numbers 43 and 58, are subsequently found to be due to acetone. A further increase in pressure would show still more fragments because of the less abundant components of the fraction. By similar comparison to reference spectra of pure samples it has been possible to identify the compounds in the various fractions (e.g., in Fig. 4).

Examination of Table 1 reveals the large number of volatile sulfur compounds found in fresh cabbage in contrast to the relatively few observed in the processed samples, suggesting the partial or complete loss of these constituents during dehydration. The release of isothiocyanates following addition of the enzyme has been demonstrated by both mass spectral and gas chromatographic techniques. Reconstitution of the dehydrated product with water and myrosinase yielded the allyl, n-butyl, and 3-butenyl isothiocyanates, proving the survival of their thioglucoside precursors in the dehydration process. Failure to observe the remaining five isothiocyanates following treatment with myrosinase may be attributed to quantities too minute for detection, or to sample variation in the processed product. Four sulfides have been identified in the enzyme-treated cabbage in addition to the single sulfide identified in the untreated dehydrated product.

The existence of isothiocyanates as natural biological materials has been amply verified by numerous investigations. The presence of allyl isothiocyanate in fresh cabbage leaves, first reported by Jensen *et al.* (1953), has been confirmed in these laboratories (Bailey *et al.*, 1957; Clapp *et al.*, 1959). Other isothiocyanates reported to be present in the fresh vegetable are 3-butenyl isothiocyanate (Jensen *et al.*, 1953) and 3-methylthiopropyl isothiocyanate (Clapp *et al.*, 1959). The present paper reports the occurrence of these same compounds and five additional isothiocyanates hitherto unrecognized in cabbage leaves. The methyl and 4-methylthiobutyl mustard oils have also been found in the seeds of other natural products (Kajaer and Gmelin, 1955; Kajaer *et al.*, 1955). The presence of these compounds suggests the occurrence of five thioglucosides in addition to sinigrin, gluconapin, and glucoiberverin, all cleaved by myrosinase to yield the mustard oils, according to the general equation



The presence of the methylthiomethyl and 4-methylthiobutyl isothiocyanates is indicated as tentative at this time because of a lack of reference spectra. Analysis of parent mass peaks and isotope ratios of the cabbage volatiles, however, has provided evidence for the probable occurrence of these compounds. Additionally, the unquestionable presence of the 3-methylthiopropyl isothiocyanate lends support to the probable existence in cabbage of methylthioalkyl homologs.

The presence of phenyl isothiocyanate was indicated repeatedly during this study. The parent mass peak of 135 and istope contributions at masses 136 and 137 strongly suggest the possible occurrence of the aromatic isothiocyanate. The very minute amount present, however, and the contribution of an unknown compound at mass 136 precludes absolute identification at this time.

The origin of sulfides may be postulated from a number of sulfur compounds, such as sulfoxides, thioglucosides, sulfur-bearing amino acids, and sulfonium compounds. The presence of carbonyl sulfide, hydrogen sulfide, and carbon disulfide may be accounted for by hydrolysis of the isothiocyanates as follows:

$$RNCS+H_2O \rightarrow RNH_2+COS \quad (1)$$
$$COS+H_2O \rightarrow H_2S+CO_2 \quad (2)$$
$$2RNCS+2H_2O \rightarrow 2RNH_2+CO_2+CS_2 \quad (3)$$

The formation of carbon disulfide may also be a consequence of the presence of hydrogen sulfide, as described above, and its subsequent reaction with unchanged isothiocyanate:

$$RNCS+H_2S \rightarrow RNHCSSH \rightarrow RNH_2+CS_2$$

The above reactions were recently reviewed by Challenger (1959).

Identification by Haas (1935) of dimethyl sulfide as the compound evolved by the red alga *Polysiphonia fastigiata* led to identification of its precursor, dimethyl-β-propiothetin, by Challenger and Simpson (1948). Cantoni and Anderson (1956) further showed that *P. lanosa* contains an enzyme capable of decomposing dimethyl-β-propiothetin to dimethyl sulfide, acrylic acid, and H^+:

$$(CH_3)_2S^+CH_2CH_2COOH \rightarrow (CH_3)_2S+CH_2CHCOOH+H^+$$

Subsequent work by other investigators demonstrated the evolution of dimethyl sulfide from other algae and various plant species, and again suggested the existence of a sulfonium compound as the precursor (Bywood *et al.*, 1951; Challenger *et al.*, 1957; Karrer and Engster, 1949; Karrer *et al.*, 1949).

McRorie *et al.* (1954) reported the existence in cabbage juice of a methionine analog, isolated as the methylmethionine sulfonium salt. This compound was considered as a possible precursor of both methionine and the dimethyl-β-propiothetin mentioned above. At about the same time, Challenger and Hayward (1954) identified a methylmethionine sulfonium salt in an asparagus extract. Decomposition of the methylmethionine sulfonium salt yielded homoserine and the characteristic odor of dimethyl sulfide. The formation of dimethyl sulfide in cabbage leaves may well be accounted for by this mechanism or by a hydrolysis mechanism, a reaction suggested by the observation of this compound as the sole component in rehydrated cabbage (Table 1). By analogy, the existence of diethyl sulfide in fresh cabbage suggests corresponding reaction processes and the possible existence of another naturally occurring sulfonium compound.

The origin of dimethyl disulfide has been shown to be *S*-methyl-L-cysteine sulfoxide (Dateo *et al.*, 1957), a compound found in cabbage by Synge and Wood (1955) and in turnips and other crucifers by Morris and Thompson (1956). It has recently been isolated, together with *S*-*n*-propylcysteine sulfoxide, from fresh, homogenized onions (Virtanen and Matikkala, 1959). A closely related compound found in onion and in garlic (Cavallito *et al.*, 1945; Stoll and Seebeck, 1948), *S*-allylcysteine sulfoxide, decomposes on distillation with steam, yielding diallyl disulfide. Identification during the present study of dipropyl disulfide and diallyl disulfide in addition to dimethyl disulfide suggests the coexistence of the corresponding sulfoxides. Ostermayer and Tarbell (1960) described the hydrolysis of *S*-methyl-L-cysteine sulfoxide by the equation

$$4CH_3\overset{\overset{O}{\uparrow}}{S}CH_2CHNH_2COOH-2H_2O \longrightarrow CH_3\overset{\overset{O}{\uparrow}}{S}SCH_3+(CH_3)_2S_2+4NH_3+4CH_3COCOOH$$

A parallel reaction doubtless occurs in the formation of dipropyl and diallyl disulfides in cabbage. If this reaction is a general one and truly accounts for the major portion of the organic sulfur of cabbage (Synge and Wood, 1955), the origin of the remaining disulfides listed in Table 1 may be due to the presence of sulfoxides not yet reported as occurring naturally.

The occurrence of organic polysulfides in cabbage juice was reported by Jirousek in 1956. Those compounds were not characterized, nor was a mechanism for their formation proposed. The production of monosulfides and disulfides from sulfonium compounds and sulfoxides suggests the possible

existence of related reactions and precursors in the formation of the trisulfides.

ACKNOWLEDGMENTS

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A Comparison of Some Taste-Test Methods [a]

N. T. GRIDGEMAN

(Manuscript received June 3, 1960)

SUMMARY

A long-term investigation into the efficacy of different egg preservatives was used to compare different taste-panel techniques. The main contrast is between *rating*, according to a specified subjective quality scale, and *multiple pair comparison*, according to flavor preference. A subsidiary contrast is between pair comparison with degrees of preference, and (on the same data) with straight binary preferences. All results are scored, presented graphically, and subjected to an analysis of variance. Pair comparison, especially with (three) degrees of freedom, proved to be more discriminatory than rating. Some aspects of the logic of the comparison of sensory scales are discussed.

INTRODUCTION

Any inquiry into food preferences is necessarily a special case of the wider problem of seeking information on the relative merits of a number of different items. Such a search must be prefaced by four decisions: 1) on the sensory basis of merit; whether taste, odor, appearance, etc., or some combination of these; 2) on the population involved, i.e., on *whose* reactions and judgments we are interested in; 3) on the importance of the problem (which will govern the amount of information sought); and 4) on how to extract the information efficiently. Of course we seldom have to make all four decisions explicit, or to dwell on each, but their fundamental status must be borne in mind during the planning stage. The fourth decision, which is bound up with experimental design, is often difficult. Much of what follows is oriented to this matter of efficiency.

In comparative taste testing, the leading methodological subdivision is between multiple pair comparison on the one hand, and some kind of rating or grading on the other. Little is known about their relative efficiency, and it may be pointless even to try to generalize. When, therefore, we were recently asked to help in an egg-storage investigation, the opportunity was taken to compare taste-test methods along with the main comparison of treatments. As will be seen below, in effect three test methods were used: two types of pair comparison (one with "straight" preferences, and the other with degrees of preference), and a rating scheme. Both methods of pair comparison were evaluated by means of a symmetrical scoring scheme and an analysis of variance of the scores. The handling is simpler than is customary, and it may be hoped that the following exposition will encourage wider use of multiple pair comparison in taste appraisal.

EXPERIMENTAL

Our primary concern being subjective testing, technical details of storage are omitted. Each treatment had two groups, making four in all. They were begun with fresh spring eggs, assigned at random to the following treatments: A_e, oiled under laboratory conditions; A_s, standard commercial oiling; B_p, sealed in bags of type "p" plastic; B_c, sealed in bags of type "c" plastic.

The sealing was done with bags large enough to hold 15 dozen eggs in Keyes trays. Air was not excluded or replaced. All eggs were put into commercial cold storage without special attention. At various times up to 6 months, random samples were withdrawn, held 7 days at 55°F to simulate marketing conditions, and then shipped to town to two taste laboratories. Simultaneously, fresh eggs were brought in for control comparison. At each sampling point, information was required on how the variously stored eggs compared with each other, and with fresh eggs. Obviously, the fresh eggs themselves would vary slightly over the half-year period (May through September), but this was inescapable, not important in itself, and irrelevant to the primary question of the comparative acceptability of the stored eggs.

[a] Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada. Issued as N.R.C. No. 6245.

Because eggs are one of the most uniform of foods, presumably giving rise to only a narrow spectrum of opinion on what constitutes good quality, it was thought sufficient to recruit tasting panels casually, without any selection device, from available staff. The use of effectively random inexpert tasters also means that inter-panel bias is unlikely. Each laboratory formed a 12-member panel, with about 8 permanent, and about 4 floating, members.

The Appraisals

On each of the six occasions—after ½, 1, 2, 4, 5, and 6 months—the two laboratories, L_1 and L_2, immediately and independently set about appraising the eggs. All judgments were made on eggs boiled 3 minutes under standardized kitchen conditions.

In laboratory L_1, *preference judgments by pair comparison* were made. Since there were 5 groups (including the fresh eggs), 10 pairings were possible. The subject was therefore presented with all 10 pairs, coded, in two sets of 5 (one on each of two successive days). Care was taken to balance the pairings so that eggs of any one group appeared as often on the left-hand side as on the right; apart from this, the order of presentation was random. The subject was asked to decide which member of the pair was more to his liking, and to indicate whether it was "slightly," "moderately," or "much" preferred. No hint was, or perhaps could have been, offered as to how these degrees might be spaced, each subject being left to construct his own hedonic scale, as it were. "Ties" were prohibited; that is to say, if the subject felt he could not choose, he was nevertheless asked to "guess" a decision (and of course to give it the lowest scale category, "slightly"). This restriction discourages taste laziness, and if in fact two members of a pair are indistinguishable, there is an even chance in favor of either. Because of this, and of the desirability of an equal-interval scale, the following senary scoring scheme was adopted:

		Score for item A	Score for item B
If item A is	much preferred	+5	−5
	moderately preferred	+3	−3
	slightly preferred	+1	−1
If item B is	slightly preferred	−1	+1
	moderately preferred	−3	+3
	much preferred	−5	+5

The data can also, obviously, be handled without regard to the *degrees* of preference: we simply score the chosen item +1 and the other item −1, thus condensing the senary scale to a binary one. In the present instance the results will be examined according to both scales, which should tell us something about the value of the extra information contained in the recording of degrees of preference.

In laboratory L_2, *rating judgments of individual items* were made. The subject was first presented with an identified fresh egg, and then with a coded hexad consisting of one egg from each of the four experimental groups, plus two fresh eggs, all in random order. He was asked to rate each egg on a loosely defined senary scale ranging from 5 (typical fresh-egg flavor) down to 0 (virtually inedible). Each of the 12 judges carried this out in duplicate, on two successive days.

RESULTS

Table 1, containing a summary of the results, should be self-explanatory.

It is reasonable to suppose that the summarized data can validly be subjected to a conventional analysis of variance. The results of such analyses are given in Table 2. But to appreciate Table 2 a picture of the dispositions is needed; hence Fig. 1. Graphs have been drawn of only the two main treatments because groups A_6 and A_8 turned out to be indistinguishable, and so did groups B_4 and B_6—see the entries labeled "groups (within treatments)" in Table 2. To assist comparison of the three methods the ordinates of the graphs have been standardized, i.e., the average scores (stored eggs minus fresh eggs) were adjusted to *equivalent residual standard deviations*, taken from Table 2. In other words, the relative lengths of unit intervals on the ordinates are:

senary preference scale	1
binary preference scale	$\sqrt{117.7/17.49}=2.59$
senary rating scale	$\sqrt{117.7/62.19}=1.38$

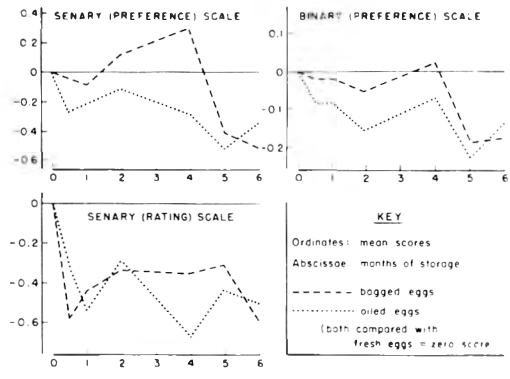

Fig. 1. Flavor scores of stored eggs.

Table 1. Stored-egg scores minus fresh-egg scores (each main entry = mean × 48).

