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CONTENTS

RESEARCH NOTE

A Biological After-Effect in Radiation-Processed Chicken Muscle

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The inhibition of bacterial growth in a food product as a result of exposure to ionizing radiations reported by Brownell *et al.* (1955) may reflect a reduced viable-cell density, an effect of the radiation on the cells themselves, or the production of a growth-inhibiting agent within the meat. Pugsley *et al.* (1935) observed a post-irradiation destruction effect when *Escherichia coli* was inoculated onto the surface of nutrient agar plates previously exposed to X-irradiation. The effect dissipated as the time between exposure and inoculation increased. A recent report by Solberg and Nickerson (1963) indicated a result somewhat comparable with that indicated by Pugsley and his associates except that this experiment involved a complex natural food, chicken, and was one of inhibition rather than destruction of the microorganisms involved. In this case there was a true inhibitory effect since uniform numbers of cells were inoculated onto the surface of the chicken meat after irradiation was completed.

EXPERIMENTS AND RESULTS

Doses of 6 megarads were administered to one set of chicken muscle samples with a 2-Mev Van de Graaff particle accelerator. Treatment at this level was carried out in a period of only a few minutes. Another group of samples were exposed to 6 megarads of gamma irradiation in a Co^{60} source. In this case approximately 36 hr were required to obtain the 6-megarad dose. The data obtained after inoculation of the treated samples with *Staphylococcus aureus* (ATCC 9664) are summarized in curves *c* and *f* of Fig. 1. There are no significant differences between the two curves, thus indicating an absence of a dose-rate effect. Two sets of controls were used, both unirradiated. One set was allowed to remain at the same temperature as existed in the Co^{60} source for 36 hr prior to inoculation; the other was inoculated at the beginning of the experiment. Logarithmic growth was similar on both sets of control samples (curves *a* and *b* of Fig. 1).

The effect of the holding time between irradiation and inoculation was also investigated. A comparison of curves *a* and *c* of Fig. 1 shows that when a 14-hr period elapsed between completion of the radiation exposure and inoculation of the samples, the adjustment phase was slightly longer than on the control samples but was substantially reduced when compared with samples inoculated immediately after exposure. (See curves *c* and *e* in Fig. 1.) The logarithmic phase growth on the irradiated chicken tissue appeared to follow the same slope as that eventually assumed by the organisms inoculated immediately after radiation exposure, as may be seen by comparison of curves *c* and *d* of Fig. 1.

The effect of the holding time between irradiation and inoculation was further investigated to determine the dissipation rate of the inhibitory effect. The results are presented in Fig. 2. It appeared that the inhibitory effect declined as the time between exposure and inoculation was increased.

DISCUSSION

The similarity of the inhibitory effect on bacterial growth in irradiated chicken tissues subjected to widely varying dose rates indicated a continual, dose-dependent build-up of the inhibitory mechanism without any dissipation as long as the radiation dose was being applied. The loss of the inhibitory effect after removal from the energy source appeared to be first order, though there is insufficient evidence to substantiate this.

The foregoing discussion suggests two avenues for further research. First, the possibility exists that foods might be preserved for short periods by applying a low-dose-rate radiation, the energy input of which is just sufficient to maintain the balance of the in-

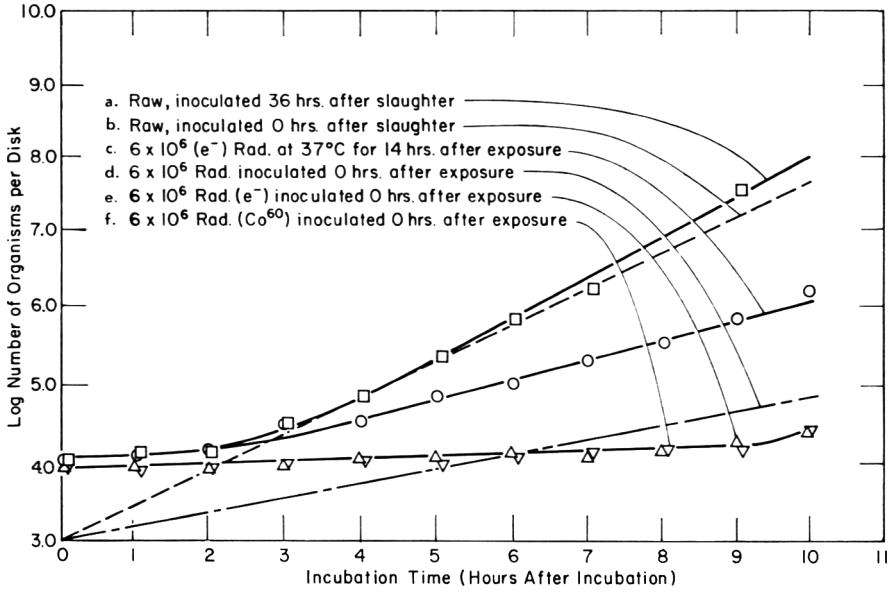

Fig. 1. Growth response of *Staphylococcus aureus* upon chicken meat subjected to various treatments.

hibitor, even though some decomposition of the inhibitor might occur during the exposure period. This dose rate may be of such magnitude that the energy available will be insufficient to initiate the chemical reactions that cause off flavors in foods. Thus, the technique may represent a food-storage device similar to a refrigerator but requiring only a low-level source of ionizing radiation as its operating mechanism.

A second possibility, also in the area of food preservation, is isolation and identification of the inhibitory agent. With this as a tool, many food items might be made into substrates temporarily unfavorable to microbial reproduction, retarding food spoilage.

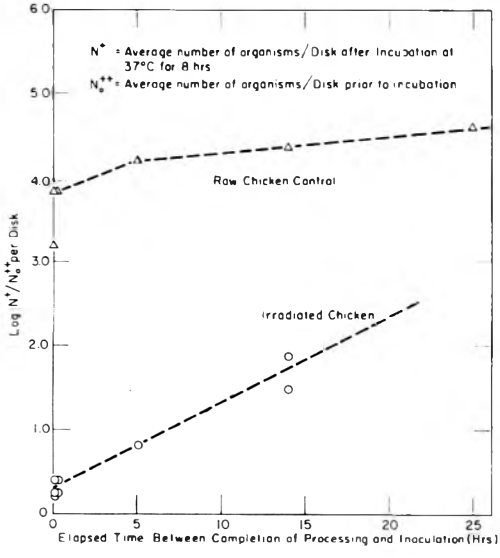

Fig. 2. Number of generations of *Staphylococcus aureus* at eight hours after inoculation of unirradiated and irradiated (6×10^6 rad) chicken muscle disks subjected to varying time delays after treatment and prior to inoculation.

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Microbiological Studies of Bruised Tissues[a,b,c]

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SUMMARY

Microbiological examination of poultry bruises revealed that 61.0–74.2% of the tissues examined harbored both aerobic and anerobic bacteria. These organisms were found, in experimentally inflicted bruises, to increase in number at the early stages of healing (1–2 days), followed by a rapid decrease to the level of the controls within 4–6 days. Age of bruise, environmental conditions (sanitation of batteries, temperature, and moisture), severity of the bruise, and hemoglobin and its degradation products were found to be among factors affecting the microbial content and their growth in bruised tissue.

A total of 86 predominant organisms were isolated from experimentally inflicted bruises; of these, 47 were Gram-positive cocci, 19 Gram-negative rods, 11 yeasts, 7 Gram-positive rods, and 2 Gram-negative cocci. Thirty-six percent of the Gram-positive cocci were found to belong to the genus *Staphylococcus*. Forty-eight percent of the *Staphylococcus* cultures were identified as *S. aureus*, and the others were *S. epidermis*. Fecal material and poultry feed were shown to be the source of the predominant organisms.

The skin of the injured tissue may be a possible portal of entry of these microorganisms. Bruising increased the permeability of the tissue as determined by dye penetration and the extent of microbial invasion.

Bruising in vertebrates is a manifestation of severe cellular injury without laceration, usually produced by a blunt object and resulting in accumulation of blood due to rupture of the vascular supply in the damaged area. Hamdy *et al.* (1957a) established that both the damaged tissue and the accumulated blood and fluid undergo, during healing, various physical and biochemical changes. Some of these changes, particularly those occurring to the extrastromal hemoglobin (namely, degradation of hemoglobin to verdohemoglobin, then to biliverdin and bilirubin), are represented by the color alterations characteristic of a bruise. It can be readily seen that the injured tissue is a complex vascular, lymphatic, and cellular response on the part of the animal as well as a highly active metabolizing area. Many authors (Boyer, 1926; Reith, 1926; Burn, 1934a,b; Adamson, 1949; Lepovetsky *et al.*, 1953; Gordon *et al.*, 1955; Fine *et al.*, 1959; Benacerraf, 1960; Frank *et al.*, 1961; Tregier and Homburger, 1961) support the concept that a few bacteria may be present in tissue, particularly following shock (Jacob *et al.*, 1954; Frank *et al.*, 1961) or irradiation (Gordon *et al.*, 1955; and Benacerraf, 1960). Ayres (1955) reported that microorganisms that gain entrance to tissues may penetrate the intestinal wall and are carried to various parts of the body by the blood. Fine *et al.* (1959) established that bacteria regularly traverse the intestinal barrier.

Bruises in livestock and poultry are condemned by Federal Meat and Poultry Inspectors. It has been estimated that in the United States loss and damage from bruising amounts to many million dollars annually

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in the livestock industry and last year reached 3 million dollars in the Georgia poultry industry alone. Even so, little information is available concerning the microbiology of bruised tissues or the adjacent areas surrounding them. These tissues may be an important source of contamination to the live animal or bird, and accordingly may be considered a health hazard after slaughter.

This paper reports experiments conducted to investigate the microbiology of bruised tissue, the possible sources of the organisms found, and factors affecting microbial activities in experimentally inflicted poultry bruises.

MATERIALS AND METHODS

Bruising and sampling. Eight- to ten-week-old birds weighing approximately 3 lb each and kept under constant environmental conditions were used in this investigation. They were offered standard rations and water *ad libitum*. Unless otherwise stated, the area contused was the breast, the pectorialis major muscle, using the standard technique previously described by Hamdy *et al.* (1961b). Symmetrically located areas on the same and/or different birds were clipped as controls (unbruised). After killing by exsanguination, both control and bruised tissues were immediately excised, sliced, minced, and thoroughly homogenized using sand or a tissue homogenizer (Servall Omnimixer) under aseptic conditions. Viable counts in the homogenates were made by pour-plate and shake-culture methods for aerobes and anaerobes (Hamdy, 1949), respectively, using tryptone glucose extract agar (TGE) containing 5% human blood; and the counts were performed after 48 hr of incubation at 37°C.

Identification of microorganisms. Morphological and biochemical activities were used for identification of the predominant organisms isolated according to *Bergey's Manual* (Breed *et al.*, 1957). Tests performed for further identification of the Gram-positive cocci isolated were: oxygen requirement, coagulase production, growth on Staphylococcus 110 media (7.5% NaCl), mannitol and glucose fermentation (aerobically and anaerobically), and hemolysis.

Measurement of skin or tissue permeability. *Skin.* Birds were bruised, the feathers covering the injured area were clipped, and 1 ml of an active *Escherichia coli* culture was placed on a sterile gauze and taped firmly to the bruised or control area. The gauze was reinoculated with active culture 4–5 times daily. At various intervals during healing, birds were sacrificed, and both control and bruised tissues were excised and examined for test organisms that may have gained entrance by penetrating the skin.

Tissue. The center of bruised and control tissues was injected intramuscularly with 0.25 ml of a 2% aqueous solution of gentian violet at a depth of 1 cm. The dimensions of the area colored by the dye were measured to reflect the extent of tissue permeability.

Preparation of standard blood pigment solutions. *Hemoglobin.* Fresh, normal, whole citrated chicken blood (5 mg sodium citrate/ml, pH 7.4) was centrifuged for 15 min at 2,000 × G to remove the plasma and buffy coat. The red-cell layer was washed with saline (pH 7.4) until the supernatant was negative for protein. The red cells were lysed and the extrastromal hemoglobin collected by centrifugation at 30,000 × G as described by Shinowara (1954). The concentration of hemoglobin from which standard solutions were prepared was determined on a dry-weight basis.

Alkaline and acid hematin. Alkaline hematin was prepared by dissolving hematin (Eastman) in phosphate buffer (pH 7.8; 0.067*M*), and standard acid hematin solution was made by dissolving the pigment in 0.1*N* NaOH and then the solution was brought to pH 5.5 using 0.067*M* acetate buffer.

Bilirubin and biliverdin; stable aqueous form. The pigments were first conjugated with serum albumin at alkaline pH, and immediately brought to neutrality with buffer as follows: Pure bilirubin or biliverdin (Eastman) was added to 4.0 ml of pH 11.3 solution prepared by diluting 1.0 ml 5*N* sodium hydroxide to 100 ml with Sørensen buffer (pH 7.40; 0.067*M*). Between 2 and 4 minutes after addition of the pH 11.3 solution, 2.0 ml of 25% normal human serum albumin (Cutter) was added and stirred. This solution was transferred to a 100-ml volumetric flask and brought to volume with Sørensen buffer (pH 7.4). The final solution had a pH of 7.40 and an albumin concentration of 0.5%.

Stable solvent form. Bilirubin was dissolved in chloroform, whereas biliverdin was prepared in absolute ethyl alcohol.

Effect of hemoglobin and its degradation products on growth of microorganism. *Media and assay procedure.* The disk sensitivity or the cup method was used for examining the effect of extrastromal hemoglobin and its degradation products (hematin, biliverdin, and bilirubin) on the growth of the predominant organisms isolated from bruised areas. TGE agar (Difco) was seeded with a 1% inoculum of a 24-hr culture of the test organism. The solution (0.05–0.10 ml), containing the desired concentration of the test substances, was added to either sterile disks or cups placed on the surface

of the agar, incubated at 37°C, and examined at 24–48 hr for growth inhibition or stimulation.

RESULTS

Microflora of bruised tissues. Bacteriological examination of bruised tissues secured randomly from broilers on a commercial processing line and from experimentally inflicted bruises (Table 1) revealed that many of these tissues harbored a microbial count relatively high both aerobically and anaerobically compared to control samples. The latter either contained relatively few organisms or were completely sterile.

Identification of the predominant organisms isolated. Twenty-six predominant organisms (group A) were isolated from bruises secured from the processing plant whereas 86 predominant bacteria (group B) were isolated from approximately 2,000 experimentally inflicted poultry bruises. Group B organisms were isolated from bruised tissues at various intervals during healing. Morphological characteristics and general classification showed that, in group A, 16 were Gram-positive cocci, 7 Gram-positive rods and 3 Gram-negative rods, whereas in group B, 47 were Gram-positive cocci, 19 Gram-positive rods, 11 yeasts, 7 Gram-negative rods, and 2 Gram-negative cocci. Thus, respectively 61 and 54% of the organisms in groups A and B were Gram-positive cocci. Thirty-six percent of the Gram-positive cocci in group B belonged to the genus *Staphylococcus*, of which 48% were identified as *S. aureus* and 52% as *S. epidermis*.

Possible sources and portal of entry. Microbial analysis showed that poultry feed and fecal material harbored a very large and heterogeneous mixture of organisms, the complete analysis of which is beyond the scope of this investigation. However, Staphylococcal cultures identical to those isolated from the bruised tissues were among the predominant organisms in all samples examined.

The greater number of organisms in bruised tissues than in unbruised tissue led to investigation of the possible portal of entry of these organisms. The assumption was made that the damaged skin might be a possible site. Hamdy *et al.* (1961c) established the presence of proteolytic enzymes in experimentally inflicted poultry bruises. It is assumed that these enzymatic activities may alter the permeability of skin and tissue at the site of the bruised area, facilitating penetration by the microorganisms. To investigate this theory, 68 birds were breast bruised, and a sterile gauze containing an *E. coli* culture was taped firmly to the bruised areas and the experiment performed as outlined in Materials and Methods. A group of 34 unbruised birds were treated similarly as controls. At various intervals during healing, bruised and control birds were sacrificed, the skin close to the inoculated gauze was removed, and the tissue was excised and assayed for the test organism. *E. coli* was present in both bruised and unbruised tissues at all stages of healing, indicating that organisms can penetrate the skin of both, the latter probably being under physical stress due to the inoculated gauze. It was also noticed that the number of *E. coli* organisms per gram of tissue was always much higher in bruised areas at the early stages of healing (6–24 hr) than at other stages of healing or than in unbruised tissue. This may be due to change of tissue permeability at that stage and/or the presence of hemoglobin and its degradation products, tissue fluid, and degenerated tissue cells in the injured area that support the growth of the test organism.

Microbiology of bruised tissue. Hamdy *et al.* (1957c; 1958; 1961a,b) observed that the time required for gross changes to occur in livestock or poultry was subject to variation, and that the extent of the damage of replicate bruises also varied with individual animals or birds but that the sequence of the visible and measurable chemical changes associated with healing was always consistent regardless of animal species, force applied, or location of the bruise. Again, Hamdy *et al.* (1961b) reported that the environmental temper-

Table 1. Incidence of bacteria in poultry tissues secured from processing plant and experimentally bruised birds.[a]

Group examined	No. of birds	Incidence of positive cultures	Range no. of organisms/g tissue in positive samples	
			Aerobes	Anaerobes
Processing plant				
Bruised	44	61%	10— >300,000	10— >300,000
Control	16	50%	10— >150	10— >200
Experimental bruises				
Bruised	58	74.2%	10— >24,850	10— >10,200
Control	45	41.1%	10— >120	10— >30

[a] These damaged tissues were contused using three blows and examined 48 hr post-bruise.

ature played an important role on the susceptibility of birds to bruising, chemistry of hemoglobin degradation, and rate of healing of the damaged areas. The following factors that may affect the microbial content of the bruised tissues were examined.

Effect of sanitary conditions and age of bruise. Two groups of 60 birds each were breast bruised. Group A was kept in dirty batteries, where the litter was removed only once every 10 days, and group B was kept in clean batteries that were changed every three days. Forty unbruised birds were kept with each group as controls. At various intervals during healing, birds from each group were sacrificed, and bruised and control tissue samples were immediately excised and examined for microbial content both aerobically and anaerobically. The results (Figs. 1, 2) indicate that the bruised tissue of both groups harbored very few aerobic and anaerobic microorganisms immediately following bruising and that these organisms increased in number at the early stages of healing (1–2 days), followed by a rapid decrease to reach the level of the controls within 4–6 days. Aerobic organisms were always greater in number than anaerobes in the damaged areas at all stages of healing, although the former decreased faster than the latter as healing progressed. Evidence of complete healing as detected morphologically was noted from 4 to 6 days after bruise contusion. Group A always contained more organisms per gram of tissue at all stages of healing. The reason is probably the constant contact of the bruised breast with fecal materials on the dropping pans of the dirty batteries. It should be noted that the microbial content (aerobes and anaerobes) in unbruised birds in both groups was relatively the same in all samples examined.

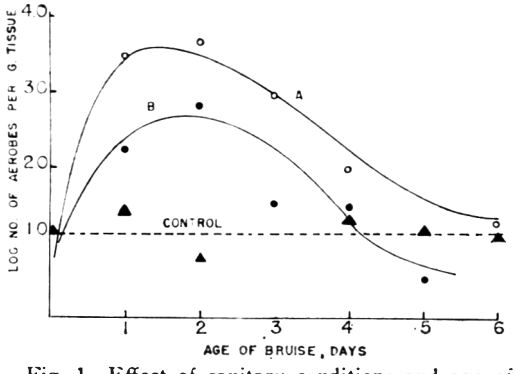

Fig. 1. Effect of sanitary conditions and age of bruise on aerobes. A) birds kept in dirty batteries; B) birds kept in clean batteries.

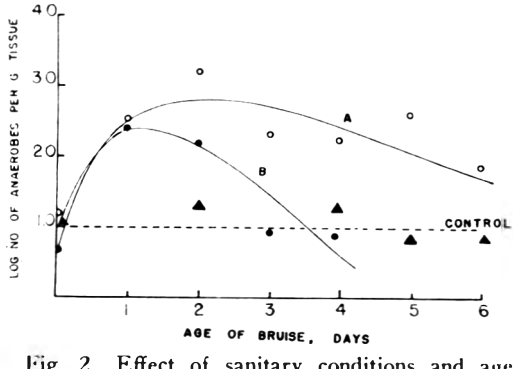

Fig. 2. Effect of sanitary conditions and age of bruise on anaerobes. A) birds kept in dirty batteries; B) birds kept in clean batteries.

Effect of environmental temperature. Hamdy *et al.* (1961b,d) showed that environmental temperature played an important role in the physiology and biochemistry of bruised tissue. To investigate the effect of the environmental temperature on microbial activities in the damaged areas, three groups (44 broilers each) were bruised. The first group was raised and kept at 30°C, the second at 19°C, and the third under ambient conditions. Twenty-four unbruised birds in each group served as controls. Bruised tissues of all groups and the corresponding controls were examined during healing for aerobic and anaerobic microbial content. The results (Figs. 3 and 4) indicated that control tissues of birds in all environmental conditions had similar low microbial counts, whereas bruised birds kept at 19°C had higher aerobic and anaerobic counts at all stages of healing than bruised tissues of birds kept at 30°C or under ambient conditions. The microbial content of bruised tissue of birds under ambient conditions fluctuated during healing, and all injured tissues reached the level of the controls upon complete healing. It is possible that holding houses at higher temperature may lead to fast drying of the fecal material and de-

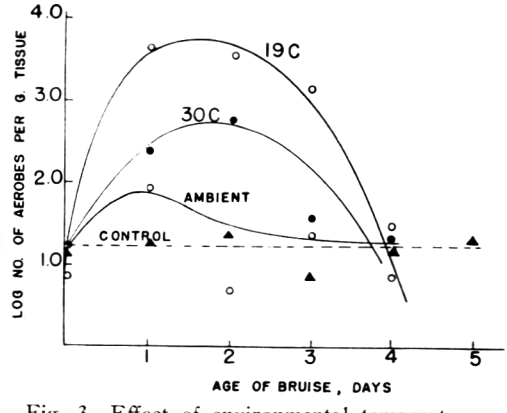

Fig. 3. Effect of environmental temperature on aerobes.

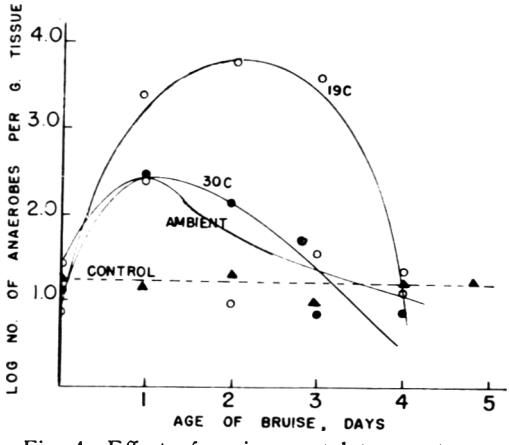

Fig. 4. Effect of environmental temperature on anaerobes.

crease viability of the organisms present accordingly.

Effect of moisture. In the previous experiment the number of microorganisms was lower at all stages of healing in bruised tissue for birds kept at higher temperature (30°C) than for birds kept at 19°C. To investigate this observation further it was decided to examine the effect of moisture on the ability of organisms to penetrate the skin of the injured area. In this experiment two groups of broilers were breast bruised and kept at 30°C. To the first group (12 birds) 1.0 ml of an active *E. coli* culture grown in nutrient broth was placed on a sterile gauze and then taped firmly to the bruised area. The second group (48 birds) was treated similarly except for repeated inoculation of the gauze, five times during a 24-hr period, with 1.0 ml of an active *E. coli* culture to which liquid gelatin (1% in the inoculated cultures) was added just prior to inoculation. Unbruised tissues in each of the groups were treated similarly as controls. Twenty-four hours after the first inoculation (48 hr post-bruise) both control and bruised tissues were excised and examined for *E. coli*. The results showed the absence of the test organisms in the tissue of the control and bruised birds in the first group, whereas *E. coli* was present in large numbers in the second group on both bruised and control tissues. Thus, moisture content and the number of viable organisms on the inoculated gauze were important factors in facilitating the invasion of both bruised and control tissue by the test organism.