Lab	Months of storage	Oiled eggs: Group A_c	Oiled eggs: Group A_s	Oiled eggs: Sum	Bagged eggs: Group B_p	Bagged eggs: Group B_c	Bagged eggs: Sum	Grand sum
	ex: preferences by pair comparison on a senary scale (−5 to +5, by 2's).							
	½	−10	−16	−26	3	− 7	− 4	− 30
	1	−20	− 1	−21	−15	6	− 9	− 30
	2	− 5	− 6	−11	− 3	14	11	0
	4	−14	−14	−28	23	5	28	0
	5	−21	−29	−50	−31	− 9	−40	− 90
	6	−28	− 7	−35	−23	−27	−50	− 85
L_1								
	ex: same preferences as above but on a binary scale (−1 and +1).							
	½	− 2	− 6	− 8	− 3	1	− 2	− 10
	1	− 8	0	− 8	− 5	3	− 2	− 10
	2	− 7	− 8	−15	− 7	2	− 5	− 20
	4	− 3	− 4	− 7	3	− 1	2	− 5
	5	− 7	−15	−22	− 4	−14	−18	− 40
	6	−10	− 3	−13	− 5	−12	−17	− 30
L_2								
	ex: quality ratings on a senary scale (0 to +5).							
	½	− 9	−23	−32	−35	−21	−56	− 88
	1	−22	−30	−52	−28	−14	−42	− 94
	2	−15	−13	−28	−13	−19	−32	− 60
	4	−30	−34	−64	−15	−19	−34	− 98
	5	−16	−26	−42	−10	−20	−30	− 72
	6	−21	−27	−48	−20	−36	−56	−104

Consequently, the areas between the curves are indices of the estimated differences between the treatments. We immediately infer from the figure that:

1) There is a general tendency for the bagged eggs to be superior to the oiled eggs, this being most pronounced on the senary preference scaling.

2) The rating method puts all the stored eggs lower down the quality scale (in terms of fresh eggs) than the preference methods.

3) Whereas the preference methods indicate a gradual quality decline, the rating method indicates a sharp initial decline without much subsequent change.

If we go on to examine the figure and Table 2 in conjunction, we additionally infer that:

4) The probability that the observed mean treatment differences are fortuitous is 0.06 with the senary preferences, 0.14 with the binary preferences, and very high ($F<1$) with the ratings.

5) The rating method, in contrast to the others, indicates no significant change in quality of the stored eggs between the first and last tests (see the entries labeled "pooled slope"), which bears out 3), above.

6) The jaggedness of the curves falls within the limits of normal variation: none of the mean squares in the interaction entries, "periods by treatments," is significantly greater than those in the "periods by groups" entries. (This is important and interesting insofar as a naive inspection of the senary preference curves would wrongly suggest that, for instance, at the 4th month the bagged eggs were superior to the fresh eggs.)

Further Analysis

The statistical structure of pair comparisons in which degrees of preference are elicited has been examined by Scheffé (1952). He has shown how the general consistency of the results can be checked. His approach, that is to say, furnishes a statistical test of the assumption of the existence of a rational hedonic continuum that is the sole, or at least the dominant, inspiration of any non-random element in the judgments.

As published, Scheffé's scheme rests on an odd-numbered scale (7- or 9-point, usually) with a central zero to allow for ties.

Table 2. Analyses of variance of the three sets of results.

Source of variance	df	Mean square	F	p
from L_1 preferences by pair comparison (senary scale)				
Treatments, "oiled" vs "bagged"				
(i) mean difference	1	477.0	4.05	0.06
(ii) slope difference	1	61.8	<1	
Pooled slope	1	799.8	6.63	0.02
Other differences between periods	4	300.1	2.55	0.08
Groups (within treatments)	2	58.7	} pooled	
Interaction, periods by treatments	4	151.1	} residual	
Interaction, periods by groups	10	116.1	} = 117.7	
from same L_1 preferences as above, but on a binary scale				
Treatments, "oiled" vs "bagged"				
(i) mean difference		40.04	2.29	0.14
(ii) slope difference	1	11.86	<1	
Pooled slope	1	100.00	5.72	0.03
Other differences between periods	4	32.55	1.86	0.16
Groups (within treatments)	2	16.71	} pooled	
Interaction, periods by treatments	4	4.84	} residual	
Interaction, periods by groups	10	22.71	} = 17.49	
from L_2 rating judgments (senary scale)				
Treatments, "oiled" vs "bagged"				
(i) mean difference	1	10.67	<1	
(ii) slope difference	1	52.37	<1	
Pooled slope	1	18.34	<1	
Other differences between periods	4	83.42	1.34	0.28
Groups (within treatments)	2	69.33	} pooled	
Interaction, periods by treatments	4	96.74	} residual	
Interaction, periods by groups	10	46.93	} = 62.19	

N.B. { df = degrees of freedom
F = variance ratio to pooled residual
p = probability of such an F (if >1) by chance

In the present experiments, for reasons already given, ties were prohibited and an even-numbered (6-point) scale was used. The analysis is easily adjustable to this mutation.

Full details of the statistical procedure (which is intricate) are given by Scheffé (1952), so they are omitted here. However, for the benefit of readers interested in the application of this extension of the method of pair comparison, the raw data from the present experiment are assembled in Table 3 as score frequencies among panel members. Thus the first entry, $\frac{110310}{012120}$, is a compacted form of the following frequency set:

score:	−5	−3	−1	+1	+3	+5
for B_e (at right)	1	1	0	3	1	0
for B_e (at left)	0	2	1	2	1	0
for both B_e	1	3	1	5	2	0

from which it will be found that the sum-score for B_e is −4 (and therefore the sum-score for B_p, the other member of this particular pair comparison, is +4). Similarly, extracting the sum-scores for the remaining six B_e entries in the first column of Table 3, we compute the over-all score for this group to be −2. And so forth.

Incidentally, the entries in the topmost section of Table 1 are obtained from Table 3 in this typical way: At the ½-month test period, the total score for B_e, as shown above, is −2, and the corresponding total score for FE (the fresh-egg group) will be found to be +12; therefore the appropriate entry for B_e in Table 1 is $[(-2)-(+12)]\ 2 = -7$.

The results of the Scheffé-type analysis of variance on the data of Table 3 are given in Table 4. Note that there is some indication

Table 3. Raw data from multiple pair comparisons (on a senary scale) among the 5 egg groups.

Group pairings		Months of storage					
		½	1	2	4	5	6
B_p	B_c	110310	102030	012210	103200	000420	014010
B_c	B_p	012120	112110	102111	011400	110202	001230
A_s	B_c	100311	020220	020211	010320	011310	004020
B_c	A_s	004110	002220	003201	031200	112110	002211
A_c	B_c	002310	002211	002400	011112	002121	101211
B_c	A_c	011220	113100	141000	011310	003120	021210
FE	B_c	002220	011220	111111	031011	112101	012210
B_c	FE	010221	102111	121200	003201	102210	011211
A_s	B_p	103020	101130	012201	002202	010311	120300
B_p	A_s	111120	101220	003210	111300	020121	011211
A_c	B_p	013020	131100	031110	004110	004020	021120
B_p	A_c	102210	022200	102210	121200	002310	003120
FE	B_p	012300	031110	022011	011031	121200	012300
B_p	FE	212100	030120	011220	111210	010221	101320
A_c	A_s	012210	102210	003111	111210	002220	010302
A_s	A_c	002130	031110	003030	120210	010410	020211
FE	A_s	031020	010320	032001	000420	031011	120201
A_s	FE	021030	011310	002220	010140	001140	020220
FE	A_c	013200	122010	002112	013110	011130	011220
A_c	FE	002040	002310	002220	011130	100311	000222

N.B. Each 6-digit entry gives the frequency distribution along the senary scale (−5, −3, −1, +1, +3, +5) of half of the panel's scores for the *right-hand* member of the pair (so that the scores for the left-hand member will be numerically identical, but with changed algebraic sign). Each double 6-digit entry constitutes the results of the whole panel on one pair comparison. FE signifies fresh-egg group.

($p = 0.08$ against chance occurrence) that the order of tasting (the chirality) within a pair had some influence on the judgment. (In practice, the order was always balanced, so that this effect, no matter how strong, would cancel out.) Note, too, that there is no evidence of "scale inconsistencies," which means that the psychosensory assumptions were apparently justified.

DISCUSSION

A discrepancy

In 2), among the inferences under Results, we noted that the rating method generally gave stored eggs lower scores than did the pair preference methods. The difference is obvious enough without statistical support (although that is available), and it constitutes an odd discrepancy because it is only the preference methods that yield evidence of a difference between the two kinds of storage. If all methods are interconsistent, whichever reveals the more marked difference between the two treatment groups should also show up the more marked difference between the pooled treatment groups and the control (fresh-egg) group. In other words, methods will naturally vary in the *spread of the differences* between a number of estimated means, but they should not reveal *differences between the differences*. Yet this is what has apparently happened here.

What is the explanation? Tentatively, we might look for it in the slight psychosensory difference between the two types of test. The subjects on the "rating" panel, being given an identified fresh egg as a preliminary, brought to their task an element of *matching* that was absent in the task of the "preference" panel. When we note that the "rating" subjects additionally always had *two* fresh eggs in the coded test hexad, it becomes con-

Table 4. Scheffé-type analysis of variance of data (see table 3) from multiple pair comparisons on a senary scale.

Source of variance	df	Mean square	F	p
Main treatments, "oiled" vs "bagged"	1	31.8	4.97	0.03
Treatments (all stored) vs "fresh"	1	30.7	4.80	0.03
Groups (within main treatments)	2	3.9		
Interaction, periods by treatments	20	7.4		
Order-of-tasting effect	10	10.8	1.69	0.08
Scale inconsistencies	6	4.9		
Residual error	680	6.40		

N.B. 1) Since the scores at each of the six testing times summed to zero, no "between period" variance could exist. 2) Column headings are as in Table 2. 3) Because the degrees of freedom for the residual errors are large (680), it is possible to use $F \sim \chi^2/df$ to obtain p.

ceivable that this emphasis on fresh eggs helped recognition and caused psychological bias. If this is correct, the preference tests are methodologically sounder than the direct ratings *with an identified item on the side*. Thus, in the present instance, as far as the comparison between the two treatments is concerned, the preference tests are constitutionally as good as, although more efficient than, the ratings. But as far as the ancillary comparison of the treatments with the controls is concerned, the ratings show what may well be a spuriously large difference. In a way this conclusion is reinforced by the observation that the scores from the rating method do not significantly change over the whole testing period, whereas the scores from the preference tests gradually fall, as is to be expected.

A consideration of the constitutional differences between the two types of 3-item sorting designs—the "triangle" test and the "duo-trio" test—has some relevance here. It is discussed in several publications, including Gridgeman (1959).

A caution

An ideal experimental comparison of the two methods would involve the same taste panel for both. This was not technically feasible in the egg work; it is to be remembered that the comparisons were parasitic on an investigation whose main object was something else again, so that the information now reported was a bonus. Formally, the possibility must be acknowledged that the pair-preference panel was more sensitive than the rating-scale panel—a circumstance that would bias the conclusions about the relative merits of the methods. But this possibility is comfortably small, partly because the panel members can be looked upon as random samples from a homogeneous egg-fancying population (see last paragraph of Introduction), and partly because of the internal evidence of a real inter-method disparity. Moreover, the findings are broadly harmonious with those of a comparable and comprehensive investigation made a few years ago, discussed below.

A comparison of comparisons

Murphy *et al.* (1957) used a strawberry palatability trial to compare ranking, rating, and multiple pair preferences as methods of taste appraisal. There were really four methods at issue, since the pairs were judged on both a binary scale and on a Scheffé septenary scale (with a central zero class). All the methods were carried out by one panel of over 30 non-experts.

It may at once be said that these trials showed that pair preferences are better than rating judgments, but it is doubtful whether a precise and meaningful *quantitative* comparison can be made. One difficulty is that we are more or less compelled to use a single *tasting* as the primary operational unit, and this is not always unambiguous. For instance, in the egg-rating trials, 7 tastings were done on 5 samples, because the control was triplicated—which, of course, is not standard practice. Another complication is that inter-taster differences were

screened from some of the strawberry trials but from none of the egg trials.

Nevertheless, a rough quantitative comparison is worth attempting. Some prefatory remarks on the general matter of scales are necessary here. In all germane sensory work there is a "natural" scale unit: it is the residual standard deviation of observations taken on the prescribed interval scale —whatever that may be, rank numbering, graded categories, or degrees of preference. That is the basis, as already stated, of the figure showing the egg results. One consequence of this natural unit is that F values (variance ratios) offer a rational device for the assessment of efficiency. From our Table 2, for example, we can assert that the relative magnitudes of the main inter-egg-treatment variances are, *on the same scale*, 4.05 −1=3.05 for the pair preferences on the senary scale, 2.29−1=1.29 for the pair preferences on the binary scale, and 0 for the rating judgments. If we also examine Tables 3 and 4 in the Murphy-Covell-Dinsmore paper in this light (and taking into account the fact that the strawberry pair comparisons took 4 times as many tastings as the ratings and the rankings), the following tabulation can be made:

	Relative efficiencies	
	Egg trials	Fruit trials
Simple pair preferences	100	100
Pair preferences with degrees	236	142
Rating	0	44
Ranking	—	59

And this is perhaps as much as we can say, quantitatively.

In the strawberry report the four methods are compared on the basis of a "sample number ratio" (SNR), attributed to Amihud Kramer, which yields relative efficiencies slightly different from those based on the F values. If, however, we look at the definitions,

$$\mathrm{SNR} = \frac{2t^2\ (\text{residual variance})}{(\text{range of sample means})^2}$$

(where t is Student's statistic), and

$$1/\mathrm{F} = \frac{\text{residual variance}}{(\text{standard deviation of sample means})^2}$$

it is apparent that, although the SNR was created to supplant the F value (on the misapprehension, it seems, that the latter is anchored to the specific test scale), the two are essentially the same (in reciprocal). When there are only two samples (as in the "condensed" egg results), the range and the standard deviation are simple factors of one another (range $= S\sqrt{2}$); when there are three or more samples the use of the range is well-known to be inferior (carrying less information) to the standard deviation.

As far as ranking is concerned, cross-comparison is impossible, since only the strawberries were so treated. Attention should perhaps be drawn to a detail of the handling of the 3-sample rankings (and of the simple pair preferences regarded as 2-sample rankings) of the strawberries. In a sense this again brings the question of scaling to the fore. To subject the results to analysis of variance, the authors transformed the ranks to "normal scores" (Fisher and Yates, 1957): thus 1 : 2 : 3 was replaced by +0.85 : 0 : −0.85; and 1 : 2 by +0.56 : −0.56. Since other instances of this practice occur, it is as well to state here that *to transform ranks to "normal scores" is pointless when the number of ranks is fewer than four*. The reason, in brief, is that normalization is aimed at scale intervals, not scale values. Only when there are more than two (necessarily symmetric) intervals can their relative magnitudes be transformed.

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Effects of Gamma Irradiation on the Chemical Properties of Actin and Actomyosin of Meats [a]

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SUMMARY

Effects of gamma irradiation on the chemical properties of actin and actomyosin during aging of meats have been studied. Meats were irradiated with 4×10^6 rad of cobalt-60 gamma rays at 40°F in three steps, i.e., immediately after slaughter, at maximum rigor, and at "rigor off." Actin and two kinds of actomyosin (AMS and AML, previously reported by authors) were isolated from irradiated and control meats. The contents of sulfhydryl groups and amino acids, viscosity, the activity of ATPase and ATP sensitivity of actin and actomyosin were determined. From the results, actin is thought to be only slightly sensitive to irradiation as compared with actomyosin, and the latter, especially AML (a long-time extracted actomyosin) is considered to be very sensitive. In this case, it is inferred that the depolymerization must have taken place in actomyosin molecule by irradiation on meats. The effects of the time of irradiation on the chemical properties of actomyosin in meats seem to be more remarkable at rigor off than at the other two steps.