Effect of bruising on the permeability of injured tissue. A group of 132 birds, kept at 19°C, were bruised, and at specific intervals during healing representative numbers (22 birds) were sacrificed. Just before killing, 0.25 ml of an aqueous solution of gentian violet was injected intramuscularly to 1 cm in the center of the bruised area. Fourteen control tissues representing unbruised birds were injected similarly. The skin over the bruised and control tissues was then removed and the areas of the dye measured. Bruised tissue stained rapidly owing to a greater permeability that allowed the gentian violet to pass into the extracapillary space. Statistical analysis revealed the regression of dye area on time post-bruise, and that this correlation coefficient (regression = 0.346) was significant at the 0.01 level of probability. The values of dye areas at various intervals during healing were mathematically treated, and the following regression equation (line of best fit) prepared:

$$\hat{Y} = 4.752 + 0.0289\ (X_1 - 67.636) - 0.000508\ (X^2_1 - 6955.63)$$

where $\hat{Y}$ = line of best fit, and X_1 is time post-bruise in hr. It can be noted (Fig. 5) that both bruising and age of the bruise affected permeability. An immediate increase in the dye area was detected ½ hr after bruise infliction, followed by an abrupt increase that reached a peak within 24–48 hr and was succeeded by a steady decrease thereafter. Complete healing as detected morphologically did not coincide with the decrease in permeability of the tissue to the dye, for morphologically healed tissue always exhibited a markedly greater dye area than did unbruised tissue. The former reached the level of the control 24 hr after complete healing.

Effect of severity of bruise on tissue permeability. Two experiments used dye penetration and extent of microbial invasion as criteria.

Dye penetration. Three groups of birds were breast bruised with different forces: 8 birds received one blow, 22 birds received three blows, and 8 birds received 5 blows. Eight unbruised birds were controls. Forty-eight hours after bruising, the birds were examined for tissue perme-

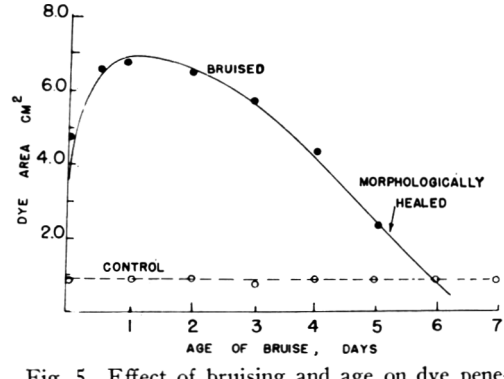

Fig. 5. Effect of bruising and age on dye penetration. Results are regression of dye area on time post-bruise.

ability by intramuscular injection of 0.25 ml gentian violet solution as described previously.

Microbial invasion. An experiment on microbial invasion was conducted as outlined immediately above except that the bruised or control tissues were assayed for microbial content 48 hr after bruising.

The results (Fig. 6) revealed that dye penetration and microbial content were directly related to the force applied. Severe bruises (contused, using 5 blows) harbored a large number of both aerobic and anaerobic organisms and had an average dye area of 11.53 cm^2.

Effect of blood pigments on growth. Hamdy *et al.* (1961c) reported that the concentration of various blood pigments in two-day-old (experimentally inflicted) poultry bruises were as follows: $0.35\pm0.11\mu M$ of extrastromal hemoglobin, $2.6\pm0.65\mu M$ of biliverdin, and $1.3\pm0.3\mu M$ of bilirubin per 100 g of bruised tissue, whereas unbruised tissue contained $0.04\pm0.01\mu M$ hemoglobin and no bilirubin or biliverdin in 100 g of tissue. The effect of hemoglobin and its degradation products (hematin, biliverdin, and bilirubin) on the growth of the predominant organisms isolated from the bruised tissues secured from processing plant revealed that hemoglobin (0.014–0.056 μM/disk) stimulated the growth of all the cultures tested whereas hematin (Table 2) at $0.474\mu M$/disk inhibited 54% of the Gram-positive cocci and 50% of the Gram-positive rods, and exerted no measurable effect on the Gram-negative rods. Biliverdin or bilirubin, in concentrations as high as $9.2\mu M$/disk, had no discernible effect on all the cultures tested.

Again, the effect of the aforementioned pigments on the growth of *S. aureus* culture numbers 63, 75, 172, and 175 isolated from experimentally inflicted bruised tissues was examined. The results (Table 3) revealed that hemoglobin stimulated the growth of all the cultures tested and that this stimulation appeared to be linearly dependent on concentration at a level of 0.014–0.056μM/disk. In each case, the diameter of the zone of growth stimulation was found to be correlated with hemoglobin concentration at a highly significant level of correlation. The extent of this stimulation on the growth of these cultures was different, although the slopes of all the graphs were almost the same. Acid hematin inhibited all cultures tested, and proportionally to concentration in the range of 0.19–0.76μM/disk. The magnitude and slope of the curves of this inhibition were about the same for cultures No. 63, 75, and 175, but not for No. 172. The last culture deviated slightly. Alkaline hematin inhibited three of the four organisms tested, and this inhibition was concentration-dependent on cultures No. 75 and 175 in the range of 0.19–0.76μM/disk but not on culture No. 63. The last organism was only slightly affected by these concentrations.

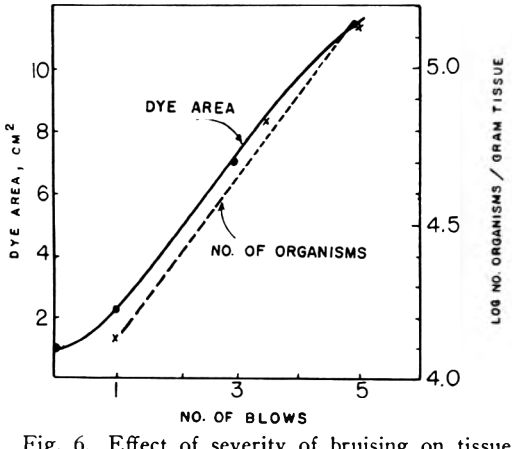

Fig. 6. Effect of severity of bruising on tissue permeability.

Table 2. Effect of hematin on the growth of predominant cultures isolated from bruised tissue secured from processing plants.

Conc. (μM/disk)	No. of organisms examined: 15 Gram-positive cocci	6 Gram-negative rods	6 Gram-positive rods
0.126	15(0)[a]	0	6(0)
0.316	5(−); 1(+)	0	3(−)
0.474	8(−); 1(+)	0	3(−)
0.790	9(−); 1(+)	0	3(−)

[a] 0 = no measurable effect; + = stimulation; − = inhibition.

Bilirubin and biliverdin, in concentrations as high as $86\mu M$/disk or cup, had no discernible effect on any of the *S. aureus* cultures examined. The reason may be selective adsorption by the filter paper or decreased diffusion of these pigments in the agar media. To overcome this difficulty, the effect of biliverdin on the growth of staphylococcus culture No. 15 was tested in liquid media (half strength of nutrient broth), and the growth was followed spectrophotometrically (optical density at 525 mμ). The results (Fig. 7) showed that biliverdin in concentrations as low as 8.6 μM inhibited the growth of the test organism.

DISCUSSION

Many authors support the concept that a few bacteria may be present in the living tissue. Frank *et al.* (1961) found that Gram-positive bacteria can be recovered from the mesenteric nodes and liver of normal rabbits and dogs for two hours after exposure to

Table 3. Effect of hemoglobin and hematin on growth of *S. aureus* cultures No. 63, 75, 172, and 175.

Pigment used	Conc. (μM/disk)	Stimulation or inhibition on *S. aureus* [a]			
		63	75	172	175
Hemoglobin	0.014	+12.8	+ 6.2	+3.3	+ 6.5
	0.028	+14.0	+ 8.1	+6.3	+ 9.2
	0.056	+18.0	+11.8	+8.7	+12.9
Alkaline hematin	0.19	− 0.6	− 2.5	0	− 8.8
	0.38	− 1.4	− 8.7	0	−11.8
	0.76	− 1.5	−12.0	0	−16.2
Acid hematin	0.19	− 2.5	− 0	−2.7	− 1.8
	0.38	− 3.1	− 2.3	−3.6	− 2.8
	0.76	− 5.1	− 4.0	−4.3	− 5.2

[a] 0 = no measurable effect; + = stimulation; − = inhibition, diameter (mm) of the zone of stimulation or inhibition minus that of the disk.

hemorrhagic shock. Those authors stated that the exposure to hemorrhagic shock so weakens the antibacterial defenses that organisms can be grown out more readily than in normal animals. The demonstration of microorganisms in bruised tissue of birds immediately after killing may have far-reaching biological significance. It definitely demonstrates that bruised tissues are not impenetrable to bacterial invasion. Almost 61–75% of the bruises examined harbored relatively large numbers of both aerobic and anerobic microorganisms. Thirty-six percent of the isolates belong to the genus *Staphylococcus,* of which forty-eight percent were identified as *S. aureus,* a food-poisoning organism. Both fecal material and poultry feed were established to be the source. Many factors (other than age of bruise) seem to affect the microbial content of the bruised tissue: sanitary conditions of the battery, environmental conditions (temperature and moisture), and severity of bruise.

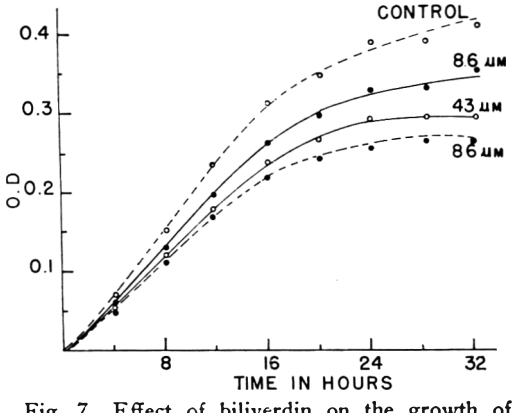

Fig. 7. Effect of biliverdin on the growth of *Staphylococcus* culture No. 15.

The increase in number of bacteria at the early stages of healing (1–2 days post-bruise) suggest that these organisms may have gained entrance to the tissue, slightly increased in number, and then failed to thrive. This failure may have two possible reasons: a) the tissue might be lacking in a nutrient essential for microbial growth, or b) the tissue might contain agents or conditions that inhibit reproduction or even survival of the parasite. Hirsch (1960) suggested that the chemical micro-environment and the macrophages play a decisive role in limiting the growth of microorganisms in inflamed or necrotic tissues. He also stated that the concentration of oxygen in the tissues undoubtedly had an effect on the fate of obligate anaerobes and that microbes that require oxygen may be limited in their growth because of a marginal oxygen tension. Skarnes and Watson (1957) and Dubos (1954) showed that antibacterial activity, of a high order under certain conditions *in vitro,* is recognized for certain porphorins related to heme. Hirsch (1960) reported that many other antibacterial substances such as lysozyme, protamines, crude extracts of platelets and lymph nodes, lactic acid, phagocytin (an acid-soluble cytoplasmic protein), and beta-lysine have been found in healthy tissues and cells of mammalian hosts. He also stated that tissue breakdown, on a micro- or macroscale, may lead to the re-

lease of other antibacterial compounds such as the basic peptides, porphorins, amines, fatty acids, and histones.

Menkin (1931, 1956) established that the increased permeability can be readily demonstrated by intravenous injection of trypan blue in rabbits. He found that the area of injury is rapidly stained, owing to outward passage of the dye, and that with inflammation caused by certain irritants, the permeability of the capillaries is enhanced about twofold. Hamdy *et al.* (1961c) reported proteolytic enzymes in bruised tissue. These enzymatic activities seem to increase the permeability of the skin and tissue at the site of the bruise, and facilitate the penetration of the bacteria into the injured tissue. This increase in permeability was established by dye penetration and extent of microbial invasion using *E. coli* as test organism, supporting the views that microorganisms are able to invade the tissue via the skin.

The effect of hemoglobin and its degradation products (hematin, biliverdin, and bilirubin) on the activities of organisms have been reported by many investigators (Brown and Hamdy, 1961; Lochead and Burton, 1953; Yegian *et al.*, 1959; Smith, 1949; Burris and Wilson, 1952; Jimenez, 1940; Gibbson and MacDonald, 1960; Porter, 1948). Synergists and antagonists for some of the blood pigments in the bruised tissue, such as hemoglobin, hematin, and biliverdin (Hamdy *et al.*, 1957a, 1961c), may be operating in the bruised tissue under investigation. It was found that hemoglobin stimulated growth of the four *S. aureus* cultures isolated from bruised tissue, but hematin (acid or alkaline) inhibited the growth of most of these organisms, and biliverdin inhibited the growth of *Straphylococcus* No. 15.

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Growth of Psychrophiles. II. Growth of Poultry Meat Spoilage Bacteria and Some Effects of Chlortetracycline[a]

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SUMMARY

The alteration of growth characteristics by environmental factors, such as growth-temperature reductions and addition of chlortetracycline to growth medium, was studied. Three bacteria that possessed different psychrophilic abilities were used as test organisms. The stresses imposed were measured by changes in growth rate, cell pigmentation, and lytic response to lysozyme. All strains grew well at 25°C, but differences began to become more pronounced at 12°C and differences were great at 4°C. Temperature fluctuations influenced subsequent growth. The effect of environmental changes on pigmentation varied with the test strain. The lytic response to lysozyme varied but was not greatly influenced by the environmental factors tested. A strain of *Brevibacterium linens* was found extremely resistant to lysozyme in a number of test systems.

INTRODUCTION

The effect of a chemical food preservative or a food preservative process, such as freezing, may be such as to cause an alteration, for one or more generations, in the usual growth pattern of the surviving bacteria. In some cases, this alteration is shown by changes in generation time. Hartsell (1951) reported changes in growth initiation of bacteria frozen in egg melange at −25°C following defrosting at 37°C. With several species the response was so changed that the frozen-defrosted cultures grew faster than the controls. The increased rate of binary fission was observed early in the storage period, and was of continued occurrence in cultures stored many months. In addition, a need was again shown for a highly nutritive medium for recovery plating of environmentally damaged cells (Curran and Evans, 1937; Nelson, 1943; Hartsell, 1944).

The studies reported here were initiated upon the premise that changes in bacterial growth rates, as well as other cell characteristics, may result from food preservative processes less drastic to the bacterium than freezing. It was thought, also, that processes less debilitating than freezing would tend to lessen the chance of observing a selective action whereby a minority of cells endowed with great resistance would be the only survivors.

The effects of the environmental stresses were measured in bacteria isolated from poultry meat by changes in growth rate, cell pigmentation, and lytic response to lysozyme.

METHODS AND MATERIALS

Test species. The bacteria used were isolated from spoiled poultry meat that had been held at 4°C. Selection and identification of the isolates able to grow well at 4°C were continued until a strain of *Pseudomonas fluorescens*, *Achromobacter guttatus*, and *Brevibacterium linens* had been obtained. One unidentified *Pseudomonas* species was also partially studied.

Growth rates. Growth rate was measured turbidimetrically at 660 mμ with a Beckman model DU spectrophotometer and was calculated by the

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method of Gunsalus (1951). The cells were grown in trypticase-yeast extract broth (2% trypticase, BBL; 0.5% yeast extract, Difco; water at pH 7.0), and the changes in their density plotted on semi-log paper against time. The growth rate was then calculated using a time (t) and a change in density (d) taken from the straight-line portion (log phase) of these curves. All figures for rate represent the means of three readings. The effect of chlortetracycline (CTC) was determined at a concentration of 1 μg/ml because this antibiotic is permitted as a shelf life extender on poultry meats. When testing was done at 4°C the inoculum was from actively growing cultures that had been transferred several times at that temperature before use. The same applied to tests carried out at 25°C. The only exceptions were those tests of the effects of temperature fluctuations.

Determination of lysis by lysozyme. Lysis is used here to indicate the decrease in optical density of the cell suspensions after incubation in the test systems.

Tubes containing 10.6 μg/ml lysozyme (crystalline from egg white, Armour Research Division, Chicago) in phosphate buffer, pH 7.0, and sufficient cells to give an optical density of 0.60, were incubated at 45°C. Readings of optical density were made at 30- and 60-minute intervals.

Nakamura's technique for lysis. Cell suspensions containing 10.6 μg/ml lysozyme in water at pH 3.5 were incubated at 45°C (Nakamura, 1923; Grula and Hartsell, 1954). Optical density was read after this incubation. Sufficient 0.1N NaOH was then added to bring the system to pH 10.0–10.5 and optical density was read again.

Lysozyme and Versene. Tubes containing 10.6 μg/ml lysozyme, 0.5 mg/ml EDTA (disodium salt of ethylenediaminetetraacetic acid), and sufficient cells to give an optical density of 0.60 were incubated at 45°C. Degree of lysis (change in optical density) was measured at 30- and 60-minute intervals.

Cell pigmentation. Changes in cell pigmentation were made by visual observation of freeze-dehydrated cells harvested during the exponential growth phase.

RESULTS

Growth rates. *P. fluorescens* grew faster at 25, 12, and 4°C than any of the other test strains (Table 1). Although the differences among all strains were small at 25°C, reductions of growth temperature tended to magnify the disparities. For example, *A. guttatus* required 0.4 hours more for each doubling at 25°C (compared to *P. fluorescens*), whereas at 4°C this strain required 5.3 more hours for each doubling. The differences between *B. linens* and *P. fluorescens* were even greater. For each doubling, *B. linens* required 0.5 hr longer at 25°C, and 16.8 hr longer at 4°C.

Table 1. Hours required for doubling of protoplasm during the exponential growth phase of several poultry spoilage bacteria.

Bacteria	Incubation temperature		
	25°C	12°C	4°C
Pseudomonas fluorescens	1.7	2.6	7.6
Achromobacter guttatus	2.1	3.4	12.9
Brevibacterium linens	2.2	3.8	24.4
Pseudomonas sp.	1.8	2.0	14.0

The effect of CTC upon the test species was determined at an antibiotic level of 1 μg/ml. This concentration was decided upon because it has been shown that poultry meat, treated at approved levels, will generally have a concentration near this for most of the storage period. This is not to say, however, that initial levels are not higher. We did not assess the effects of these higher initial concentrations. Growth rates were measured after the cells had overcome any initial inhibition and were growing in the exponential growth phase.

At 25°C, the rates for *P. fluorescens* and *B. linens* were equal to their rates without CTC, whereas the rate for *A. guttatus* was found to be 0.6 hours longer (Table 2).

Table 2. Hours required for doubling of protoplasm during the exponential growth phase of several poultry bacteria in the presence of 1 ppm chlortetracycline.

Bacteria	Incubation temperature	
	25°C	4°C
Pseudomonas fluorescens	1.7	7.8
Achromobacter guttatus	2.7	12.9
Brevibacterium linens	2.2	Neg. in 35 days
Pseudomonas sp.	1.9	....

A combination of temperature reduction (25 to 4°C) and addition of CTC had a greater effect on *B. linens* than on the other test strains. The antibiotic added at the lower temperature suppressed growth for over 30 days. CTC at the 1 μg/ml level did not appear to have an inhibitory effect upon the exponential growth rate of the two psychrophilic strains.

Temperature fluctuations. Consideration was given to the effects of temperature fluctuations on growth during succeeding transfer since the organisms finding their way to the surface of processed poultry could have come from several environmental levels.

When *P. fluorescens* was grown first at 25°C and then transferred to 25°C, the exponential growth rate did not differ greatly from the rate when transferred from 4°C to 25°C (Table 3).

Table 3. Effect of prior growth conditions on first-transfer cells of two psychrophilic bacteria.

Cells first grown at:	Growth rate determined at:	Hours per doubling: *Pseudomonas fluorescens*	Hours per doubling: *Achromobacter guttatus*
25°C	25°C	1.7	2.1
25°C	4°C	7.6	12.9
4°C	4°C	4.1	14.7
4°C	25°C	1.6	3.9

An unexpected result came in transferring from 4°C to 4°C; the rate of growth was found to be faster than from the transfer of 25°C to 4°C. Either a selection of more hardy cells resulted from the prior growth at 4°C or first transfer to 4°C from 25°C did not allow a full shifting of those mechanisms that would result in better growth at the lower temperature. The authors favor the latter concept.

Temperature fluctuations affected *A. guttatus* differently than *P. fluorescens*. For the pseudomonad, growth at 25°C was nearly the same whether transfer was from the same or a lower temperature. With *A. guttatus*, transfer from 4°C to 25°C resulted in a slower growth rate than transfer from 25°C to 25°C. Of even greater significance is the fact that transfer from 4°C to 4°C had the effect of increasing doubling time. These changes were generally noticeable only on first transfer; later generations of the cultures grew at rates characteristic for a given temperature. It is intriguing to consider the multi-modality concept of Quesnel (1960) wherein a long, long, short generation time may prevail as the temperature is cycled.

Because antibiotic would be an additional environmental factor in poultry processing plants using CTC, additional stress in this form was applied. In these studies, the cultures were grown at a given temperature in the presence of 1 μg/ml CTC. Transfer was then made to a similar medium free of antibiotic. The growth rates (doubling time) were essentially the same as when CTC was not present (Table 4).

Table 4. Effect of prior growth in presence of chlortetracycline on growth rate at first transfer of two psychrophiles.

Cells first grown at:	Growth rate determined at:	Hours per doubling: *Pseudomonas fluorescens*	Hours per doubling: *Achromobacter guttatus*
25°C	25°C	1.4	2.6
25°C	4°C	7.8	12.8
4°C	4°C	4.1	14.7
4°C	25°C	1.5	3.3

Changes in pigmentation. In examination of culture plates of poultry meat isolates, changes in pigmented colonies are often noted. These differences may be interpreted as indicating a change in the types of bacteria growing on the meats. Because growth rates are influenced by environmental factors, a close examination was made of changes in pigmented cells as an indication of species or generic change in bacteria on refrigerated foods (Table 5).

Table 5. Pigmentation of bacteria isolated from poultry meat and grown under different conditions.

Strain	Growth condition	Pigmentation
Pseudomonans fluorescens	4°C	tan
	4°C, 1 ppm CTC[a]	tan
	25°C	tan
	25°C, 1 ppm CTC	tan
	25°C, 2.5 ppm CTC	off-white
	25°C, 5.0 ppm CTC	peach
	25°C, 10.0 ppm CTC	off-white
Achromobacter guttatus	4°C	light cream
	4°C, 1 ppm CTC	light cream
	25°C	light cream
	25°C, 1 ppm CTC	light cream
Brevibacterium linens	4°C	light yellow
	25°C	lemon yellow
	25°C, 1 ppm CTC	white

[a] Chlortetracycline.

The intensity of pigmentation elicited by *P. fluorescens* (a light tan) was the same whether the cells were grown at 4 or at 25°C. This strain also retained its characteristic color even in the presence of CTC at 1 μg/ml. An increase in the concentration of CTC, however, resulted in changes in pigmentation of this culture: 2.5 μg/ml changed the tan to a color best described as "off-white." Increasing the CTC concentration further (5 μg/ml) resulted in a culture that appeared "peach" colored. A further increase in CTC to 10 μg/ml resulted in a reversion to "off-white."

B. linens possessed a decided lemon-yellow color at 25°C. At 4°C, the color faded to a light yellow. The addition of CTC at 1 μg/ml at 25°C resulted in a culture that grew without noticeable pigment (white). Obviously, these changes indicate the lack of reliability of chromogenesis as a useful criterion for speciation.

Lysis by lysozyme. Previous work (not published) in the Purdue University poultry products laboratories has shown that a lytic factor for some strains of bacteria is often found in extracts of chicken skin. The bacterial species found to be most sensitive were those that were rich in the mucopolysaccharide substrate of lysozyme.

Because *Pseudomonas* strains have been shown to be sensitive to the action of lysozyme under certain conditions (Shively and Hartsell, 1961) cultures grown under the influence of the environmental stresses previously described were tested for lysozymic sensitivity. Conventional lysozyme assay techniques in a buffered system at pH 7.0 were used.

P. fluorescens was especially sensitive to lysis, but the response did not appear to be specific for lysozyme. *A. guttatus* responded only slightly to the action of lysozyme, and *B. linens* was completely insensitive to the test system.