When meat was irradiated with necessary doses of gamma rays from a cobalt-60 source to obtain effective preservation, it was recognized that irradiated meat was accompanied with the development of irradiation odors and off-flavor (Batzer and Doty, 1955; Batzer *et al.*, 1957, 1959 a, b; Cain *et al.*, 1958; Pearson *et al.*, 1959; Sliwinski and Doty, 1958), color changes (Ginger and Schweigert, 1956; Hougham and Watts, 1959; Watts *et al.*, 1955), destruction of vitamins (Batzer *et al.*, 1959 a) and oxidation of fats (Licciardello *et al.*, 1959; Tappel *et al.*, 1955; Tarladgis *et al.*, 1959), which may lower food acceptance and values.

So, it seems necessary to use gamma irradiation as the preservation method to promote sterilizing effects at lower doses and/or to minimize spoilage of meats at high doses of irradiation (Matsuyama *et al.*, 1959).

Protein in food contributes measurably to the flavor, texture, and appearance of food (bases of consumer acceptance); and changes in foods that have been irradiated seem to be partly related to changes in the protein.

It is generally recognized that irradiation with relatively high doses is accompanied by the denaturation of protein—a splitting of protein molecules or the association of these molecular fractions; and denaturation of protein from irradiation has been reported, including changes of viscosity, solubility (Crowther and Liebmann, 1939; Fricke, 1952; McArdle and Desrosier, 1955), ultraviolet absorption spectra (Alexander *et al.*, 1956), and the action of proteolytic enzymes (Doty and Wachter, 1955).

But studies on irradiation of meat proteins seem to be mostly restricted to changes of the pigment protein.

This is a report on the effects of gamma irradiation on the chemical property of fibrous protein in the muscle, actin and actomyosin, during aging of meats.

EXPERIMENTAL

Meat (longissimus dorsi muscle of rabbit) was irradiated with gamma rays from a cobalt-60 source at dose levels of about four million rad (40°F for 44 hours).

Meat was irradiated at three times: immediately after slaughter, at maximum rigor, and at rigor off as shown in Figure 1.

Actin and two kinds of actomyosin (named AMS

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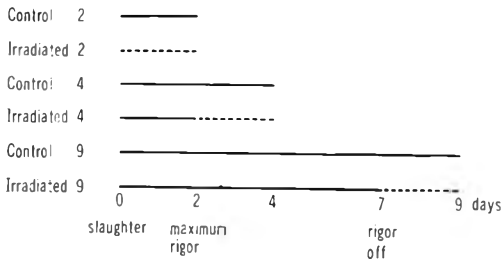

FIG. 1. Irradiation of meat in three steps during aging.

and AML, previously reported) were isolated from both irradiated and unirradiated meats.

Preparation of actin was carried out by the method of Bárány *et al.* (1957). To the required weight of the sample (longissimus dorsi muscle of rabbit) were added 20 volumes of 0.4% sodium bicarbonate solution and the mixture was stirred for 20 minutes at room temperature and centrifuged, and the supernatant was discarded. Again the same volumes of 0.4% sodium bicarbonate solution were added to the residue, and the mixture was stirred and centrifuged repeatedly.

Thereafter, 15 volumes of distilled water were added to the residue, and the procedure was carried out as previously.

Ten volumes of acetone were added to the residue, and the mixture was filtered by suction after stirring. Similarly, 3 volumes of acetone were added to the residue, and the same procedure was carried out four times successively. This acetone-treated residue was allowed to stand overnight at room temperature and dried.

Twenty-five volumes of 0.3*M* potassium iodide solution containing $5 \times 10^{-4}M$ ATP were added to the definite quantity of the acetone-dried muscle powder. After extraction for 30 minutes, the mixture was centrifuged, and to the supernatant were added 9 volumes of distilled water and one-hundredth volume of 1*M* acetate buffer (pH 4.7). The mixture was allowed to stand for 10–20 minutes in order to precipitate the actin, and was centrifuged.

To the precipitate (crude actin) was added the same volume of 0.3*M* potassium iodide solution as mentioned above, and actin was dissolved. Thereafter, the procedure was repeated twice.

The supernatant has been confirmed not to contain alkaline earth metal ion. Finally, the precipitate was dissolved by the addition of 0.3*M* potassium iodide solution and used as refined actin solution (3 mg/ml). Two kinds of actomyosin (named AMS and AML) were prepared, as follows. The name of AMS was given to actomyosin, that is obtained by extracting meat for a short period with 0.6*M* potassium chloride solution and then precipitating by dilution to an ionic strength of 0.1 with redistilled water, and refined.

AML is another kind of actomyosin, extracted with Weber-Edsall solution, for a long period from the residue remaining after extraction of AMS, and precipitated by dilution to an ionic strength of 0.1 with redistilled water, and so refined (Fujimaki, 1958a; Hasselbach and Schneider, 1951).

pH values of the muscle. To 1 g of the sample was added 5 ml of distilled water and stirred well, and then pH values of the supernatant were measured by electric pH meter with glass electrode.

Relative viscosity. Viscosity of actin (3 mg/ml) and actomyosin solution (2.5 mg/ml) was measured at 34°F with an Ostwald viscosimeter.

ATP sensitivity (Hasselbach and Schneider, 1951; Weber and Portzehl, 1951). To 5 ml of actomyosin solution in potassium chloride (3 mg/ml) were added 0.25 ml of $2 \times 10^{-2}M$ magnesium chloride solution and 0.5 ml of neutral sodium ATP solution. After it was mixed thoroughly, the relative viscosity $\eta_{rel\,(ATP)}$ was measured.

ATP sensitivity was calculated from the following equation:

$$\text{ATP sensitivity} = \frac{\log \eta_{rel} - \log \eta_{rel\,(ATP)}}{\log \eta_{rel\,(ATP)}} \times 100\%$$

ATPase activity (Fujimaki, 1958b). The reaction mixture—which contained a final volume of 3.0 ml, composed of 1.0 ml of 0.2*M* tris(hydroxymethyl) aminomethane-maleate buffer solution (pH 6.5 for AMS, pH 6.2 for AML), 0.5 ml of $6 \times 10^{-2}M$ calcium chloride solution, 0.5 ml of 1.2*M* potassium chloride solution, 0.5 ml of actomyosin solution (2.5 mg/ml), and 0.5 ml of $5 \times 10^{-3}M$ neutral sodium ATP solution—was incubated at 78°F. At measured intervals of time, to aliquots of the reaction mixture was added 1.0 ml of 10% trichloroacetic acid solution, and the protein precipitated was filtered off through a dry filter paper. The amount of inorganic phosphorus liberated in 1 ml of the filtrate was determined colorimetrically with a modification of the Allen method.

One unit of ATPase activity was defined as the amount of enzyme that liberated 1 μg of inorganic phosphorus under the above conditions.

The estimation of sulfhydryl was carried out by the method of Harold Edelhoch *et al.* (1953). To 5 ml of protein solution was added 2 ml of 5*M* guanidine bromide solution (pH 10.0).

A drop of 10% sodium nitroprusside solution was added to the solution, and the solution was then titrated with $10^{-2}M$ salyrgan solution to disappearance of the purple. The titration was carried out at 34°F.

Tryptophan was determined by the glyoxzylic

acid method of Carpenter (1948), tyrosine by the Folin method revised by Hagiwara and Akabori (1955), methionine by the nitroprusside method of Block and Bolling (1951), arginine by Sakaguchi's method (1950), and glutamic acid by Warburg's Respiratory Manometer method (Umbreit *et al.*, 1957).

Quantitative determination of alkaline earth metals was carried out by the method of Arnold *et al.* (1956).

To the sample were added ammonium buffer solution (the mixture of 1 volume of 1*M* ammonium chloride with 5 volumes of 1*M* ammonium hydroxide solution) and a drop of Eriochrome black T solution (2 g of Dotite B. T. [1-(1-hydroxy-2-naphthylazo)-5-nitro-2-naphthol-4-sulfonic acid sodium salt] dissolved in 50 ml of methanol containing 2 g of hydroxylamine hydrochloride. Then, the solution was titrated with $1 \times 10^{-3}M$ EDTA solution (sodium salt of ethylenediamine tetraacetic acid).

The quantitative determination of calcium was carried out by the method of Jenness (1953).

To the sample, 2*N* sodium hydroxide solution was added to make the pH of the solution above 10, and it was titrated with $1 \times 10^{-2}M$ EDTA solution using about 0.2 g of Dotite N. N. [2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid] as the indicator.

Water-holding capacity was measured by the method of Wierbicki *et al.* (1957a).

RESULTS AND DISCUSSION

pH values of meats

It has been shown that irradiation decreases the hydrogen-ion concentration of meat, and that a dose of 10 megarads will raise the pH of meat by approximately 1.0 unit (Evans and Batzer, 1960).

But as Batzer *et al.* (1959a) have reported, there seems to be no significant difference between the pH value of the control meat and that of irradiated meat, as shown in Table 1.

Table 1. Effects of irradiation on pH values of muscle.

Muscle	Age in days	Control	Irradiated
A	0	6.67	.....
	2	5.84	6.06
	4	5.88	5.85
	9	6.38	6.21
B	0	.....	.....
	2	6.05	5.93
	4	5.74	5.83
	9	5.98	5.82

Relative viscosity, ATP sensitivity, and ATPase activity of actomyosin

McArdle and Desrosier (1955) reported that the radiation dose of 0.5×10^6 rad on aqueous casein solution resulted in a slight decrease in relative viscosity, showing the increase of the viscosity with increase of the dose above this level, like the changes of the viscosity of egg albumin solution.

It has been considered that a splitting of casein molecules into smaller fractions occurred when the purified protein was irradiated in aqueous solution, and further irradiation caused the association of these molecular fractions until complete coagulation took place.

Sumiki (1959) reported that changes in the ultraviolet absorption spectra of egg albumin and casein were qualitatively similar, and that the relative viscosity of a 3% casein solution increased slightly with doses up to 1×10^6 rad, and markedly above 1.5×10^6 rad, whereas no significant change was found in the egg albumin solution irradiated with doses of 0.5 to 3×10^6 rad.

Schweigert (1959) showed that irradiation resulted in increases in viscosity of protein solutions and decreases in solubility.

As shown in Table 2, the difference between irradiated and control meats in viscosity of AMS was small at the early step of aging; it became larger with aging. With AML, however, irrespective of the step of aging, irradiation influenced much its viscosity, and the viscosity of AML from irradiated meats was considerably lower than that from control meats.

ATP sensitivity of AMS and AML from irradiated meats was much lower than that from control meats throughout the step of aging, as shown in the same table.

ATPase activity of AML from irradiated meats seemed to be slightly different from that from control meats throughout the step of aging, and the difference between ATPase activity of AMS from irradiated and control meats was not significant except at 9 days of aging.

Evans and Batzer (1960) wrote that in simple systems some enzymes are readily

Table 2. Effects of irradiation of muscle on relative viscosity, ATP sensitivity, ATPase activity, and content of alkaline earth metals and calcium of actomyosin.

Age (days)	Relative viscosity		ATP sensitivity (%)		ATPase activity [a]		Alkaline earth metals [b]		Calcium [b]	
	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated
AMS										
0	1.48	.....	.....	.....	3.15	.....	1.0	.....	1.0	.....
2	1.41	1.45	.....	5.6	1.81	1.96	1.5	1.9	1.2	0.4
4	1.69	1.50	31.9	6.7	1.77	1.71	16.8	11.4	3.6	2.3
9	1.75	1.39	34.8	2.3	2.01	0.86	14.1	6.3	1.5	1.1
AML										
0	2.20	.....	25.4	.....	3.08	.....	3.3	.....	1.8	.....
2	2.56	1.91	43.0	23.5	3.12	2.91	6.6	5.3	.....	.....
4	2.50	1.99	46.6	25.5	3.20	2.70	26.8	9.4	3.6	1.8
9	2.18	1.65	42.1	21.7	2.47	2.33	16.7	5.8	1.7	.....

[a] The values are shown in liberated p (μg/min).
[b] The values are shown in mM/100 g of protein.

inactivated well below the sterilizing level of irradiation, but in meat *per se*, doses well above the sterilizing level are necessary for inactivation.

Doty and Wachter (1955) reported that irradiation at 1.6×10^6 rad reduced the apparent proteinase activity of beef muscle about 50%, and that at lower irradiation doses (5×10^5 rad) there was little reduction in proteinase activity.

While the proteinase activity of muscle should reduce apparently at the level of doses (4×10^6 rad), ATPase activity of actomyosin reduced scarcely at the same doses except in the case of AMS from irradiated meat at 9 days of aging.

The reason why the ATPase activity of AMS from irradiated meat was reduced only at 9 days of aging, remains unknown, but the protein of AMS at this step of aging is thought to be very sensitive to irradiation.

From the results of viscosity and ATP sensitivity, it is inferred that the depolymerization must have taken place in actomyosin molecule by irradiation of meats.

Relative viscosity and the content of sulfhydryl groups of actin

As shown in Table 3, there seemed to be no significant difference between the relative viscosity and the content of sulfhydryl groups of actin from irradiated and control meats throughout each step of aging.

McArdle and Desrosier (1955) reported that irradiation of casein and egg albumin solution caused a substantial increase in free sulfhydryl groups, with no increase in free amino nitrogen.

Also, Kraybill *et al.* (1960) reported that gamma irradiation (to 9.3 megarad) resulted in dose-related increases in viscosity, and in sulfhydryl and disulfide content of raw skim milk.

On the other hand, Erdmann and Watts (1957) reported that doses ranging from 2×10^5 to 2×10^6 rad caused a loss of free sulfhydryl groups and marked changes in nitrite in cured meat.

From the results that there is no significant difference between the viscosity and the content of sulfhydryl groups of actin from irradiated and control meats, it is considered that actin is relatively stable to irradiation, compared to actomyosin, especially AML.

Amino acid composition of actomyosin and actin

In this experiment, both irradiated and control meats at 9 days of aging were used.

Schweigert (1959) wrote that little destruction was noted with sterilization doses of amino acids in intact proteins, and that there was no major loss in amino acid contents although changes were profound in the physical and biological characteristics of the proteins irradiated.

Read (1959) has shown that amino acids were much more radio-resistant when they were bound together in protein molecules, and that even at a dose of 5.3 megarad, the

Table 3. Effects of irradiation of muscle on the relative viscosity and content of sulfhydryl groups, alkaline earth metals, and calcium of actin.

Age (days)	Relative viscosity		SH-groups content [a]		Alkaline earth metals [b]		Calcium [b]	
	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated
2	1.57	1.56	1.67	1.69	3.9	2.5	2.0	1.6
4	1.52	1.48	1.20	1.39	5.1	4.4	2.3	3.0
9	1.52	1.56	1.41	1.55			3.1	2.5

[a] The values are shown in M per 57,000 g of actin.
[b] The values are shown in mM per 100 g of protein.

maximum loss was only 6.92% of tryptophan.