Nakamura lysis. As was found with the neutral system, *P. fluorescens* was lysed readily but the lysis could not be attributed to lysozyme. *P. fluorescens* was lysed after incubation by the addition of alkali alone. Little was left that could be calculated as being due to lysozyme effect. *A. guttatus* exhibited a 50% lysis by the Nakamura technique (acid plus lysozyme, then alkali), which could be attributed to lysozyme. This lysis was not enhanced or suppressed by the cultural conditions under which the strain was grown.

B. linens was as indifferent to lysozyme in the Nakamura system as in the neutral system. No lysis was observed. Reports concerning the effect of lysozyme on *B. linens* could not be found in the literature; therefore, the high degree of resistance of this strain cannot be compared to that of others.

EDTA and lysozyme. Again, *P. fluorescens* was so sensitive to lysis in the controls that the effect of lysozyme could not be measured. From all appearances the strain used in these studies is highly unstable. The combination of EDTA and lysozyme did not result in a greater lytic response than could be obtained by lysozyme alone on those cells grown in the absence of CTC. Lysis was increased only slightly in cells of *A. guttatus* grown in the presence of CTC. EDTA did not effect a lytic response with *B. linens*.

DISCUSSION

Some of the reasons for the predominance of the *Pseudomonas-Achromobacter* types on refrigerated poultry meat may be related to an indifference of these types to wide environmental changes. For example, *P. fluorescens* and *B. linens* differ greatly in growth rate at 4°C. Further, it has been shown (Wells *et al.*, 1963) that there is a great increase in lipid in *B. linens* at 4°C (from 6.7% at 25°C to 16.7% at 4°C) whereas *P. fluorescens* exhibits little change over the amount present at more nearly optimum conditions (12.2% at 25°C and 11.8% at 4°C). The rates, however, were slowed slightly at 4°C from the influence of CTC. Since *A. guttatus* generally followed the same course as that taken by *P. fluorescens*, one can only wonder about the relationship of increased lipids and growth rates of poultry spoilage bacteria at refrigerator temperatures.

The greater effectiveness of CTC for the preservation of fresh poultry meat at the lower storage temperatures has been shown by Wells *et al.* (1957) and Shannon and Stadelman (1957). Is it possible that lipid increase has an effect upon cellular absorptive properties? If such is the case, the nonpsychrophiles would find themselves at a physiological disadvantage on refrigerated meats because of altered absorptive properties due to increased cell lipid. Further, it may be, as stated by Lacey (1961), that "a change in permeability could lead to a change in enzymatic activity through a change in access or release of any factor influencing the action of enzymes in solution."

Little need be said concerning changes in cell pigmentation as an indication of the course of bacterial changes on stored meat—except that the data show such evaluations to be subject to great error.

The effects of temperature fluctuations upon poultry spoilage bacteria, as well as the effects of prior growth in the presence of CTC, indicate the importance of the source of contamination of fresh poultry meats.

Lytic response to lysozyme, which had been expected to reflect alteration(s) in cell wall orientation, indicated that this lytic system could not be used to evaluate the response of spoilage bacteria from refrigerated poultry meats. Neither did it indicate that the environmental stresses applied predisposed the test strains to sensitivity to lytic substances that might be found in chicken skin.

The *Achromobacter* as represented by *A. guttatus*, do not appear to be as active at psychrophilic growth temperatures as were pseudomonads as represented by *P. fluorescens*. Although *A. guttatus* exhibits lipid changes similar to those of *P. fluorescens*, it possesses, under all conditions, a higher lipid content. *B. linens* is a poor psychrophile and shows a greater tendency to increased lipids and slower growth rates as growth temperature is depressed. Perhaps an ability to resist making changes in cellular organizations that influence growth rates (exclusive of slower enzymatic turnover rates) is one prime characteristic of the psychrophilic bacteria associated with spoilage of poultry meat held at refrigerator temperatures.

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The Microflora Within the Tissue of Fruits and Vegetables*

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SUMMARY

Bacteria occur within normal, sound fresh fruit tissues. They are mostly gram-negative motile rods, representatives of the Pseudomonadaceae and the Enterobacteriaceae. Lactic-acid-forming bacteria are found on the surface of crops. In different crops and in different varieties, bacteria may appear abundantly in one field and rarely in others. They are found more frequently in low-growing vegetables than in tree-borne fruits. In cucumbers the bacteria are more often in the tissue close to the periphery and less often in the central core. In tomatoes their frequency is highest close to the stem-scar and the central core of the fruit, decreasing toward the fruit periphery.

It appears that the bacteria can enter the living plant tissue by different pathways and may persist there as harmless commensals. When the vegetables are brined, the bacteria multiply in the tissue as well as in the brine. Lactobacilli penetrate brined tomatoes primarily through the stem-scar and multiply more rapidly in the fruit than in the brine. During fermentation of tomatoes and cucumbers the Enterobacteriaceae are mostly suppressed by the lactic-acid-forming bacteria. However, if the latter are excluded by surface disinfection of the fruits, the Enterobacteria continue to multiply, causing internal bloaters, an increase in pH, and, ultimately, putrefaction.

The presence of bacteria within fruit tissue is generally associated with pathologic conditions. In healthy fruits the bacterial flora is assumed to be limited to the surface, while the inner tissue remains sterile. There is, however, little experimental confirmation in literature. Literature is scarce on the subject of microflora in normal, sound tissue, and may be divided into three groups with respect to conclusions. A few writers (Allen and Allen, 1950; Burcik, 1949; Duggeli, 1904; Fernback, 1888; Fischer, 1950) state that plant tissue is sterile and that the occurrence of bacteria represents a pathological condition; they assume that positive findings of others were based on faulty technique.

A second group (Hennig and Villforth, 1940; Hollis, 1951; Ingram and Riches, 1951; Marcus, 1942; Sanford, 1948; Smith and Niven, 1957; Szilvasi, 1942; Tervet and Hollis, 1948; Tonzig and Bracci-Orsenigo, 1955) is made up of scientists who reported the presence of microorganisms in the tissue of different crops and plant parts, such as potatoes, carrots, peas, cucumbers, as well as a long list of other plant tissue. They reported the facts and made no conjectures as to the possible origin or role of these microorganisms.

The third group (Buchta, 1948; Dawid, 1957; Rusch and Kolb, 1950; Schanderl, 1953, 1962) found bacteria regularly in the tissue of different plants and concluded that bacteria are natural inhabitants of the plant cell and can multiply when the normal cell organization is destroyed. According to those authors, bacteria should be found in every specimen, and the same species of bacteria would develop in the same tissue.

The problem came to our attention during a study of the microflora of fermenting cucumbers when internal cavities were found to be caused by bacteria within cucumbers (Samish and Dimant, 1957, 1959). The scope of our study was broadened thanks to a grant from the Agricultural Research Service of the U. S. Department of Agriculture ("Fundamental Studies of Microbial Flora within the Tissues of Fruits and Veg-

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The approach taken in this study consisted at first of surveying a number of fruit and vegetable crops of widely differing origin so as to find whether microorganisms could be found in their tissue, and their exact location and identity.

This could be established only after the proper procedures were developed for isolating the fruit tissue free of bacterial contamination. Subsequently, the role of the internal microflora in the progress of fermentation in brined vegetables was investigated.

METHODS

Different techniques of sterilization were compared in order to find ways to eliminate all chance of contaminating the samples either from the fruit surface or from the atmosphere (Samish *et al.*, 1961). The procedure was then standardized as follows: The fruits were washed thoroughly first under running tap water, then scrubbed with a commercial detergent, immersed in a fresh detergent solution, rinsed again in tap water, immersed for 15–20 min in a solution of sodium hypochloride containing 300 mg available chlorine, immersed for 5 min in a weak solution of sodium thiosulfate, again rinsed with sterile water, and finally immersed for 30 min in a solution containing 80% alcohol and 0.02% iodine.

After this treatment the fruits were flamed and immediately dissected in order to remove specimens for plating. The manipulations were carried out in a special transfer room sterilized daily with a u.v. lamp for 2–3 hours. At the end of each trial similar manipulations were carried out as controls, with autoclaved fruits or sterile peptone water exposed for the maximum time that would be required for the preparation and plating of the fresh fruits (Samish *et al.*, 1961).

In the early trials, surface-sterilized fruits, particularly tomatoes and cucumbers, were pierced with sterile pipettes, which were rotated so as to macerate the inner tissue, and aliquots of about 1 ml pulp were extracted and transferred to nutrient media.

With this technique the time required for manipulation was somewhat longer, and gram-positive bacteria belonging to the atmospheric flora were also encountered, though they are not reported in our findings. In later trials we used mostly the technique of direct swabs and smears from the freshly cut fruit surface upon solid nutrient media (yeast dextrose agar), which excluded practically all danger of contamination. The presence of bacteria within the inner tissue could thus be proven with reasonable certainty, because the smears often showed many colonies of either one or two species of gram-negative bacteria that were not present in the atmosphere. Density of bacteria was determined by transferring the pulpy juice with a glass pipette and diluting it with sterile water before plating. The isolated bacteria were identified as to genus according to "Bergey's Manual of Determinative Bacteriology." (Breed *et al.*, 1957). The pH of the samples was measured with a Beckman potentiometer.

With the smear technique, however, only very small samples could be examined, which were not necessarily representative for the entire fruit. Later trials showed that the distribution of bacteria within the tissue was not uniform.

The main source of doubt, which we encountered at first in trying to determine the sterility or nonsterility of fruit tissue, was that results could not always be confirmed when a trial was repeated. Only prolonged trials helped to prove conclusively that the variability of results was not due to faulty technique, but was inherent in the samples themselves.

RESULTS

Fresh fruits and vegetables. Ten fruits and vegetables were studied: tomatoes, peas, beans, cucumbers, citrus fruits, grapes (1500–3000 fruits each), some 300–500 melons and olives, and peaches and bananas (about 200 each). Vegetables had a higher incidence of bacteria than tree-borne fruits. Bacteria—most often gram-negative motile rods—were found frequently within tomatoes, cucumbers, garden peas, and green beans, and less often in melons and bananas. Grapes, citrus fruits, olives, and peaches rarely contained bacteria.

Results varied greatly with samples from different farms. In some fields many fruits contained bacteria, and in others only a few (Table 1).

The bacterial content of the fruits seems to be affected not only by growth conditions but also by varietal characteristics (Table 2). Thus, the oc-

Table 1. Frequency of bacteria in crops from different fields.

Crop	Number of		% of fruits containing bacteria	
	Samples	Fields	Av.	Highest
Cucumbers, central core	344	3	13	47
Tomatoes, pulp	570	6	25	90
Broad beans, seeds	250	3	32	60
Garden peas, seeds	345	3	15	78

Table 2. Effect of maturity upon bacterial content of tomatoes.

Variety	No. of tomatoes sampled	% of tomatoes containing gram-negative bacteria	
		Green-yellow	Pink-red
Tamar	169	9.3	4.8
Marmand	915	22.5	21.0
Moneymaker	58	33.3	17.6

currence of bacteria may differ widely with different species and varieties, and even with the same variety grown in different fields. In addition, different parts of a single fruit often differed greatly in bacterial content (Table 3). However, green and red tomatoes collected from the same plants did not differ widely in bacterial content.

Surface-disinfected green beans, broad beans, and garden peas contained bacteria more often in their pods than in the seeds.

When short, cylindrical pieces were excised from fresh, surface-disinfected cucumbers with sterile cork borers of three diameters, the central cylinder (diameter of corkborer, 17 mm) contained gram-negative bacteria in 9% of the samples, but the median hollow cylinder (diameter of corkborer, 24 mm) did in over 50%. In the outermost ring, including the surface disinfected peel, 88% of the samples had gram-negative bacteria. Thus, bacteria were more abundant at the fruit periphery, and less so near to the central axis.

The pattern of distribution was different in tomatoes, however. The tomatoes were dissected and swabs made at eight locations, as indicated in the schematic drawing of Fig. 1.

Bacteria were found most often in the stem-scar and in the underlying central core. They were less frequent near the stylar end and near the fruit periphery. Here again, bacteria in tomatoes of the same variety were more frequent in fruit from some farms than from others.

We thus can recognize a gradient of density of bacterial population, which may be possibly indicative of the way of entry of the microorganisms: in the tomato from the stem-scar to the core and to the endocarp; and in cucumbers from the cuticula toward the core.

Table 4 lists the bacterial flora found in different locations in tomatoes.