But, according to Tsien and Johnson (1959), glutamic acid, aspartic acid, serine, and glycine were most seriously reduced by irradiation (2.8–9.3 million rad) in both beef and milk.

Also, Hedin *et al.* (1960) reported that when the water-soluble, non-dialyzable fraction of beef, thought to be a mixture of at least two electrophoretically separable proteins, was irradiated (5 megarads), cystein and methionine could no longer be detected, while the content of histidine decreased by approximately 25% as a result of irradiation of the protein.

So, different results have been reported on the retention of amino acids present in protein molecules irradiated with sterilization doses.

But as shown in Table 4, there seemed to be no significant difference between irradiated and control meats in the amino acid composition (tryptophan, tryosine, methionine, arginine, and glutamic acid) of actomyosin and actin.

On the contents of free amino acids in the muscle, as shown in Table 5, the contents of free tryosine, methionine, arginine, and nonprotein nitrogen were found to be less in irradiated meats than in control meats.

The losses of free amino acids and nonprotein nitrogen in irradiated meats will be due to deamination or decarboxylation by irradiation, as Schweigert (1959) and Read (1959) have stated.

The content of alkaline earth metals and calcium of actomysin, actin, and globular protein

As shown in Table 2, the contents of bound alkaline earth metals and calcium of actomyosin showed considerable changes throughout the step of aging, and significant differences were seen between those of irradiated and control meats. Also, the differences seemed to be slight at the early step of aging and became remarkable as aging progressed.

But, as shown in Tables 3 and 6, no significant difference was seen between the contents of bound alkaline earth metals and calcium of actin and globular protein from irradiated and control meats except those of globular protein from samples at 9 days of aging.

Arnold *et al.* (1956) reported that, during the aging of beef, sodium and calcium ions are continuously released by muscle proteins, potassium ions are absorbed after the first 24 hr, and magnesium ions are released during the first 24 hr and also between 6 and 13 days, followed by decreases in released magnesium ions.

Table 4. Effects of irradiation on amino acid composition of AMS, AML, and actin (%).

Amino acid	AMS		AML		Actin	
	Control	Irradiated	Control	Irradiated	Control	Irradiated
Try	1.33	1.35	0.94	0.93	.....	.....
Tyr	3.02	3.19	3.28	3.41	3.16	3.04
Met	5.25	5.45	5.30	5.94	7.92	7.76
Arg	9.50	9.63	10.33	9.63	9.14	8.85
Glu	22.6	22.7	24.8	25.2	16.6	16.4

Table 5. Effects of irradiation on the contents of free amino acids in the muscle (mg per 100 g of muscle).

Amino acid	Control	Irradiated
Try	trace	trace
Tyr	2.37	2.05
Met	52.0	39.2
Arg	0.52	0.31
Glu	trace	trace
Non-Prot-N	295	373

Table 6. Effects of irradiation of muscle on the contents of alkaline earth metals and calcium of globular protein (m*M* per 100 g protein).

Age (days)	Alkaline earth metals		Calcium	
	Control	Irradiated	Control	Irradiated
0	0.88	.....	0.31	.....
2	0.83	0.88	0.24	0.23
4	0.96	0.77	0.24	0.26
9	1.37	0.74	0.37	0.27

In further studies on the role of ions in protein hydration and tenderness, Wierbicki *et al.* (1957 a, b) confirmed the dynamic shifts of the potassium, sodium, calcium, and magnesium ions involved in the hydration of proteins. Also, it has been found that the added chlorides of sodium, potassium, magnesium, or calcium increase the water-holding capacity of beef protein on cooking and on freezing and thawing.

Wierbicki *et al.* (1957 b) found that addition of calcium ions to meat increases the water-holding capacity of cooked beef, whereas Grau and Hamm (Gran *et al.*, 1953; Hamm, 1955, 1956, 1958) reported opposite results.

They found that the removal of calcium ions by a complexing agent, such as phosphate, ATP, EDTA, etc., or by ion exchanger, increases the water-binding capacity of sausage. They also reported that salts of the alkali metals, especially sodium chloride, as well as phosphates, increase the water-binding properties of pork and beef.

So, it may be considered that there are intimate relations between the water-holding capacity and the content of alkaline earth metals of meat.

Hashimoto *et al.* (1960) reported that actomyosin in particular has strong connection with water-holding capacity among the muscle proteins.

From the results of actin and globular protein on the contents of bound alkaline earth metals and calcium, those proteins are thought not to be so much related with the water-holding capacity and not to be so sensitive to irradiation.

Therefore, it will be important to examine the relation between the contents of bound alkaline earth metals and/or calcium to actomyosin and the water-holding capacity of meats.

Though Schweigert (1959) noted that irradiated meat seemed to have a reduced water-holding capacity, presumably from alteration in the affinity of denatured protein for water, irradiated meat has shown the increase of the water-holding capacity, compared with that of control meat at 9 days of aging (meats will he very sensitive to irradiation at this step of aging): the juice released from 10 g of muscle on heating at 178°F was 3.8 ml in the control, compared with 3.2 ml in irradiated meat by the method of Wierbicki *et al.* (1957 a).

Considering the reduction of the content of bound alkaline earth metals and calcium of actomyosin and increase in the water-holding capacity of meat by irradiation, we may be able to ascertain the view that the release of alkaline earth metals and/or calcium from meat proteins, especially actomyosin, will increase the water-holding capacity of meat.

From the above-mentioned results, it may be assumed that the use of irradiation in meat preservation should better be held at the early step of aging from the viewpoint of chemical properties of meat proteins.

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The Oxidation of Lipids in Thin Films[a]

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SUMMARY

A model system of thin films of lipid supported on dry gelatin plates was used for studying rates of lipoxidation. Variations in the nature of the gelatins used in the supporting surfaces showed significant differences in protective action against lipid oxidation. It is suggested that the observed differences may be due to differences in the orienting effects of the surfaces. Gamma-tocopherol was found to be an effective inhibitor of the oxidation of the films. Synthetic phenolic antioxidants were not nearly as effective. Phospholipid in large concentration was also an effective inhibitor.

When food is dehydrated, the natural aqueous state of the components is upset and there are new orientations of the lipid component in relation to the other constituents. One important effect is the creation of new surfaces upon which the lipid lies and spreads. Since other investigators have found the spread of lipid on surfaces to be conducive to oxidation (Lea, 1934, 1939), an understanding of the nature of these effects would be helpful in preventing lipid deterioration in dehydrated foods.

This paper reports the reactions of lipids in the presence of other food components at low moisture levels. A model-system approach was devised employing thin films. Food components were selected and combined under controlled conditions for better understanding of their effects on lipid oxidation. This research was conducted to corroborate results obtained through a model-system study on freeze-dried emulsions, reported on earlier (Bishov *et al.*, 1960).

EXPERIMENTAL

Thin films of the components used in the model system were prepared on flat glass Petri dishes 135–145 mm inside diameter. Films were prepared by pipetting a solution of material to be studied on the plate surface at room temperature and slowly evaporating the solvent. When multi-layers of different components were prepared, each layer was made and dried before the succeeding layer (dissolved in a solvent that would have minimum effect on the preceding layer) was pipetted and dried.

The lipid materials used are listed in Table 1.

Table 1. Lipids used in model system.

Lipid	Treatment
Cottonseed oil, refined (Wesson)	
Cottonseed oil, refined, tocopherol-free	Tocopherols removed at 175–200°C, 6.5 microns. on centrifugal cyclic-batch molecular still (DPI).
Trilinolein (Hormel)	
Soy phospholipid, Gliddex "P" (Glidden)	

Films of these lipid materials were prepared from 1% solutions of lipid in benzene. Solvent was evaporated from the Petri dish at room temperature in a hood to obtain a slow movement of air over the dish.

Gelatin solutions for thin film preparation were made by adding the dry gelatin to cold water and stirring while warming on a hot plate. Thin films from these solutions were prepared by adding

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1 cc of a 3% solution of the gelatin for every 10 sq cm of plate surface and drying 2 hr in a 75°C draft oven. To prevent cracking of the film during drying, 1% glycerine (solution basis) was added to the gelatin solution as a plasticizer. Gelatins used in the experiments are summarized in Table 2. The phospholipid films were prepared in the same manner as the lipid films using a 1% solution in methanol–petroleum ether or aqueous ethanol.

Table 2. Properties of gelatins used in surface experiments.

Gelatin sample	Type	pH	Bloom	Viscosity	% Ash
1 (deionized)	A	6.4	high	....	0.08
2 (deionized)	A	7.6	low	....	0.16
3	A	4.3	205	42	0.74
4	A	4.2	275	53	0.66
5	A	5.0	110	29	0.94
6 (deionized)[a]	A	4.1	high	....	0.14
7 (deionized)	B	4.9	low	....	0.03
8	B	5.5	210	46	1.69
9	B	5.6	240	47	1.56
10	B	6.7	100	34	1.72
11	B	6.7	35	19	1.87

[a] Gelatin #6 was deionized, and pH then adjusted.

In preparing multi-layers of gelatin, cottonseed oil, and soy phospholipid, the following procedure was used: When cottonseed oil was to be the film adjacent to the pre-formed protein film (see above), the oil solution in benzene was pipetted on the dry protein surface and solvent evaporated as previously described. To minimize mixing with the pre-formed lipid film, the phospholipid was dissolved in 92% ethanol or methanol–petroleum ether mixture (60:40), in which the lipid was sparingly soluble. When the cottonseed oil–phospholipid film positions were reversed, cottonseed oil was dissolved in acetone, in which the dried phospholipid film was sparingly soluble.

The mixture of phospholipid and gelatin [(c/g + p) in Table 6] was made by dispersing 0.1 g of the phospholipid in 1.0 g of glycerine, and this dispersion was added to 100 ml of 3% gelatin solution. The mixture of phospholipid and cottonseed oil was made by dissolving 1% of each lipid into petroleum ether solvent. The above-prepared thin films were exposed in a forced-draft oven at 75°C. After exposure, the lipids were removed by extracting repeatedly with chloroform. Peroxide values were run by the method of Wheeler (1932). Diene conjugation measurements were made by the method of Mitchell *et al.* (1943) on iso-octane solutions of the oxidized fat.

RESULTS AND DISCUSSIONS

In a preliminary experiment on surface oxidation, filter paper (Whatman No. 1, 4.25 cm diameter) was used as a surface for the lipids. Concentrations of cottonseed oil on filter paper of uniform size were varied by dipping the papers for about five minutes into different concentrations of cottonseed oil in benzene. Evaporating the benzene left a film of oil on the tared paper, which was reweighed to determine the weight of oil, and then oxidized 16 hours in an oven at 45°C. The papers were extracted with benzene and peroxide (Hills and Thiel, 1946), and carbonyl (Henick *et al.*, 1954) values were determined on the extract. The results (Table 3) show that as the amount of oil decreases on the paper, or, in other words, as the amount of total surface area to quantity of oil increases, there is an increase in the peroxide value within experimental limits. The carbonyl values also increased with increasing ratio of surface area to oil quantity except for the two lowest oil additions. A possible explanation is that the oil film was discontinuous, which limited the effect of free-radical chain reactions.

At the lowest concentrations, recovery of the oil by benzene extraction was insufficient for determination of the peroxide value. Because of this and the fact that the capillarity of the paper leads to uneven distribution of the oil, making surface-area measure-

Table 3. The oxidation of cottonseed oil on filter paper.

% oil in benzene	0.01	0.1	1	3	5	10	20	50	100
Mg oil adsorbed on filter paper	0.9	1.1	2.8	6.2	9.4	19.5	37.5	109	135
PV after 16 hours at 45°C millimols/kg	insufficient sample			2400	385	49	38	14	12
Carbonyl value micromols/g	700	675	2905	1330	156	51	22	14	11

ments unreliable, the previously described model system was devised using flat glass Petri dishes.

Effect of spreading of lipids. Fig. 1 shows the effect that decreasing the weight of oil per given surface area has on the oxidation of cottonseed oil on glass and gelatin surfaces. The amount of oxidation was found to be dependent on the relative amount of

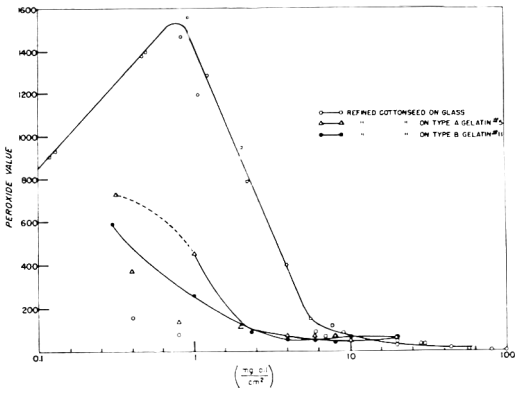

FIG. 1. The relation between peroxide values and film thickness of refined cottonseed oil on glass and gelatin surfaces after heating for 16 hours in the draft oven at 75°C.

oil exposed to air. The maximum peroxide value appeared at 1 mg oil/sq cm of glass surface. No measure of film thickness was made, but it was calculated that the lipid films were several hundred molecules in thickness. The thickness also probably varied from hills and valleys in the protein surface. However, at lower weight lipid/sq cm surface, oxidation had undoubtedly proceeded beyond the measured peroxide value, since insoluble polymeric substances, formed from peroxide interaction or decomposition, were found on the surface.

The difference between the oxidation of cottonseed oil on glass and on gelatin surfaces is clearly seen in Fig. 1. The protein surface greatly reduced the rate of oil oxidation. Two possible explanations are: 1) hills and valleys in protein film reduce surface area of oil, and 2) orientation effect of protein on oil reduces susceptibility to oxygen attack.

Fig. 2 shows the differences in rate of peroxide formation in refined and molecularly distilled cottonseed oils on glass and

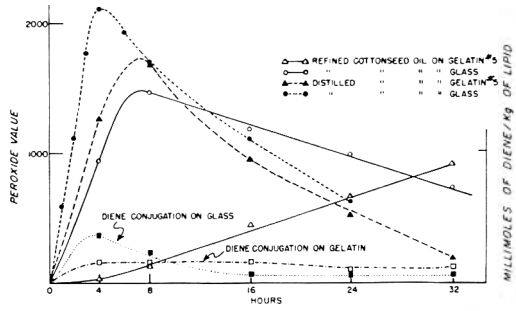

FIG. 2. Rates of autoxidation of cottonseed oil (1 mg/sq cm) on glass or gelatin surface in a draft oven at 75°C. Diene conjugation rate curves are the iso-octane solutions from the distilled cottonseed oil samples. Conjugated diene was calculated from the absorption at 233 mmc of 1 g/liter using the specific absorption of Brice *et al.* (1952), as follows: diene (millimols/kg) = A/0.026333.

gelatin surfaces at a concentration of 1 mg oil/sq cm. The oxidation of refined cottonseed oil on glass was without an induction period. On the other hand, an induction period of about 150 minutes was observed for oxidation of the refined cottonseed oil on a gelatin surface, and had no similarity to the rate on a glass surface.

Oxidations carried out using a tocopherol-free (molecularly distilled) oil did not show the vast differences in rates of oxidation on glass and gelatin surfaces, as did the unaltered oil (Fig. 2). This suggests that, in part, the protective action of protein against autoxidation of cottonseed oil was due to enhancement of the antioxidant activity of the tocopherols.

Diene conjugation of the recovered distilled oils from these surfaces is also shown in Fig. 2. On a glass surface, diene conjugation increased rapidly to a maximum and then gradually decreased. This was found to precede the maximum peroxide value attained by the oil. The disappearance of diene conjugation has been attributed by other workers to a termination reaction of the free-radical chain, involving the formation of polymers (Bradley and Richardson, 1940). The tacky substances found on the highly oxidized surfaces were probably the result of this reaction. Quantitative measurement of oil recovered from glass plates showed a low yield by cold chloroform ex-

traction. The tacky insoluble substance was tightly bound to the glass and could be removed only by heating with chloroform. A chloroform-insoluble film was also found on gelatin plates exposed for 16 hours or more. These films became visible after the plate was filled with hot water and the film allowed to separate from the water-soluble gelatin. When picked up with a pair of tweezers, the film formed fibrous strands. Qualitative tests showed no nitrogen present in the chloroform-insoluble film.

Effect of antioxidants. To study the effect of the natural antioxidants in cottonseed oil, 0.1% γ-tocopherol (D.P.I., Eastman) was added to samples of the cottonseed oil, stripped of tocopherol by molecular distillation, and trilinolein. Thin films of fortified and unfortified lipids were prepared on glass and gelatin surfaces and held at 75°C in a draft oven. Samples were removed after set intervals, and peroxide values determined. This study revealed that γ-tocopherol significantly inhibited the oxidation of these lipid films on glass as well as on gelatin (Fig. 3 and 4). Protective effect was greater for the gelatin surface than for the glass surface. There was little difference between glass and gelatin surfaces, however, for the unfortified trilinolein film (Fig. 4). The greater effectiveness of the gelatin surface in protecting cottonseed oil may be due to the presence of some residual tocopherol in the oil. That this is not the entire reason is seen in Table 4 and discussed below.

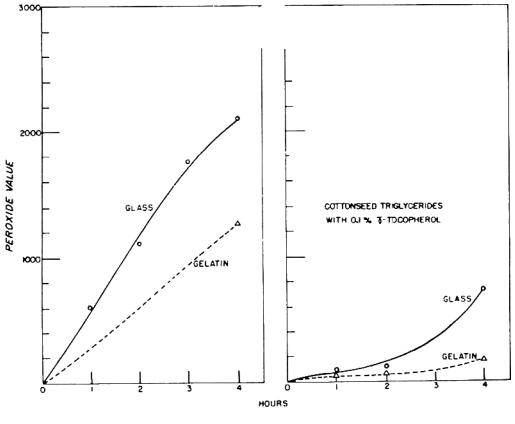

FIG. 3. Comparison of the rate of autoxidation of distilled cottonseed oil films with and without γ-tocopherol (1 mg/sq cm).

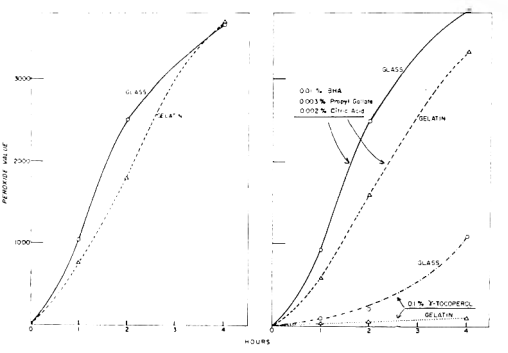

FIG. 4. Comparison of the rate of autoxidation of trilinolein films with and without antioxidants added (1 mg/sq cm).

In another series of tests a commercial antioxidant preparation (see Fig. 4) was added to trilinolein and subjected to heating in the draft oven. The results indicated that the antioxidant mixture was without significant effect in inhibiting film autoxidation.

Effect of gelatin on lipid oxidation. As mentioned above, gelatin possessed a significant inhibitory effect on lipid film oxidation. The question arose, however, whether this effect was only enhancement of the tocopherol effect. Consequently, a series of experiments were carried out with eleven different gelatin surfaces, each prepared as described under Experimental. Property differences of these gelatins are listed in Table 2. As indicated in Table 4, samples 1–6 were acid-processed and samples 7–11 were lime-processed gelatins. The tests using refined cottonseed oil films were run for 16 hours at 75°C, whereas the distilled cottonseed oil was heated for only 4 hours at 75°C because of the break in the peroxide curve at 6–7 hours (Fig. 2).

The results in Table 4 show that there was a wide variation in protective effect of gelatin films. Inspection of Tables 2 and 4 shows that there is no correlation between the ash content of the gelatin and peroxide formation in the lipid film. The observed variation may be due to the protein surface characteristics, since the same distilled oil was used throughout the different experiments. This indicates that the protective effect of gelatin may not be entirely attributable to enhancement of tocopherol antioxidant activity. These results and those in

Table 4. A comparison of the autoxidation of refined and tocopherol-free cottonseed oil films on 11 gelatin surfaces.

Gelatin	Refined cottonseed oil PV after 4 hr at 75°C	Tocopherol-free cottonseed oil PV after 16 hr at 75°C
Acid-processed		
1	416	1180
2	378	1650
5	452	1267
3	336	1720
4	299	1709
6	294	1195
Lime-processed		
7	385	1433
8	488	1412
9	426	1638
10	421	1602
11	374	1759

Table 5, in which various antioxidants were tested for their protective effect against distilled cottonseed oil films on gelatin, make it questionable that tocopherol merely acts as a free-radical acceptor. At considerably lower equivalent phenolic hydroxyl concentration (Table 5), the larger tocopherol molecule had a much greater protective effect. Thus the effectiveness of tocopherol may be due to physical orientation as well as stopping free-radical chain reactions.

Also observed in Table 4 is a difference in protective effect of the gelatin samples due to processing conditions. Certain samples of the acid-processed gelatins gave significantly greater protection against oxidation than any of the lime-processed gelatins.

Effect of phospholipid. In a recent publication, Bishov *et al.* (1960) reported that phospholipid significantly enhances the protective effect of protein against lipid oxidation in a dehydrated emulsion system. Similar protective effects were noted with thin film studies. Table 6 shows the effect of position of phospholipid layer in relation to refined cottonseed oil layer on the extent of oxidation after heating for 16 hours at 75°C in a draft oven. Positioning of the various films was accomplished as described under Experimental.

The results indicate that the stabilizing effect of a phospholipid film was greatest when the film was on the gelatin surface with the cottonseed oil exposed to air [c/p/g]. Reversal of the lipid positions [p/c/g] resulted in a slight decrease in the protection. A mixture of the phospholipid with the cottonseed oil [(c+p)/g] showed a protection equivalent to [c/p/g] orientation. A mixture of the phospholipid with the gelatin solution [c/(p + g)], however, had negligible effect when compared to cottonseed oil films on gelatin surfaces. The most striking observation

Table 5. A comparison of the effect of various antioxidants in inhibiting the oxidation of thin films of distilled cottonseed oil on gelatin surfaces (1 mg oil/sq cm). Samples were heated 4 hours at 75°C.

Antioxidant treatment	Peroxide value		Mol wt anti-oxidant	Equivalents [a] phenolic hydroxyl (10^6) 100 g oil
Untreated control	1634.2	1564.8	0	0
0.1% BHA	750.2	715.0	236.1	4.23
0.1% BHT	1001.9	1125.4	220.1	4.54
0.1% NDGA	176.3	126.7	292.1	6.85
0.1% α-toc.	151.50	153.9	430.7	2.32
0.1% γ-toc.	173.9	137.5	416.7	2.40

[a] Equivalent phenolic hydroxyl/100 g oil was determined as follows: (g antioxidant/100 g) (No. of phenol hydroxyls in antioxidant/mol wt).

Table 6. Effect of phospholipid position on the autoxidation of cottonseed oil.

	p/c/g [a]	c/p/g	(c+p)/g	c/(p+g)	p/g	c/g
Gliddex "P" (in 92% ethanol)						
PV	78.7	56.7	61.7	373.6	42.0	320.3
millimols diene / kg oil	52.1	43.2	33.4	362	.....	358
Gliddex "P" (in 60:40 methanol & pet. ether)						
PV	33.6	26.4	17.4	165.9	53.7	184.3

[a] p/c/g indicates position of lipid layers: e.g., phospholipid (p) layer on a cottonseed oil (c) layer on gelatin (g). The concentrations of phospholipid and cottonseed oil were made 1 mg/cm² each on all surfaces.

was the over-all improvement in protection of the cottonseed oil by the addition of phospholipid compared with the gelatin surface alone. No polymer formations were observed on the surfaces treated with phospholipid, in contrast to polymers found on glass and on gelatin surfaces in the previously mentioned rate studies (Fig. 2).

Using the [c/p/g] orientation, the effect of phospholipid concentration is shown in Fig. 5 on both glass and gelatin surfaces. A simi-

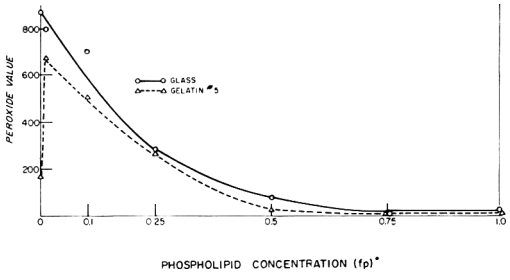

FIG. 5. The effect of phospholipid concentration (f_p) on the autoxidation of refined cottonseed oil on glass and gelatin surfaces. Samples were heated 16 hours at 75°C. Phospholipid concentrations were varied as follows: $f_p =$

$$\frac{(\text{mg phospholipid/sq cm})}{(\text{mg phospholipid/sq cm}) + (\text{mg cottonseed oil/sq cm})}$$

larity in the protection on both glass and gelatin was observed as the weight fraction (f_p) was increased. Maximum protection was achieved at f_p of 0.75, which corresponds to 3 parts of phospholipid to 1 part of cottonseed oil.

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Autoxidation of Fish Oils. I. Identification of Volatile Monocarbonyl Compounds from Autoxidized Salmon Oil [a, b, c]

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SUMMARY

Isolation and identification of a number of volatile monocarbonyls from autoxidized salmon oil were accomplished. The carbonyls, volatile by vacuum steam distillation, were reacted with 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazones were separated by partition and paper chromatograpy. Spectrophotometric analyses and melting-point determinations were made. The data show that, in addition to malonaldehyde previously reported, positive identification was made on 15 monocarbonyls: the C_2, C_3, C_5, C_6, C_7, C_8, and C_9 alkanals; the C_3, C_4, C_5, C_6, C_8, C_9, and C_{10} 2-enals; and hept-2,4-dienal. Tentative identification of butanal, dec-2,4-dienal, and undec-2-enal was based upon a comparison of chromatographic behavior and absorption spectra of the authentic with the unknown. The isolated ketones, tentatively identified as 2-hexanone, 2-octanone, 2-nonanone, and 2-decanone, were characterized by the preceding methods and by their behavior in alcoholic potassium hydroxide. Five additional compounds of unknown identity were isolated; three provided sufficient material for melting-point determinations. Including the forerun, at least 28 carbonyls were found in the volatile portion of rancid salmon oil.

Fats or oils in fresh seafoods are devoid of flavor, or at most have a very bland or mild taste. In a short time, however, varying with the method of handling or processing, odors or flavors develop that are considered characteristic of the particular product. Aside from the action of microorganisms or the products of their metabolism, the action of oxygen on the highly unsaturated lipids and phospholipids is believed to give rise to many of these flavors. This flavor development may be considered the first state of oxidation, and is initiated by an energy source such as radiant energy.

If the reaction continues, undesirable flavors soon result from the decomposition of hydroperoxides and the formation of carbonyls (Lea, 1953). The product may then be considered to be in the stage of off-flavor development. The flavor that these carbonyl compounds impart to the seafood or fat is described by taste panels as "rancid," "fishy," "cod-liver-oil-like," or "painty." The typically described odor of oxidized fish oils, so called "fishy odor," although long known and recognized, has yet to be elucidated (Lea, 1939). Animal nutritionists have learned that, although fishery products are valuable as nutritive supplements and sources of proteins, the levels at which they can be fed are definitely limited. A fishy taste that develops in cooked pork that has been stored has been attributed to excessive feeding of fish oil or fish meal (Anglemier and Oldfield, 1957).

Among the seafoods used for human consumption, frozen fishery products are especially susceptible to oxidative rancidity (Yu and Sinnhuber, 1957). This may be attributed to the unsaturated fats they contain as well as to the rather low level of natural antioxidants. In many of these seafood items that are relatively high in unsaturated fat, such as salmon, one may encounter considerable fat oxidation.

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In frozen fish scrap used for animal feeding or in rations that contain unsaturated fats such as those with horsemeat or cod liver oil, one occasionally finds severe oxidative rancidity. The 2-thioharbituric acid (TBA) test is used as a measure of the degree of oxidative rancidity in fish and other food products (Tarladgis *et al.*, 1960, Yu and Sinnhuber, 1957). The TBA number is a measure of the milligrams of malonaldehyde per 1000 g of sample (Sinnhuber and Yu, 1958a). It is not unusual to encounter TBA numbers of 20–100 in frozen seafoods (Sinnhuber and Yu, unpublished). Assuming an oil content in salmon of 10%, it is apparent that oxidation has produced 200–1000 mg of malonaldehyde per 1000 g of oil. Since this is only one carbonyl, it seems reasonable to expect that oxidation of this extent must yield even larger amounts of other carbonyls. For example, Day and Lillard (1960) found 87 mg of total volatile carbonyls in oxidized milk fat, with a corresponding TBA number of 15. If the relation is similar in seafoods, the level of carbonyls could conceivably affect the nutritive value of the food. The disease condition known as steatitis or "yellow fat disease," encountered in mink, (Mason and Hartsough, 1951) swine, (Davis and Gorham, 1954) and cats (Cordy, 1954), is caused by the feeding of diets high in rancid or oxidized fats, complicated by a lack of vitamin E. Matsuo (1954) and Kaneda and co-workers (1955) found oxidized fish oil toxic to rats. Recent studies on the nutrition of salmon demonstrated that oxidized salmon oil is toxic to young salmon (Sinnhuber *et al.*, in preparation). Considerable research has been directed toward problems involving the nutrition of autoxidized fats (Andrews *et al.*, 1960; Kaunitz *et al.*, 1959; Machlin *et al.*, 1959). The exact material responsible for the toxic properties has not been elucidated. Holman found that methyl oleate and ethyl linoleate peroxides were nontoxic to rats when administered orally (Holman and Greenberg, 1958).