Representatives of three families were primarily present within the tissue: Pseudomonadaceae were encountered most often (genus *Pseudomonas* and genus *Xanthomonas*), and Enterobacteriaceae (genus *Aerobacter*) and Corynebacteriaceae (genus *Corynebacterium*) less frequently. The stem-scar contained in addition Bacilli, micrococci, yeasts, and molds, although rarely and at low concentrations (Table 4).

The characteristics of four species of Pseudomonadaceae, which were found most often in the tomato tissue, have been described in a previous paper (Samish *et al.*, 1961).

The bacteria that generally produce lactic acid in brined vegetables—lactobacilli and streptococci—were not found inside the fresh fruit tissue of the crops examined. They apparently exist only on the surface of the fresh fruit.

Brined vegetables. When cucumbers or tomatoes surface-sterilized with hypochlorite were shaken in nutrient broth and removed, the broth remained sterile. But when they were retained in the broth, gram-negative bacteria developed, although with a delay of 7–10 days.

When green-ripe tomatoes were covered with sterile 5% salt solution and *Lactobacillus plantarum* starter was added, these bacteria readily penetrated the fruits (Table 5).

Within 24 hr of brining, the lactobacilli multiplied very rapidly in part of the fruits; differences between individual fruits were very high, even between fruits kept in the same container. Seven days after brining, the lactobacilli were abundant in every tomato and their concentration was higher within the fruits than within the brine.

The distribution of the lactobacilli in different locations within the brined tomatoes is illustrated in Fig. 2. These results seem to indicate that the lactobacilli enter the tomatoes largely through

Table 3. Frequency of bacteria in different fruit parts.

Crops	No. of fruits sampled	% of samples containing bacteria		
		Peripheral hollow cylinder with peel	Median hollow cylinder 17–24 mm	Central core 17 mm
Cucumbers	125	88	51	9
		Surface	Pods	Seeds
Green beans	498	0	75	27
Broad beans	290	2	60	22
Garden peas	508	5	49	20

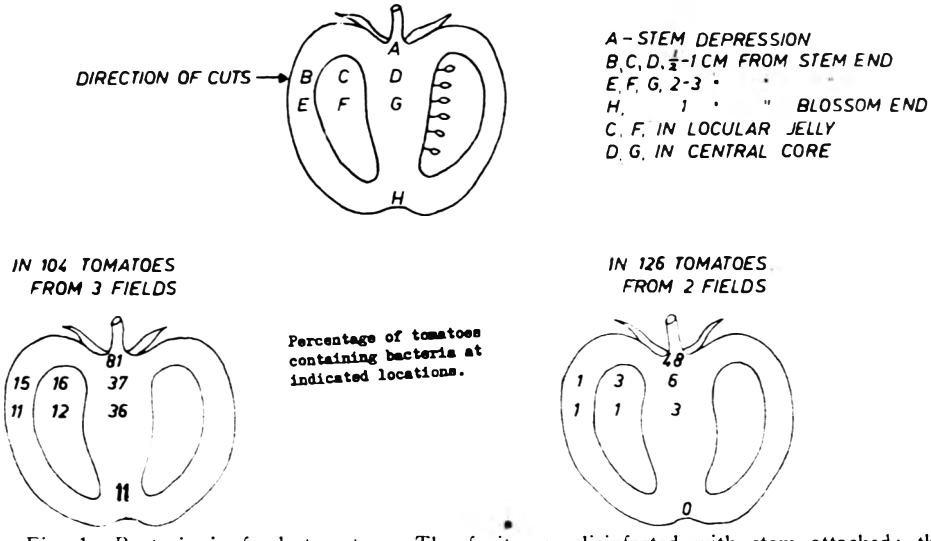

Fig. 1. Bacteria in fresh tomatoes. The fruit was disinfected with stem attached; the stem was then pulled off, and a smear was taken of the resulting stem-scar.

the stem-scar to the central core, a distribution pattern similar to that found with the indigenous bacteria.

When green tomatoes were brined without starters, however, the progress of fermentation was not only slow but also less uniform. The Enterobacteriaceae generally multiplied in the brined fruit as well as in the brine, Pseudomonadaceae multiplied only in the brine and rarely in the fruit, and Corynebacteriaceae were never found in either fruit or brine. When true acid formers appeared, they gradually superseded the gram-negative forms. Such delayed fermentation still produced well fermented tomatoes.

Table 6 illustrates how the progress of fermentation may be affected by the origin of the fruits as well as by the original concentration of lactobacilli in the brine.

Tomatoes from a lot with only a few bacteria in the fresh state and brined with added starter, fermented rapidly. Within one week, all gram-negative bacteria had disappeared and the acidity was high in the brine as well as in the tomatoes. But without starters the *Acrobacter* species continued to multiply and acidity accumulated only slowly.

When tomatoes were taken from lots with bacteria-rich fruits, however, the indigenous bacteria continued to multiply even when *Lactobacillus plantarum* starter had been added.

With added starter the tomatoes usually, though not always, fermented well—although with a delay of about two weeks. But when such tomatoes were brined and no starters had been added, the fruit spoiled generally within 2–3 weeks. Spoilage was even more rapid whenever the acid formers had been largely removed from the fruit surface by thorough washing of the fresh fruits before brining and when no starters were added at the time of brining. The *Acrobacter* species, having no competition, multiplied very rapidly and often formed gas pockets in the locular jelly, and white

Table 4. Bacterial flora in different locations within 250 fresh tomatoes.

Fruit part	% of fruits containing bacteria					Total
	Pseudom.	*Enterob.*	*Coryn.*	Others		
Stem depression	49	13	10	Micrococci	4	57.5
				Bacilli	6	
				Molds	2	
				Yeasts	0.1	
Pericarp	7	1	4	0		7
Locular jelly	8	3	4	0		13
Central core	28	7	4	0		34
Blossom end	3	1	3	0		4

Table 5. Density of population of *L. plantarum* (no. bacteria in 1 ml) in fermenting tomatoes (*L. plantarum* starter added).

Days after brining	Brine	Tomato pulp		
		Av.	Minimum	Maximum
1	2×10^5	9×10^5	0	2.5×10^8
2	2.5×10^6	1.6×10^7	0	6.6×10^7
7	9×10^7	3.9×10^8	3×10^6	2.4×10^9

spots containing Enterobacteriaceae on the fruit surface. Such fruits generally spoiled within one week.

According to the data in Tables 5 and 6 the lactobacilli tend to multiply more rapidly in the fruit than in the brine. This may possibly be due to the higher concentration of nutrients in the tissue or to their preference for a less salty substrate and their ability to tolerate a lower pH and a reduced air supply.

Analyses of NaCl in the brine and in tomato pulp as determined by the Volhard Ferric Alum volumetric method (Winton, 1945) proved that penetration of the brined tomatoes by salt is indeed very slow, as shown in Table 7. Tomatoes covered with a 5% brine contained only about 0.4% NaCl four days later, and about 1.0% NaCl in two weeks. Salt penetration was slower into red fruits than into green ones.

When cucumbers were brined with 5% salt solution, the gram-negative bacteria multiplied in the fruit and in the brine even when *L. plantarum* starter had been added. Contrary to tomatoes, however, the microflora was concentrated more highly in the brine than in the fruit, and the acidity of the brine increased more rapidly than that of the fruit (Table 8). With or without added starters, both the fruit and the brine were densely populated with lactobacilli about 10 days after brining, whereas the gram-negative bacteria had disappeared. But when fresh cucumbers had been immersed for 2 hr in a quaternary ammonium compound (0.1% benzalkonium chloride), rinsed, and brined, gram-positive acid-forming bacteria never appeared whereas gram-negative bacteria multiplied rapidly. Within three days every single cucumber had abnormally large hollows, and Enterobacteriaceae were found highly concentrated in the hollows as well as in the brine. But even where the cucumbers were either thoroughly washed in tap water before brining or dipped in a detergent only shortly, fermentation did not progress normally, *Aerobacter* species multiplied rapidly, producing hollows and a repulsive odor.

DISCUSSION

Our data indicate that different bacterial species of the epiphytal flora are also often present within the tissue of fruits and vegetables. They were unevenly distributed in different crops as well as in different parts

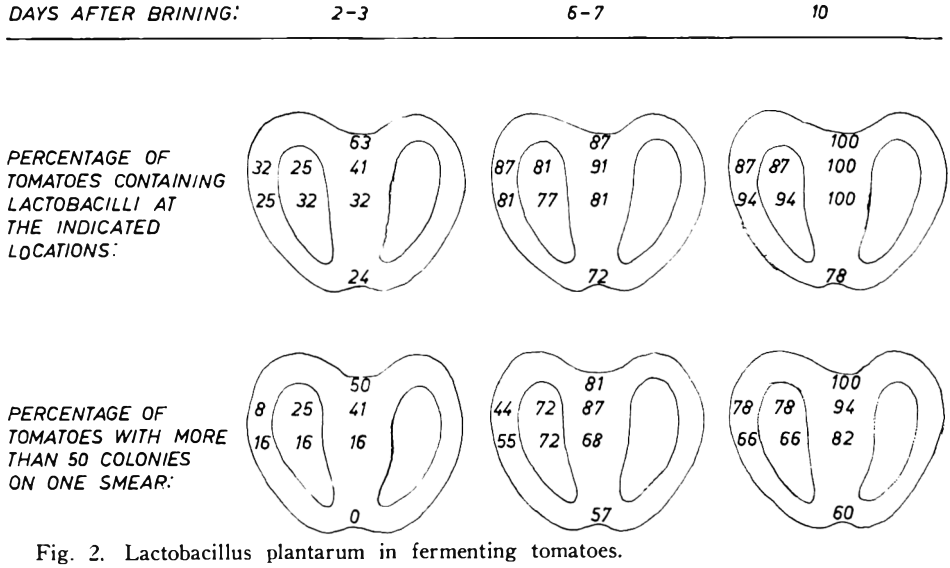

Fig. 2. Lactobacillus plantarum in fermenting tomatoes.

Table 6. Microflora in brined tomatoes with and without added starter; 5–6 replications; log of population/ml of juice.

Days after brining		Gram-negative bacteria in *fresh* tomatoes							
		Few bacteria				Many bacteria			
		Without starter		With starter		Without starter		With starter	
		Tomato	Brine	Tomato	Brine	Tomato	Brine	Tomato	Brine
2	Lactob.	1.0	2.0	7.3	4.5	4.5	2.7	5.1	3.7
	Strept.	no	3.0	no	no	5.7	3.0	5.5	4.3
	Aerob.	2.0	6.4	3.5	4.0	6.8	6.3	6.6	4.6
	Pseudom.	no	no	no	no	no	no	no	no
	pH	4.4	6.7	3.9	6.7	4.6	6.7	4.3	6.7
7	Lactob.	6.5	5.7	8.5	7.7	7.2	6.7	9.5	7.4
	Strept.	6.8	6.7	no	6.3	5.8	5.7	no	6.0
	Aerob.	2.5	7.6	no	no	9.0	6.0	7.4	6.7
	Pseudom.	no	no	no	no	no	5.7	no	7.0
	pH	4.3	6.7	3.7	4.2	4.4	6.7	4.1	6.7

Table 7. Salt content of the brine and the tomato pulp of fermenting tomatoes.

Days after brining	% NaCl		
		In tomato pulp	
	In brine	Av.	Range
0	5.0	0.1	
5– 6	4.8	0.4	0.2–0.7
7– 8	4.1	0.5	0.3–0.8
9–10	4.0	0.6	0.4–1.0
11–12	3.8	0.7	0.6–1.0
13–14	3.8	0.9	0.6–1.5

of the fruit. This finding seems hardly compatible with systematic occurrence of the bacteria. The fact that we found considerable differences in density of bacterial population in fruits from different fields may indicate an effect of agrotechnical practices or of climate, which it might be worthwhile to investigate.

Our data do not seem to justify conclusions as to the method of entrance or the role of the microflora in the plant tissue, but the pattern of distribution observed with two crops may suggest a possible explanation. Some members of the epiphytal flora may reach the tissue because of their smallness and motility, through natural apertures such as stomata, lenticels, broken hairlets, connecting tissue, or other wall perforations.