Isolation and characterization of the autoxidation products of fats and oils have been the subject of considerable research in the past decade (Badings, 1959; Brekke and MacKinney, 1950; Chang and Kummerow, 1954; Gaddis and Ellis, 1959b; Gaddis *et al.*, 1959; Johnson *et al.*, 1953; Swift *et al.*, 1948, 1949). The reagent 2,4-dinitrophenylhydrazine (DNP) forms crystalline derivatives with aldehydes and ketones, with characteristic melting points. This property, along with absorption spectra, has been used to identify aldehydes and ketones (Day and Lillard, 1960; Day *et al.*, 1960; Forss *et al.*, 1960; Klein and deJong, 1956). Gaddis and Ellis (1959b) described paper chromatography methods used for the class separation of carbonyls of rancid pork fat. Pippen *et al.* (1958) isolated a number of carbonyls from the volatiles of cooked poultry. Chipault (1959), working on the odor problem of oxidized fish oils, has reported tentative identification, by column and paper chromatography and ultraviolet spectra, of the C_1 through C_6 alkanals; the C_3, C_4, and C_5 methyl ketones; and glyoxal, methyl glyoxal, and diacetyl. Recent work by Day and Lillard (1960) on the identification and characterization of carbonyls from oxidized milk fat suggested a likely approach to the isolation of carbonyls from fish oils.

The research described in this paper was designed to isolate and characterize the volatile monocarbonyls present in autoxidized salmon oil. Evidence is presented for the isolation of 28 compounds, and 21 are characterized by absorption spectra and chromatographic behavior on partition columns and paper in comparison with authentic compounds. Fifteen are positively identified by comparing their melting points with those of authentic compounds.

EXPERIMENTAL

Solvents and reagents. Low-boiling petroleum ether (37–40°C) and hexane were purified as described by Day *et al.* (1960). A.C.S. grades of chloroform, methanol, and commercial-grade absolute ethanol were treated for removal of trace amounts of carbonyl compounds by refluxing with DNP and trichloroacetic acid, followed by distillation. Nitromethane (Eastman grade) was redistilled.

Materials and equipment. The Celite was analytical grade. The chromatographic paper was Whatman No. 3. The absorption spectra of the DNP-hydrazones dissolved in chloroform were ob-

tained with a Beckman DK-1 recording spectrophotometer. The melting points were taken on a Kofler micro-hot stage and recorded as corrected melting points.

Authentic carbonyl compounds. These were obtained as described by Day and Lillard (1960).

METHODS

Peroxide value. Peroxides, determined by the A.O.C.S. method (Am. Oil Chemists' Soc., 1959), are expressed as meq of peroxide per kg of fat. *2-thiobarbituric acid number (TBA no.).* The procedure used was that of Sinnhuber and Yu (1958a).

Procedure.

A. Oxidation of salmon oil

Fresh salmon oil, edible grade, was prepared by the usual commercial process: This is accomplished by pressure cooking. The cannery trimmings were cooked 45 minutes at 240°F with an equal volume of water in a pressure cooker. The oil was removed by flotation, washed with water, and centrifuged at 200°F in a continuous high-speed centrifuge.

Oxidized salmon oil was prepared by passing oxygen into 8 L of the oil in a sealed bottle at room temperature until there was a slight positive pressure of oxygen. The bottle was shaken daily and the slight positive oxygen pressure maintained. At 40 days, the oil was considered highly rancid. The color had changed from a dark red to a pale yellow. The peroxide value, 2.4 at the beginning, was 162 at the end of the oxidation period. The TBA number had changed from 40.2 to 1800.

B. Isolation of volatile carbonyl compounds

Rancid salmon oil (500 g) was vacuum steam-distilled at 40°C; the volatile carbonyls were collected and reacted with DNP-hydrazine; and the monocarbonyl derivatives were isolated as previously described (Day and Lillard, 1960).

C. Separation of dnp-hydrazone mixture

The column partition method by Day *et al.* (1960) was used for initial separation of the mixture into 10 major chromatographic bands and a forerun fraction. Twenty columns were required to separate the quantity of DNP-hydrazones obtained from the 500 g of oil. The common bands from each column were pooled and rechromatographed separately to ensure elimination of contamination from adjacent bands.

Each of the column chromatographic bands contained more than one class of DNP-hydrazones (Table 1). The multi-class mixtures composing each band were separated, and purification was effected by combinations of column and paper chromatography techniques. Bands 6 and 9 (Table 1) were fractionated by column partition chromatography alone, giving hexanal in Band 6 and *n*-propanal and but-2-enal in Band 9. Fractions having absorption maxima the same as the authentic DNP-hydrazones were crystallized and identified. Further details of the procedure have been reported (Day and Lillard, 1960).

The column procedure for fractionating the initial 10 bands was slow, and in a number of cases, particularly with those bands containing alka-2,4-dienals, purification of some compounds did not appear possible. The paper method of Gaddis and Ellis (1959a) for separating DNP-hydrazones into classes was used for fractionation of each band. The procedure was modified by using a solid stopper in place of the mercury pressure-controlling device, and the chromatographs were developed at 2–5°C. In some cases, 1% chloroform reduced streaking and caused faster development of the spots. Obtaining sufficient quantities of each compound for melting-point determinations required 24–48 paper strip chromatograms. To save time, two spots, side by side, were applied to each paper strip. The classes of DNP-hydrazones were separated, and the spots representing a particular DNP-hydrazone were cut from the paper. The derivative was eluted with chloroform, and the chloroform was removed at reduced pressure. The dry DNP-hydrazone thus obtained usually gave oily, ill defined crystals. The material could usually be purified by passing it through a small partition column before crystallization.

Many chromatographic procedures, including the aforementioned, were used in an attempt to resolve fractions b and c of Band 6. The only solvent that gave a good separation was methanol, substituted for petroleum ether in the Gaddis and Ellis procedure (1959a).

Identification of DNP-hydrazones

Data to establish the identity of the DNP-hydrazones were obtained in the following manner. Chain lengths were ascertained by observing the chromatographic behavior of the authentic and the unknown compounds with column (Day and Lillard, 1960) and paper methods (Huelin, 1952; Klein and de Jong, 1956). The class of the hydrazone was established by absorption spectrophotometry of chloroform solutions. The hydrazones of ketones were differentiated by measurement of fading rates in alcoholic KOH as described by Jones *et al.* (1956). Melting points and mixture melting points were taken and recorded as corrected melting points.

RESULTS AND DISCUSSION

Table 1 summarizes the data obtained for the DNP-hydrazones of the volatile mono-

Table 1. Physical data for the 2,4-dinitrophenylhydrazones of volatile monocarbonyl compounds from autoxidized salmon oil.

Column band no.		DNP hydrazone	Isolation Method [a]	Absorption max. (mμ) Found	Authentic	Melting point (° C) Found	Authentic	Mixture
Forerun		Unidentified Compounds	A					
1	a	2-decanone	C	362				
	b	Unknown	C	377				
2	a	2-nonanone	B	364	363			
	b	Undec-2-enal	B	373	373			
3	a	2-octanone	C	359				
	b	n-nonanal	C	358	358	107–107.5	107	106–107
	3	Dec-2-enal	C	373	373	124–126	126–128	
4	a	*n*-octanal	C	358	358	105.5–107.5	107	105.5–107.5
	b	Non-2-enal	C	373	373	125.5–127.5	126–127	125–127
5	a	2-hexanone	C	360	363			
	b	*n*-heptanal	C	358	358	105–107	107–108	104–107
	c	Oct-2-enal	C	373	373	126–128	128–129	126–129
	d	Dec-2,4-dienal	C	390	390			
6	a	*n*-hexanal	A	358	358	108.5–110	108–109	108–109
	b	Unknown	D	373		108–109		
	c	Unknown	D	390		178–179		
7	a	*n*-pentanal	B	358	358	105–107	106.5–107.5	105–107
	b	Hex-2-enal	B	373	373	144	143–144	142–144
8	a	Unknown	B	354				
	b	*n*-butanal	B	358	358			
	c	Pent-2-enal	B	373	373	155–158	158–159	155–158
	d	Hept-2,4-dienal	B	390	390	152–155	153–156	152–156
9	a	*n*-propanal	A	359	358	153–154	154–155	152–154
	b	But-2-enal	A	375	373	182–187	189–190	
10	a	*n*-ethanal	C	356	356	162–164	166	160–163
	b	Prop-2-enal	C	364	365	162–165	165	161–165
	c	Unknown	C	353				

[a] Isolation method: A) Partition column. B) Partition column; paper chromatography; solvent petroleum ether. C) Partition column; paper chromatography; solvents petroleum ether + 1% chloroform. D) Partition column; paper chromatography; solvent methanol.

carbonyls from oxidized salmon oil. The DNP-hydrazone mixture was resolved into 28 fractions.

Hydrazones constituting 15 of these fractions were conclusively identified. Fractions 1a, 2a, 3a, and 5a were tentatively identified as 2-decanone, 2-nonanone, 2-octanone, and 2-hexanone, respectively, on the basis of chromatographic behavior, absorption spectra in chloroform, and the stability of their absorption peaks at 535 mμ in alcoholic KOH (Jones *et al.*, 1956). Quantities of the ketones were insufficient for crystallization, being only trace concentrations in the mixture.

Fraction 1b was not characterized. Only trace amounts were present. The absorption spectrum would suggest a mixture of a dienal with other hydrazones having absorption peaks at a lower wavelength. The C_{11} alkanal, C_{12} enal, and C_{14} dienal could exist in this fraction.

Fractions 2b, 5d, and 8b were tentatively identified by chromatographic behavior and absorption spectra. The limited quantities of these fractions prohibited melting-point determinations. Conclusive identification will be attempted in subsequent studies.

The absorption spectra of fractions 6b and 6c are respectively identical to alk-2-enals and alk-2,4-dienals. The compounds that would correspond to these fractions would

be hept-2-enal and non-2,4-dienal. The chromatographic behavior of the two fractions are identical to the enal and dienal. The melting points of the unknowns, however, do not agree with those of authentic hept-2-enal (M.P. 127–128°C) and non-2,4-dienal (M.P. 138–141°C). It is notable that a compound bearing similar properties to fraction 6c was recently isolated from "fishy" flavored butterfat (Forss *et al.*, 1960).

The absorption maximum of fraction 8a is similar to that of the hydrazone of ethanal (356 mμ) or methanal (348 mμ), but the fraction moved at a rate comparable to a ketone by the Gaddis and Ellis method (1959a). This fraction occurred in trace amounts.

The evidence obtained for fraction 10c is indicative of the impure hydrazone of methanal.

It is of interest that the types of carbonyl compounds encountered in autoxidized salmon oil are similar to those found in other oxidized (Badings, 1959; Chipault, 1959; Day and Lillard, 1960; Forss *et al.*, 1960) lipid systems (Gaddis and Ellis, 1959b; Gaddis *et al.*, 1959; Kawahara and Dutton, 1952; Kawahara *et al.*, 1952; Pippen *et al.*, 1958; Swift *et al.*, 1949). The data presented here are not complete, but a comparison of the compounds isolated from salmon oil and fishy-flavored butterfat are strikingly similar (Forss *et al.*, 1960). When the individual DNP-hydrazone fractions (Table 1) were regenerated and the odor of the free carbonyls observed, no particular fraction resembled the fishy odor. It thus appears that more than one carbonyl compound is involved, and that a proper quantitative relationship is necessary to achieve this typical odor. Indirect evidence supporting this view is as follows. The fishy odor was almost completely removed from the oxidized salmon oil by distillation and was collected in the distillate. The odor of the distillate was almost completely destroyed by reaction with acidified DNP-hydrazine.

Conclusions must await characterization of the unidentified fractions in Table 1, as well as the dicarbonyl mixture, and determination of the quantitative relationships. It is notable that substantial amounts of the bis-DNP-hydrazones of dicarbonyls were obtained, though characterization of this fraction was not included in this study. Sinnhuber and Yu (1958b) reported the isolation of malonaldehyde from the volatile fraction of rancid salmon oil. This compound would be expected to occur in the dicarbonyl mixture.

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Oxidation of Fat in Model Systems Related to Dehydrated Foods.[a] II. Composition and Position of Dispersed Lipid Components and Their Effect on Oxidation Rates

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SUMMARY

Oxidation rates at elevated temperatures of model dehydrated systems containing dispersed lipids are influenced by concentration of the components, type of dispersing medium, and position of the lipid film with respect to the dispersing medium. Generally, the proteins decreased, and polymeric carbohydrates accelerated, oxidation rates. Arginine and lysine salts of safflower fatty acids were extremely stable. Phospholipids had a stabilizing influence when dispersed with the fat and the dispersing medium prior to freeze-drying or when applied as a film between the dry medium and the fat film. When the positions of the fat and the phospholipid films were reversed, the protective action of the phospholipids on the stability of the system decreased markedly.

INTRODUCTION

This paper continues investigations into the stability of model systems related to dehydrated foods. These simplified systems were designed to contain lipid, protein, and carbohydrate components in varying concentrations for purposes of studying the interactions occurring under accelerated storage conditions at elevated temperatures.

In a previous publication (Bishov *et al.*, 1960) we noted that dehydrated fatty emulsions dispersed with an inert polymeric carbohydrate, carboxymethyl cellulose (CMC), could be used to evaluate the role of proteins, phospholipids, and heme compounds in lipid oxidation. Other investigators have also noted that phospholipids alone or with proteins acted as antioxidants (Dutton and Edwards, 1945; Henick *et al.*, 1958; Lea, 1939; Olcott and Mattill, 1936; Patrick and Morgan, 1944), whereas heme components acted as catalytic pro-oxidants (Brown and Tappel, 1958; Robinson, 1924; Tappel, 1953; Watts, 1954). The present paper continues these investigations with special emphasis on determining the influence of component concentration, types of dispersing media, and the position of lipid with respect to the dispersing media, on oxidation rates in dehydrated model systems. The relationships established in these oxidation studies emphasize the quantitative nature of the reactions involved. Togashi *et al.* (1959) recently noted that variations in the chemical nature at the supporting surfaces for thin fat films showed significant differences in protective action against lipid oxidation. They also observed that phospholipids were effective as inhibitors of the surface oxidative reaction. Chargaff (1949) noted that ionic charge alteration probably plays an important role in the protein and lipid interactions, and Dotty and Schulman (1949) demonstrated the existence of lipid-bound protein at charged interfaces. Luck (1949) concluded that the binding of fatty acid anions by the positively charged amino acid residues is due to electrostatic attraction and van der

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Waal forces. It is probable that some of the proposed mechanisms and postulates apply to the experimental evidence presented herein. The experimental design was necessarily exploratory in nature since the reactions of fat and other food components in the newly developed freeze-dried rations have not been studied to any extent.

It is hoped that the results of these investigations may facilitate new approaches to extending the storage life of the recently developed freeze-dried rations for the Armed Forces.