It may well be that the bacteria enter the fruit tissue more readily in the early stages of fruit development, at a time when the different channels are not yet covered by corky or waxy materials.

The tomato skin is quite impervious to bacteria. The entrance of bacteria into the fruit seems to take place most often from the stem depression and the connecting tissue, though the permeability of the stem depres-

Table 8. Microflora in brined cucumbers. Log of population in one ml liquid.

Days after brining	Treatment	None		*L. plantarum* starter added		Treated with Benzalkon-cl.	
		Cucumbers	Brine	Cucumbers	Brine	Cucumbers	Brine
3	gram +	no	7.2	2.5	8.6	no	no
	gram –	5.4	7.6	3.6	7.5	4.4	7.7
	pH	5.4	4.5	4.5	4.2	5.8	5.7
7	gram +	4.5	8.6	4.6	9.9	no	no
	gram –	1.8	7.4	no.	3.6	>10	9.7
	pH	4.2	4.1	3.6	3.9	5.6	5.7
11	gram +	>10	>10	>10	>10	no	no
	gram –	no	no	no	no	>10	>10
	pH	3.5	3.3	3.5	3.2	5.2	5.3

sion of individual fruits does not seem to be uniform.

We found, indeed, that the rate of fermentation of brined fruits was conspicuously delayed when the stem-scar had been previously closed with balsam. This observation agrees with that of Wahrman and coworkers (1957), who reported that the ripening of detached fruits was much delayed when the stem-scar had been closed with wax. The gas exchange of the tomato apparently also takes place primarily through the stem-scar.

The gradient of density of bacterial population is almost opposite in cucumbers to that in the tomato, i.e., decreasing from the periphery toward the center. The cuticula seems to be a less efficient barrier against the penetration of bacteria, possibly because of the abundance of stomata and broken hairlets.

When nutrient broth is shaken with thoroughly surface-disinfected fruits it remains sterile, but when such fruit is kept in it the bacteria start to multiply. They had been presumably imbedded below the epidermis in subcutaneous cavities or adjoining intercellular spaces.

But, whatever the mode of entrance of the bacteria, it seems rather certain that they make only a limited development in the healthy tissue. Apparently these "entrapped" microorganisms do not find congenial environmental conditions for their development in healthy plant tissue, and remain harmless commensals.

But when the structure of the fruit tissue is disturbed by injury or maceration, by changes in temperature or of air supply, or by plasmolysis through brining, these bacteria may multiply very rapidly when conditions are favorable for their growth.

This theory finds support from studies carried out with animal tissue. Some authors (Burrows, 1955; Dubos, 1954; Hare, 1956; Hirsch, 1959) report that bacteria can persist in healthy animal tissue in a latent state because of the inhibiting action of the healthy cells, but may be activated by some physiological disturbance of the tissue.

In our studies some of the indigenous bacteria, such as the corynebacteria, did not survive after brining. The pseudomonads, the most common species encountered in the fruits, apparently found conditions more favorable for their development in the liquid phase than within the brined fruit. The Enterobacteriaceae, however, multipled in the fruit as well as in the brine, and, if they did not encounter competition from acid formers, had a deleterious effect upon the progress of fermentation.

The presence of *Aerobacter* species in the brine of fermenting vegetables soon after brining has been reported by Etchells, Pederson, and others (Etchells and Jones, 1945; Pederson and Albury, 1961; Vaughn *et al.*, 1954). Our data confirm these findings and seem to show that they multiply not only in the brine but also within the brined fruit and may cause internal bloaters as well as spoilage.

When the acid-forming bacteria were removed from the surface of either tomatoes or cucumbers before brining, with the help of detergents, the indigenous *Aerobacter* species invariably became very active. They liberated gas in the brine and in the fruits, formed large internal bloaters, and subsequently caused complete deterioration.

When acid-forming bacteria were present, however, they suppressed the gram-negative groups, invading and superseding them more rapidly in the tomatoes than in cucumbers.

Our findings may become of interest to the food technologist dealing with microbial problems during fermentation as well as during storage, chilling, freezing, pasteurization, or aseptic canning of fruits and vegetables. Mechanical means of inactivating the microflora on the surface of the fruits may not always be equally effective for microflora imbedded in the tissue. The food technologist may wish to know to what extent the bacterial makeup of the fresh vegetables, which is itself affected by agrotechnical practices, may in turn affect the industrial processes.

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Chemical Preservatives as Inhibitors of Yeast Growth

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SUMMARY

The resistances of 37 yeast cultures to 5 chemical preservatives (sorbic, benzoic, sulfurous, salicylic, and formic acids) in concentrations increased by steps were treated statistically by the method of distribution curves, plotting the surviving cultures against the concentration of each preservative. The area confined by each curve and the coordinate axes was found to be inversely proportional to the potency of each preservative. This treatment of data seems to be a new way of evaluating chemical preservatives, and possibly antibiotics.

Chemical preservatives are continuously succeeded in food processing by other substances of greater effectiveness and acceptability. Chemical preservatives have been criticized for centuries as toxic, and more recently have been suspected of having carcinogenic properties (Saint-Rat, 1956).

Nevertheless, they are used in tremendous amounts in both home and factory to preserve raw materials and finished food items. In the Mediterranean area (and in Germany) such materials are important in industrial food processing, including wines, fruit juices (single-strength or concentrated), pulps of different types (Exarchos and Balatsouras, 1958, 1961), and olives stuffed with anchovies. To improve fermentation, microbial flora are selected by the use of sulfur dioxide and sorbic acid (Costilow, 1957). In recent years, investigators have developed methods of comparing chemical preservatives in the prevention of microbial spoilage. The test organisms have been few (Von Schelborn, 1953), a single organism (Karl and Busch, 1957), or only some specific enzymes of the microbial cell (Luck, 1958). The basis for comparison has been the potency of the undissociated part of the preservative (Von Schelborn, 1953), the microbial crop (Karl and Busch, 1957), or catalase activity in liberating oxygen from a substrate of hydrogen peroxide and peroxidase in liberating oxygen from the same substrate and oxidizing phenolic substances.

Since the resistance of microorganisms to chemical preservatives has been claimed to vary considerably with genus, species, and even strain, comparative evaluations based on a limited number of microorganisms or specific enzymes would seem to be of limited value.

EXPERIMENTAL

Test organisms. The organisms tested were 36 yeast isolates belonging to seven genera (*Hansenula, Pichia, Debaryomyces, Sporobolomyces, Candida, Torulopsis,* and *Rhodotorula*) and the yeastlike organism *Oospora lactis.* The source was three substrates susceptible to yeast spoilage: unpasteurized citrus juice and olive and cucumber brines (Vaughn *et al.,* 1960). Most of them have been found in those substrates by other investigators (Recca and Mrak, 1952; Mrak *et al.,* 1956; Gonzalez, 1960); others are reported from these substrates for the first time. Since the yeasts originated from three substrates of quite different chemical compositions, they presumably constitute a random sample.

Materials. The chemicals compared are five preservatives in current use in Greece, either officially or unofficially: sorbic, benzoic, sulfurous, salicylic, and formic acids. The basal media were single-strength orange juice and potato infusion. Both media have been shown to be adequate in buffer capacity and nutrient supply. The pH was not appreciably changed by differing preservative doses or by the growth of the test organisms. The orange juice pH was adjusted, when necessary, with a concentrated solution of sodium hydroxide. Also added was 4% glucose. The potato infusion was obtained by boiling 200 g of peeled potatoes, filtering through cheesecloth, and making up the volume to 1 L. Additions were 80 g of glucose and 3.5 g of citric acid monohydrate.

Methods. The nonvolatile preservatives (sorbic, benzoic, and salicylic acids) were added to the

basal medium before sterilization. (The sorbic and salicylic acids had previously been dissolved in a small volume of pure alcohol). After the preservatives were added, the medium was stirred vigorously, poured into 16 × 150-mm test tubes, and sterilized 15 min at a pressure of 15 psi.

The volatile preservatives (sulfurous and formic acids) were sterilized before being added to the basal medium. Portions of the stock solutions, 75% sulfurous acid and pure formic acid, were withdrawn aseptically and diluted with sterile water in 100-ml volumetric flasks so that 1 ml of solution added to 9 ml of basal medium in screw-cap tubes gave the desired concentration of preservative.

Each test was begun by inoculating 37 tubes containing only basal medium with cells from 48-hr slants of the mother culture on potato-dextrose agar. When growth was evident (in another 48 hr), a loopful was taken and inoculated into each tube of the next series of 37 (basal medium plus lowest concentration of preservative). The tubes were incubated at room temperatures or in a laboratory incubator (below 22°C). Growth was observed every other day in terms of gas production, turbidity, film, or sediment, depending on the yeast strain. Gas was observed in Durham vials inverted into the medium, or was presumed when the Vaspar layer of the stratified tubes was displaced upward.

When volatile preservatives were tested, the tubes inoculated with fermentative strains were stratified with a Vaspar layer. Oxidative strains, however, were always inoculated into screw-cap tubes. The tightly screwed caps were further sealed with a strip of cellotape, which was made airtight by solidified Vaspar. The air in the tube was sufficient to support the growth of the aerobic strains when the concentration of preservative was lower than that of the "hem-dosis" (meaning the concentration of each preservative that prevents any growth of all test cultures during one month of incubation). Blanks were run to exclude lack of oxygen as a factor preventing growth of the oxidative strains.

Growth was usually evident in 48–72 hr. Then a loopful was transferred from positive tubes to tubes containing the next-higher concentration of the same preservative. The steps of increase of preservative were by double, half, or a quarter of the initial level, depending on the individual preservative.

Tubes were characterized as negative after one month of incubation without showing growth. Excess of glucose in the basal medium promoted rapid growth and production of a high volume of gas.

No attempt was made to inoculate tubes with a uniform number of cells; other investigators (Karl and Busch, 1957) found such a procedure laborious and of little significance in this type of study. If cell crops are compared at a definite time after inoculation, of course, the number of cells is very important, but in our tests the end point was not a quantity of cells but the concentration of preservative that permitted no growth in one month. Tubes containing the "hem-dosis" of each preservative showed no growth when the inoculum was doubled or tripled. Therefore, in all regular tests the inoculum was standardized at one loopful.

Sorbic acid was the only preservative tested also in combination with 8% sodium chloride. The question was whether the salt would enhance its inhibitory effect.

RESULTS AND DISCUSSION

Resistance to sorbic acid. Table 1 shows the resistance of test cultures to sorbic acid. Though not a typical preservative, since it is metabolized by molds and catalase-negative bacteria (York and Vaughn, 1955), sorbic acid appears to have a preservative value comparable to that of common preservatives. No isolate grew when the concentration was as high as 1000 ppm, and resistance among the yeasts varied less than with the other preservatives. The most resistant organisms appear to be strains of *Hansenula subpellicosa, Candida pulcherrima,* and *Oospora lactis.* NaCl at 8% enhanced the effect of sorbic acid. This combination would be of practical value in lactic acid fermentation, which is not inhibited by sorbic acid in low concentrations (Emard and Vaughn, 1952).

Although some yeasts are reported to metabolize sorbic acid (Alderton and Lewis, 1958), in our experiments sorbic acid, at a level specific for each yeast strain, inhibited growth independently of inoculum dose and incubation time.

Resistance to benzoic acid. Table 2 indicates that benzoic acid at 300 ppm prevented growth of all three isolates. Those three (two *Pichia membranaefaciens* and one *Candida krusei*) withstood a double dose or even higher. All *Hansenula* were unable to withstand a concentration higher than 200 ppm.