EXPERIMENTAL

A) *Materials and methods*

The methods have been described (Bishov *et al.*, 1960). The techniques for preparation and freeze-drying the emulsions (Rolfes *et al.*, 1955), accelerated aging at elevated temperatures in the rancimeter (James, 1945; Salwin, 1954), and the measurement of oxidation products as peroxides and as volatile carbonyl and other components (Hills and Thiel, 1945; Sidwell *et al.*, 1958) were similar to those in the first paper of this series (Bishov *et al.*, 1960).

The following materials were used: carboxymethyl cellulose gum (CMC); soy oil, refined edible; soy phospholipids; hemoglobin; potato starch, 10 g boiled 10 min in 300 ml H_2O; egg white, sugar-free spray-dried; amylose starch, Stein Hill & Co., Inc., 10 g boiled 10 min in 300 ml H_2O; ovalbumin, water-soluble, de-fatted freeze-dried whole egg; gelatin, type B, pH 6.8, p.i., 4.9, Atlantic Gelatin Co.; aqueous beef extract, clear soluble fraction, freeze-dried; cottonseed oil, refined; lard, refined; L-arginine safflate and L-lysine safflate, General Mills; dialdehyde starch (Sumstar), Sumner Chemical Co.

B) *Preparation of dehydrated emulsions*

The emulsions used to obtain data for the graphic material in the following figures are briefly described. Detailed descriptions appear in the earlier paper.

Five grams of the dispersing medium were suspended in 300 ml water for one minute in a Waring Blendor. The other components were added in the sequence as follows: heme compounds, phospholipids (50 mg/g fat), and fatty materials (10 g), blended for one minute after each addition. The emulsions were shell-frozen in a 5-L boiling flask and freeze-dried. The dried emulsions were ground in a sharp-bladed Waring Blendor, and refrigerated at −20°C until needed.

Samples that involved film studies were prepared as follows: (a) One gram of soy oil was dissolved in twenty ml acetone. This was then adsorbed on 500 mg freeze-dried CMC dispersing medium. The ratio of fat to dispersing medium was 2:1 for all experiments. (b) 50 mg of phospholipids were dissolved in 20 ml petroleum ether and adsorbed on the CMC dispersing medium. (c) Phospholipids were placed on the oil film of (a) from a solution containing 50 mg of phospholipids in a 3:1 methyl alcohol–petroleum ether mixture in which the oil was sparingly soluble. (d) The oil was placed on the phospholipid film by proceeding as in (a). In all cases above, the solvent was evaporated by air-drying in a beaker.

C) *Accelerated storage*

Samples containing 1.0 g of fatty material and the other components, as described, were heated in a stream of air in rancimeter tubes for accelerated aging at 95°C (James, 1945). These samples were heated continuously for the periods indicated, and the volatile components were collected during each 150 minutes. Unless otherwise indicated, the data are plotted in integrated form as shown.

RESULTS AND DISCUSSION

I. *Effects of Variations of Fat and Other Food Substances on Rate of Oxidation in Dehydrated Emulsions*

It was previously shown (Bishov *et al.*, 1960) that carboxymethyl cellulose gum could be used as a dispersing medium in investigating oxidation characteristics of dehydrated emulsions containing fat, phospholipids and heme compounds. The present studies include investigation of several polymeric carbohydrates besides carboxymethyl cellulose, such as potato, starch, and dialdehyde starch; and proteins, such as gelatin, egg white, and ovalbumin as dispersing

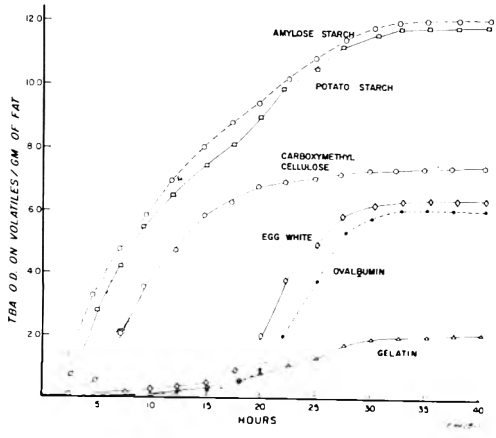

FIG. 1. Effect of some dispersing media on fat oxidation in dehydrated emulsions.

media. These are shown in Fig. 1. The data indicate that oxidation was significantly higher and the induction periods shorter for the carbohydrates than for the proteins. As shown in Fig. 2 and discussed in the first paper, heme substances, such as myoglobin from beef extract when added to CMC soy oil in dehydrated emulsions, also appear to accelerate the oxidation rate of fat-containing emulsions. This probably results in the reduced acceptability of some of the freeze-dried meat items. Increasing quantities of the extract significantly accelerated the oxidative reaction with shorter induction periods, resulting in a more rapid formation of

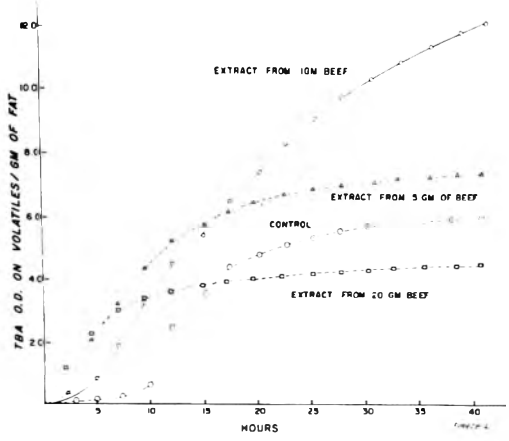

FIG. 2. Effect of myoglobin from beef extract on fat oxidation in dehydrated emulsions.

carbonyl compounds. The lower levels of total thiobarbituric acid (TBA) reactive volatile substances at the end of aerating at 95°C suggest that with increasing beef extract concentration, increasing proportions of peroxides break down into compounds other than carbonyls.

The sequence of formation and breakdown of peroxides in the model dehydrated fat emulsions is shown in Fig. 3. The data show that, initially, in oxidation of fat, peroxides are formed at a very rapid rate, reaching a maximum after five hours at 95°C. The groups assayed by the TBA test did not reach their maximum for more than 20 hr at that temperature. These data suggest that the volatile substances measured with the TBA reagent are peroxide breakdown products. A similar sequence of reactions with

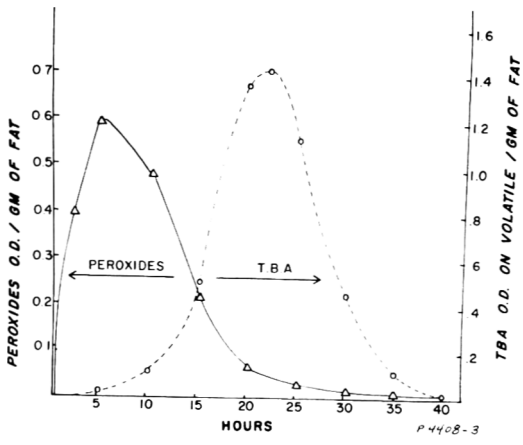

FIG. 3. Oxidation at elevated temperatures of fat in dehydrated emulsions as measured by peroxides and TBA.

increasing temperatures at a constant exposure interval was reported in the earlier paper.

Oxidation rates are significantly influenced by the nature of the fatty substances dispersed in the dehydrated emulsions. Oxidation rates of several fatty substances in dehydrated CMC emulsions are shown in Fig. 4. The data indicate that L-arginine- and L-lysine-safflates were extremely stable. Data not presented here indicate that even in the presence of oxidation-promoting hemoglobin the safflates retained significantly low rates of oxidation, whereas fats such as lard, soy oil, and cottonseed oil were oxidized very

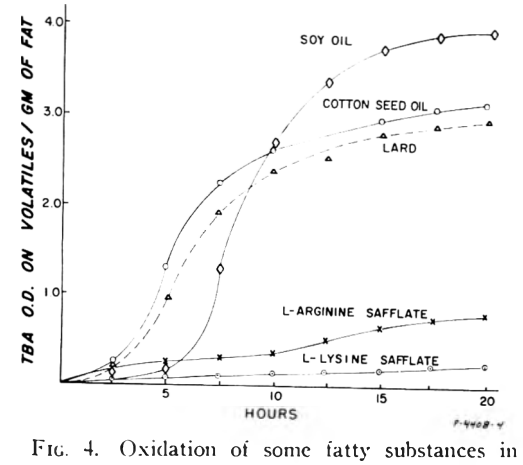

FIG. 4. Oxidation of some fatty substances in dehydrated carboxymethyl cellulose CMC emulsions.

rapidly. These safflates were, however, normal substrates for lipoxidase-catalyzed oxidation in aqueous suspension.

II. *Effects of Concentration of Lipid Films and Dispersions on Oxidation in Dehydrated Emulsions*

The data presented thus far indicate that oxidation interactions in the dehydrated-food-like model-system emulsions are significantly influenced by the composition of all the components that come into contact with each other. The data that follow demonstrate that concentration and position of the lipid components are also significant factors. The phenomena discussed here are probably those of surface interactions. The structures in the model systems are similar to those of freeze-dehydrated food in that the solids remaining after crystallized ice is sublimed, have a flaky structure whose volume is equivalent to that of the wet food. These structures provide tremendous areas for spreading lipid materials as films or dispersions. Oxidation reactions similar to those with thin lipid films probably take place, as suggested by the work of Togashi *et al.* (1959).

A. *Effect of Concentration of Lipids as Dispersions and as Added Films on Oxidation in Dehydrated Emulsions*

The data in Fig. 5 indicate that, for a given quantity of dispersed fat, when emulsified with CMC prior to dehydration, oxidation increases with increasing quantity of the dispersing (CMC) medium. The higher oxidation levels are accompanied by decreasing induction periods. This suggests that the greater the dispersion of the available fat the more rapid is the oxidation. As shown by the TBA values in Fig. 5 the oxidation levels increased fivefold with a twenty-fivefold increase in the quantity of the dispersing medium. The data in Fig. 6 show that when fat was applied as a film on the freeze-dried carboxymethyl cellulose by evaporation from petroleum ether, the oxidation rate did not vary significantly among the emulsions for the first 10 hours of aeration. For aeration periods of more than 10 hours, the extent of oxidation decreased with increasing dispersion provided by the increasing quantities of the CMC.

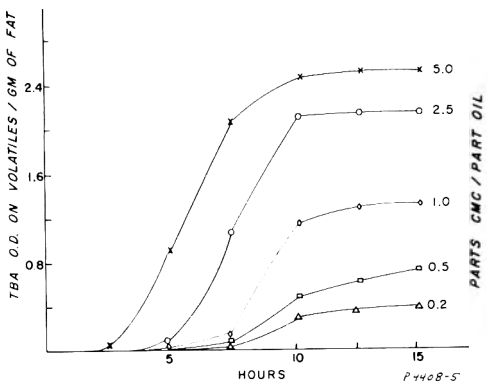

FIG. 5. Effect on oxidation of concentration of fat dispersed with CMC in dehydrated emulsions.

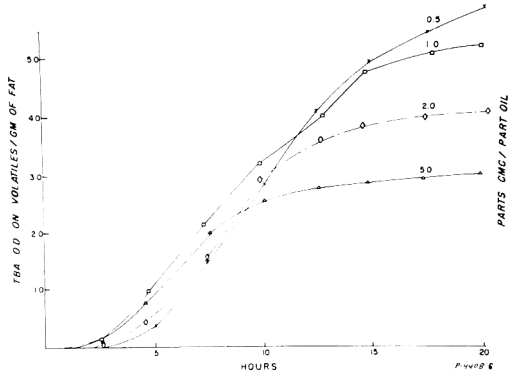

FIG. 6. Effect on oxidation of concentration of fat film added on dehydrated CMC.

Comparison of Figs. 5 and 6 indicates that induction periods for fat film applied on freeze-dried CMC (Fig. 6) are significantly shorter than for those when fat and CMC were dispersed before freeze-drying. The diminution in the total accumulated TBA reactants with increasing dispersion may, in part, be attributed to the physical protection afforded some of the oil by the capillarity of the freeze-dried CMC. The degree of this protection was constant and independent of the amount applied, therefore increasing the percentage of the oil protected as the ratio of CMC to oil was increasing. Further examination of the data in Fig. 6 suggests that the extent of oxidation was greater probably because of the mobility of the increasingly thinner fat film

as quantities of the dispersing medium decreased. This observation is in agreement with those of Togashi *et al.* (1959) in their investigations of thin films.

B. *Effect of Added Phospholipid Film on Oxidation Rates of Dehydrated Emulsion Containing Fat or Insoluble Fat Residue*

In a previous paper it was noted that phospholipids had protective effects against fat oxidation in dehydrated model systems containing fat. Fig. 7 illustrates this protective

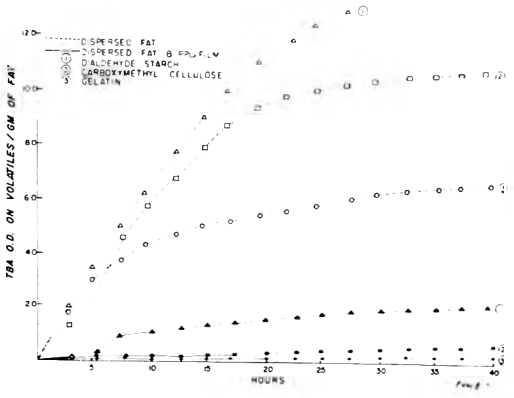

FIG. 7. Effect of added phospholipid film on fat oxidation in dehydrated emulsions containing whole fat.

action on fat with three different types of dispersing media. The protective action of phospholipids is shown by the drop in TBA

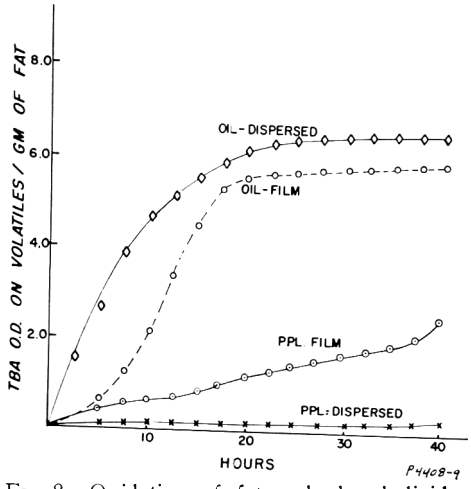

FIG 8. Oxidation of fat and phospholipids as films and as dispersions in dehydrated emulsions.

levels to less than a fifth of that attained without phospholipid protection.

Fig. 8 shows that the oxidation of fat is less rapid when applied as a film than when dispersed with CMC, while oxidation of phospholipids is more rapid when it is applied as a film than when dispersed. The differences may be due to structural and position differences of the fat and phospholipids under investigation.

III. *Effect of Phospholipid and Fat Film Positions on Oxidation Rates in Dehydrated Emulsions*

Significant differences in the protective action of phospholipids on fat oxidation were noted when the position of each was reversed in relation to the dispersing medium CMC. (This is sufficient evidence that the two exist as separate films). These observations confirmed those made by Togashi *et al.* in investigations into the chemical nature of surfaces and their effect on fat oxidation.

Fig. 9 indicates that when the oil film was placed between CMC and the phospholipid film (as in curve A) the phospholipid appeared to lose a great deal of the

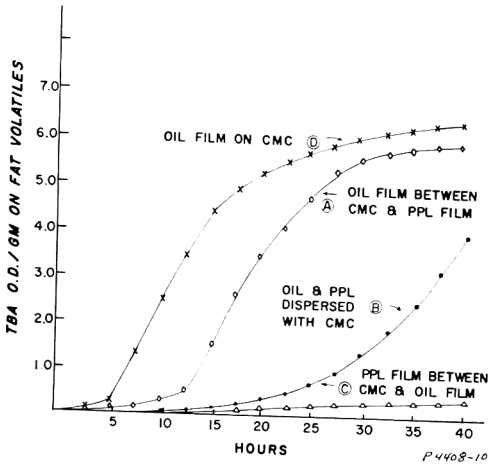

FIG. 9. Effect of phospholipid and fat film positions on oxidation in dehydrated emulsions.

protective action it had when the positions of phospholipid and oil films were the reverse (curve C).

The protective action of phospholipid and oil dispersed with aqueous CMC (curve B) falls between that of curve A and curve C.

Oxidation rates in oil film alone, curve D, without the antioxidant action of either dispersed or film phospholipids, are significantly higher accompanied by shorter induction periods.

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Survival of Bacillus stearothermophilus Spores as a Means of Measuring Effective Electron-Beam Dosage [a, b]

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SUMMARY

Because of the limited penetrating ability of low-voltage electrons, depth and density are necessarily major considerations in the experimental irradiation of food by this means. In this study, survival of bacteria dispersed in agar was used to indicate the effective amounts of irradiation resulting at various depths from various electron doses to the surface. The per cent survival at each depth was plotted against the dose received as measured by cobalt glass dosimeters located at the same depth. These had been calibrated in terms of dose response by means of gamma radiation delivered by a source accurately calibrated by ferrous dosimetry. Within the 3-cm depth studied, a linear relationship was found between dose and log per cent survival.

INTRODUCTION

One of the drawbacks of irradiation of foods with relatively low-voltage electrons has been their limited depth penetration, making thickness of the food product an important factor to consider for uniform dose distribution. A depth-dose curve is often used in determining optimum thickness of material to be irradiated. Depth-dose curves for electron sources have been obtained with small ionization chambers in water (Skaggs, 1949) or with films in a wood product having a density close to 1.0 (Hsieh and Uhlmann, 1956; Pollock, 1953). Glass dosimeters have also been described (Kreidl and Blair, 1956a, b) for estimating dose received from both electron and gamma ray sources. Change in optical density of cobalt glass has been used to estimate dose received by a food product irradiated with electrons (Caldwell and Frainey, 1958). Recently, paper discs impregnated with suspensions of bacterial spores have been described for use as a microbiological monitor system for electron sterilization of plastic materials (Chandler, 1959).

In the studies to be described, *Bacillus stearothermophilus* was used as a test organism for determining the effective doses at various depths from given electron doses at the surface. Cobalt glass dosimeters were inserted between half-centimeter layers of agar containing spores of *B. stearothermophilus* and irradiated with electrons at various surface dose levels. Plate counts of viable *B. stearothermophilus* spores in each layer were made before and after irradiation of the agar. Agar layers were also irradiated with the same requested doses with gamma radiation from a spent fuel-rod source accurately calibrated by ferrous dosimetry. The apparent dose as measured by the change in optical density of the cobalt glass was then compared with the per cent survival of the *B. stearothermophilus* spores.

MATERIALS AND METHODS

Agar medium. Thirty grams of Difco Bacto-Agar were dissolved in 1 L of distilled water by

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[b] Presented at the Twentieth Annual Meeting, Institute of Food Technologists, San Francisco, California, May 15-19, 1960.

boiling and then autoclaving 40 minutes at 245°F. After being cooled to 55°C the melted agar was inoculated with spores of *B. stearothermophilus* and maintained at 55°C in a water bath. Layers of agar ½-cm thick were formed by pouring 35 ml of inoculated medium into a Petri dish 9 cm in diameter and removed onto a circle of 1-mil polyethylene film after the agar had hardened. Six of these layers were "sandwiched" together, with 2 cobalt glass dosimeters per 0.5 cm depth of agar. To minimize error due to the density of the glass, care was taken not to place dosimeters beneath one another.

The sets of agar circles to be irradiated with electrons were enclosed in heat-sealed pouches made of Mylar-Saran-polyethylene film, and were refrigerated until irradiated. For gamma irradiation, two agar circles were packaged in heat-sealed pouches, which were in turn sealed in No. 2 cans so that the circles and dosimeters would be at right angles to the spent fuel rods in the source. The cans were also refrigerated until irradiation, and both sets of agars were irradiated within one week of preparation.

Spore Suspension. *Bacillus stearothermophilus* was grown 96 hours at 131°F on nutrient agar slants, and, after being checked microscopically for sporulation, the growth was washed from the agar with sterile distilled water. This suspension was heated 10 min at 100°C to kill the vegetative cells, and was then used to inoculate the agar. *B. stearothermophilus* was used because it is an easily grown heat-resistant organism that allowed maintenance of the agar at 55°C during preparation of the agar layers.

Bacteria Counts. All estimates of organisms surviving irradiation were made by taking duplicate samples of the agar layers with a sterilized cookie cutter 3.5 cm in diameter. The samples were blended 1 min with 100 ml sterile distilled water in a Waring Blendor, and appropriate dilutions were plated with Difco dextrose tryptone agar. The mean count of seven unirradiated samples was used in calculating per cent survival at the various doses and depths. Count of the unirradiated agar was 18×10^4 spores per 3.5×0.5-cm circle. Error in the sampling and counting procedure was estimated to be about 15%.

Cobalt dosimeter glasses. Cobalt glasses measuring $6 \times 15 \times 1.5$ mm were purchased from Bausch and Lomb Optical Company. Optical densities of all glasses were read at 500 mμ in a Bausch and Lomb Spectronic 20 colorimeter before and within 3 hours of irradiation. A special dosimeter holder was used, as previously described (Caldwell and Frainey, 1958).

EXPERIMENTAL

Electron irradiation, at 9 requested dose levels ranging from 0.02 to 2.0×10^6 rads, was done at the Midwest Irradiation Center, Rockford, Illinois, using a linear accelerator operated at 7.0 Mev. The turntable was 14 in. from the window. Beam spread across the window had up to 10% fall-off, according to calculations at the source.

On the following day a set of samples was irradiated with the same requested doses at the Argonne National Laboratory Gamma facility. The reciprocal of the net OD of the gamma-irradiated cobalt glasses was plotted against the reciprocal of the dose delivered as determined by ferrous dosimetry at the facility (Swope, 1958). The resulting curve (Fig. 1) was used as the reference curve from which

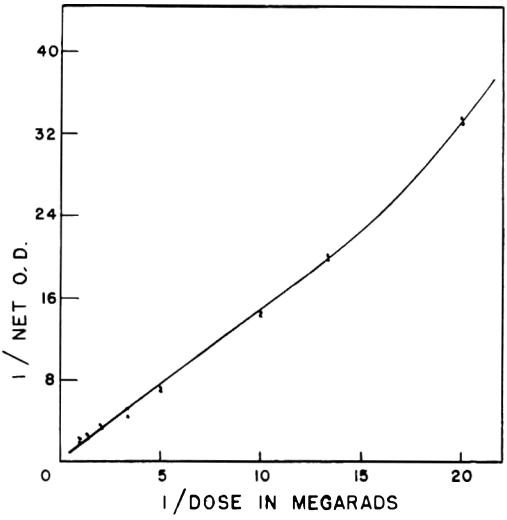

Fig. 1. Relationship between the reciprocals of net optical density (OD) of cobalt glass at 500 mμ and gamma dosage as determined by ferrous dosimetry.

were calculated the doses received at the electron source.

Optical density change in response to irradiation of cobalt glass dosimeters was used for calculating dose received at various depths resulting from electron-beam irradiation of the agar circles. Since the reference curve used for calculation (Fig. 1) was itself based on ferrous dosimetry, a comparison could be made between gamma and electron-beam radiation in terms of per cent survival, assuming the dose response of cobalt glass to be the same for both types of radiation.

Doses resulting from given amounts of electron-beam surface irradiation are plotted at various depths of penetration in Fig. 2. The dose values here were obtained directly from cobalt glasses

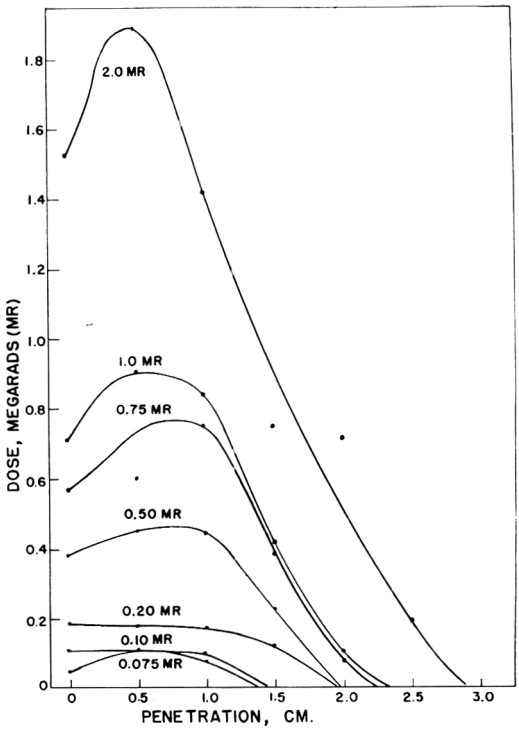

Fig. 2. Penetration of electron beam into agar medium as measured by cobalt glass dosimetry, showing the effect of varying the surface dose. Curve labels indicate the surface dose in megarads as calculated independently by glass dosimetry at the electron source.

located at the depths shown. These curves all show a shape that is typical of measurements of electron penetration into a liquid medium, consisting of an initial increase in dose just below the surface, probably due to secondary ionization in the medium, followed by rapid attentuation at increasing depths (Caldwell and Frainey, 1958; Hsieh and Uhlmann, 1956; Pollock, 1953; Skaggs, 1949).

Fig. 3 shows effective dose penetration as indicated by bacterial survival. These curves reflect the cobalt glass dose penetration measurements, including both the increase and decrease in dose at the corresponding agar depths. The surface dose values used to label the curves in Figs. 2 and 3 were calculated independently by glass dosimetry at the electron source.

Data from Figs. 2 and 3 were replotted in Fig. 4 to show the relationsip between per cent survival and electron dose at any depth. Dose values were obtained from Fig. 2 by interpolation. Also shown in Fig. 4 is bacterial response to gamma radiation in terms of dose as determined by ferrous dosimetry at the source. These data were obtained from the same agar layers used for preparation of the dosimetry reference curve given in Fig. 1. Bacterial survival response to similar doses of irradiation from the two sources appeared to be in reasonable agreement.

DISCUSSION

The relationship between dose and log per cent survival as shown in Fig. 4 appeared to be linear, with some scattering of points in the direction of high survival. The latter may be explained at least in part by the 10% fall-off in dose at the edge of the field. The glass dosimeters were placed toward the center of the circles, but the

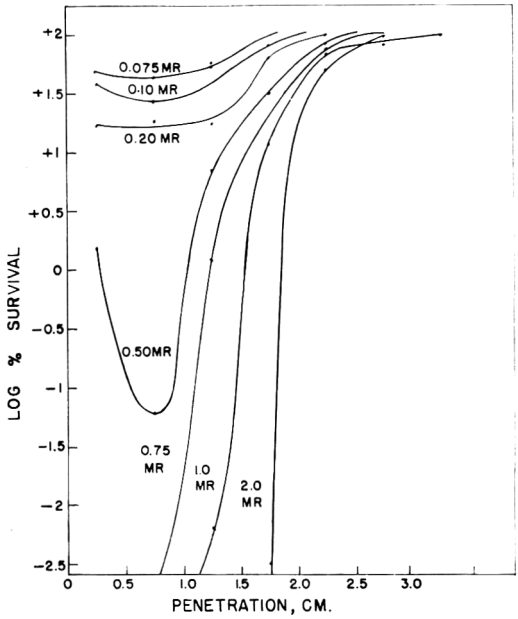

Fig. 3. Penetration of electron beam into agar medium as measured by per cent survival of *B. stearothermophilus* spores, showing the effect of varying the surface dose. Curve labels indicate surface dose in megarads as calculated independently by glass dosimetry at the electron source.

samples for bacteria counts were taken from the entire layer, representing areas receiving 90–100% of the requested dose. A study of effective dose distribution over the width of the agar as well as the depth would be necessary to eliminate this source of error.

The dose vs survival curve shown in Fig. 4 is in good agreement with the curve obtained by Morgan and Reed (1954) for a comparable initial spore population of *B.*

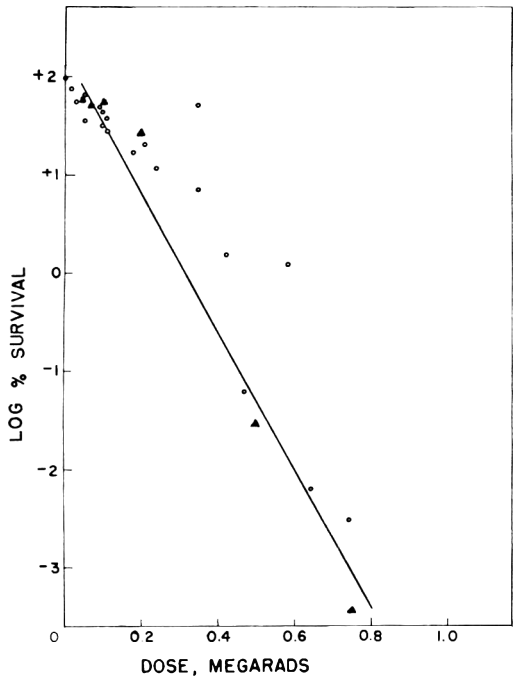

Fig. 4. Relationship between dose received at various depths in an agar medium as measured by cobalt glass dosimetry and the per cent survival of *B. stearothermophilus* at equivalent depths. Circle, electron beam; triangle, gamma rays.

coagulans irradiated with varying gamma ray doses. They pointed out that the conditions of spore production and irradiation greatly affected their radiation resistance. These are factors that must be considered not only in determining a sterilizing radiation dose for a particular organism in a particular food but also in correlating radiation dose and per cent survival of organisms.

If inoculated agar were formed into the same shape and thickness as the food to be processed, survival of bacteria could be applied in experimental irradiation of foods with electrons to determine uniformity of dose distribution within a product normally being monitored by physical dosimeters. A correlation between biological and physical methods of measurement would thus provide valuable information about the over-all sterilizing effectiveness of a dose that would be physically measured in a relatively small area in the product.

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