From our results 1000 ppm benzoic acid should be considered enough to prevent

Isolate no.	Genus and species	100 ppm		250 ppm		400 ppm		600 ppm		800 ppm		1000 ppm	
		− NaCl	+ NaCl	− NaCl	+ NaCl	− NaCl	+ NaCl	− NaCl	+ NaCl	− NaCl	+ NaCl	− NaCl	+ NaCl
6	*Hansenula subpelliculosa*	+	+	+	+	+	−						
13	*Hansenula subpelliculosa*	+	+	+	+	−							
14	*Hansenula subpelliculosa*	+	+	+	+	−							
24	*Hansenula subpelliculosa*	+	+	+	+	+	−						
26	*Hansenula subpelliculosa*	+	+	+	+	−	−	−	−				
35	*Hansenula subpelliculosa*	+	+	+	+	+	−	+	−	−	−		
50	*Hansenula subpelliculosa*	+	+	+	+	+	−	+	−	+	−		
51	*Hansenula subpelliculosa*	+	+	−									
42	*Hansenula anomala*	+	+	+	+	−							
10	*Hansenula anomala* var. *ciferrii*	+	+	−									
9	*Hansenula suaveolens*	+	+	+	+	−							
30	*Hansenula minuta*	+	+	+	+	+	−						
43	*Hansenula minuta*	+	+	+	+	+	−						
28	*Pichia membranaefaciens*	+	+	−									
29	*Pichia membranaefaciens*	+	+	+	+	−							
48	*Pichia (Saccb.) pastori*	+	+	+	+	−							
40	*Debaryomyces nicotianae*	+	+	−									
57	*Sporobolomyces roseus*	+	+										
3	*Candida krusei*	+	−										
15	*Candida krusei*	+	−										
27	*Candida pulcherrima*	+	+	+	+	+	−	+	−	+	−		
34	*Candida pulcherrima*	+	+	+	+	+	−	+	−	+	−		
17	*Candida mycoderma*	+	+	+	+	−							
56	*Candida mycoderma*	+	+	+	+	+	−						
7	*Candida parapsilosis*	+	+	+	+	+	−						
21	*Candida melinii*	+	+	+	+	−							
63	*Candida melibiosi*	+	+	+	+	+	−						
41	*Torulopsis stellata*	+	+	−									
55	*Torulopsis stellata*	+	−	−									
54	*Torulopsis stellata*	+	−	−									
62	*Torulopsis stellata*	+	+	−									
45	*Torulopsis magnoliae*	+	+	+	+	−							
36	*Torulopsis inconspicua*	+	−										
46	*Torulopsis bacillaris*	+	+	+	+	−							
44	*Rhodotorula minuta*	+	+	−									
49	*Rhodotorula mucilaginosa*	+	+	−									
61	*Oospora lactis*	+	+	+	+	+	−	+	−	+	−	+	
	Number growing	37	32	23	23	12	0	5	0	4	0	1	

Table 2. Tolerance of the isolates to benzoic acid in 7 concentrations (ppm).

Isolate no.	Genus and species	100	200	300	400	500	600	700
6	*Hansenula subpelliculosa*	+	+	−				
13	*Hansenula subpelliculosa*	+	−					
14	*Hansenula subpelliculosa*	+	−					
24	*Hansenula subpelliculosa*	+	+	−				
26	*Hansenula subpelliculosa*	+	+	−				
35	*Hansenula subpelliculosa*	+	+	−				
50	*Hansenula subpelliculosa*	+	+	−				
51	*Hansenula subpelliculosa*	+	+	−				
42	*Hansenula anomala*	+	+	−				
10	*Hansenula anomala* var. *ciferrii*	+	+	−				
9	*Hansenula suaveolens*	+	+	−				
30	*Hansenula minuta*	+	+	−				
43	*Hansenula minuta*	+	+	−				
28	*Pichia membranaefaciens*	+	+	+	+	+	+	−
29	*Pichia membranaefaciens*	+	+	+	+	+	+	+
48	*Pichia (Sacch.) pastori*	+	+	−				
40	*Debaryomyces nicotianae*	+	+	−				
57	*Sporobolomyces roseus*	+	−					
3	*Candida krusei*	+	+	+	+	+	+	
15	*Candida krusei*	+	+	−				
27	*Candida pulcherrima*	+	+	−				
34	*Candida pulcherrima*	+	+	−				
17	*Candida mycoderma*	+	−					
56	*Candida mycoderma*	+	+	−				
7	*Candida parapsilosis*	+	−					
21	*Candida melinii*	+	−					
63	*Candida melibiosi*	+	+	−				
41	*Torulopsis stellata*	+	+	−				
55	*Torulopsis stellata*	+	−					
59	*Torulopsis stellata*	+	+	+	−			
62	*Torulopsis stellata*	+	+	−				
45	*Torulopsis magnoliae*	+	+	+	+	−		
36	*Torulopsis inconspicua*	−	−					
46	*Torulopsis bacillaris*	+	+	−				
44	*Rhodotorula minuta*	−	−					
49	*Rhodotorula mucilaginosa*	+	−					
61	*Oospora lactis*	+	+	−				
	Number growing	35	27	5	4	3	3	1

yeast growth in an acid medium. Bell and Etchells (1952) reported that at pH 3.68 a much higher concentration of benzoic acid was necessary to prevent the growth of spoilage yeasts. Our work did not show such high tolerance. Some yeasts (e.g., *Rhodotorula minuta* and *Torulopsis inconspicua*) were unable to stand even traces of that chemical. Since most, however, grew with 200 ppm benzoic acid, that level should be considered insufficient.

Resistance to sulfur dioxide. Table 3 indicates that sulfur dioxide at 500 ppm prevented the growth of all isolates at 3.6 pH and 12–13% soluble solids in the medium. Both pH and soluble solids critically affect its efficiency (Cruess *et al.*, 1931) since the action is confined to the uncombined and dissociated portion of the inhibitory agent.

Resistance to sulfur dioxide varied considerably with genus, species, and strain. Generally speaking, the oxidative species (*Pichia membranaefaciens, Debaryomyces nicotianae, Sporobolomyces roseus, Rhodotorula* spp., *Oospora lactis*, etc.) were in-

Table 3. Tolerance of the isolates to SO_2 in 8 concentrations (ppm).

Isolate no.	Genus and species	50	100	150	200	300	400	500	600
6	*Hansenula subpelliculosa*	+	+	+	+	+	+	−	
13	*Hansenula subpelliculosa*	+	+	+	+	+	−		
14	*Hansenula subpelliculosa*	+	+	+	+	−			
24	*Hansenula subpelliculosa*	+	+	+	+	+	+	−	
26	*Hansenula subpelliculosa*	+	+	+	+	+	+	−	
35	*Hansenula subpelliculosa*	+	+	+	+	+	−		
50	*Hansenula subpelliculosa*	+	+	+	+	+	+	−	
51	*Hansenula subpelliculosa*	+	+	+	+	+	+	−	
42	*Hansenula anomala*	+	+	+	+	−			
10	*Hansenula anomala* var. *ciferrii*	+	+	−					
9	*Hansenula suaveolens*	+	+	+	+	−			
30	*Hansenula minuta*	+	+	+	+	+	+	−	
43	*Hansenula minuta*	+	+	+	+	+	+	+	−
28	*Pichia membranaefaciens*	+	+	−					
29	*Pichia membranaefaciens*	+	+	+	+	−			
48	*Pichia (Sacch.) pastori*	+	+	+	+	+	+	−	
40	*Debaryomyces nicotianae*	+	−						
57	*Sporobolomyces roseus*	+	−						
3	*Candida krusei*	+	+	−					
15	*Candida krusei*	+	+	+	+	−			
27	*Candida pulcherrima*	+	+	+	+	+	−		
34	*Candida pulcherrima*								
17	*Candida mycoderma*	+	+	+	+	−			
56	*Candida mycoderma*	+	+	−					
7	*Candida parapsilosis*	+	+	+	+	+	+	−	
21	*Candida melinii*	+	+	−					
63	*Candida melibiosi*	+	+	+	−				
41	*Torulopsis stellata*	+	−	−					
55	*Torulopsis stellata*	+	+	−	+	−			
59	*Torulopsis stellata*	+	+	+	−				
62	*Torulopsis stellata*	+	−	−					
45	*Torulopsis magnoliae*	+	+	−					
36	*Torulopsis inconspicua*	+	−	−					
46	*Torulopsis bacillaris*	+	+	+	+	−			
44	*Rhodotorula minuta*	+	+	−					
49	*Rhodotorula mucilaginosa*	+	−	−					
61	*Oospora lactis*	+	+	−					
	Number growing	37	31	22	21	13	10	1	0

hibited at 100 ppm. The fermentative species were more resistant. The question is whether the difference is due to the preservative *per se* or to the value of oxidation-reduction potential.

Pichia and *Hansenula* have been reported (Cruess *et al.*, 1931; Scheffer and Mrak, 1951) to be rather sensitive to SO_2. In our experiments some members of the genus *Hansenula* withstood 500 ppm. Of the five preservatives tested, sulfur dioxide was the only one that reacted partially with the reducing substances of the basal medium to give compounds of sulfonic acid (lacking preservative value). In our comparative evaluation, however, only the total SO_2 originally added to the substrate was considered.

Resistance to salicylic acid. Table 4 shows that all 37 cultures were completely inhibited by salicylic acid at 400 ppm. No strains with unusual resistance were encountered.

Von Schelborn (1953) reported that salicylic acid has high efficiency, with a strong tendency toward dissociation and specificity

Table 4. Tolerance of the isolates to salicylic acid in 9 concentrations (ppm).

Isolate no.	Genus and species	50	100	150	200	250	300	350	400	500
6	*Hansenula subpelliculosa*	+	+	+	+	+	+	−		
13	*Hansenula subpelliculosa*	+	+	+						
14	*Hansenula subpelliculosa*	+	+	+	−					
24	*Hansenula subpelliculosa*	+	+	+	+	+	−			
26	*Hansenula subpelliculosa*	+	+	+	−					
35	*Hansenula subpelliculosa*	+	+	+	+					
50	*Hansenula subpelliculosa*	+	+	+	−					
51	*Hansenula subpelliculosa*	+	+	−						
42	*Hansenula anomala*	+	+	+	+					
10	*Hansenula anomala* var. *ciferrii*	+	+	+	−					
9	*Hansenula suaveolens*	+	+	+	−					
30	*Hansenula minuta*	+	+	+	+					
43	*Hansenula minuta*	+	+	+	+	+	−			
28	*Pichia membranaefaciens*	+	+	+	+	+	+	+	−	
29	*Pichia membranaefaciens*	+	+	+	+	+	−			
48	*Pichia (Sacch.) pastori*	+	+	+	+	+	−			
40	*Debaryomyces nicotianae*	+	+	+	+	−				
57	*Sporobolomyces roseus*	+	+	−						
3	*Candida krusei*	+	+	+	+	+	+	+	+	−
15	*Candida krusei*	+	+	+	+	+	+	+	+	−
27	*Candida pulcherrima*	+	+	+	−					
34	*Candida pulcherrima*	+	+	+	−					
17	*Candida mycoderma*	+	+	+	+					
56	*Candida mycoderma*	+	+	+	+	+	−			
7	*Candida parapsilosis*	+	+	+	+	+	+	−		
21	*Candida melinii*	+	+	+	+	−				
63	*Candida melibiosi*	+	+	+	+	+	−			
41	*Torulopsis stellata*	+	+	−	−					
55	*Torulopsis stellata*	+	+	+	−					
59	*Torulopsis stellata*	+	+	+	−					
62	*Torulopsis stellata*	+	+	−						
45	*Torulopsis magnoliae*	+	+	+	+	−				
36	*Torulopsis inconspicua*	−	−	−	−	−				
46	*Torulopsis bacillaris*	+	+	+	+	−				
44	*Rhodotorula minuta*	+	−	−						
49	*Rhodotorula mucilaginosa*	+	+	+	+	−				
61	*Oospora lactis*	+	+	−						
	Number growing	36	35	30	20	11	5	3	2	0

against bacteria. Our data indicate that this chemical is an excellent yeast inhibitor in acid media. A concentration of 500 ppm should be sufficient under commercial practices. Resistance varied again with genus, species, and strain, but with no apparent relation to oxidative or fermentative faculty. The most sensitive and the most resistant were oxidative.

Resistance to formic acid. Table 5 shows the results for formic acid. Though this preservative is permitted in soft drinks in Greece and in fruit juices and confitures in Switzerland and Germany and other European countries (Toubeau, 1952), it was quite ineffective against yeast. Resistance against 1000 ppm was very common, and only 1600 ppm suppressed growth of the whole group.

Luck (1958) reported that formic acid is a relatively strong inhibitor of the heminenzymes catalase and peroxidase, which are not critically important to yeast growth. Possibly that is why it is inefficient against yeasts.

Isolate no.	Genus and species	100	200	300	400	600	800	1000	1250	1500	1600	1800
6	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	−			
13	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	+	+	−	
14	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	−			
24	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	+	−		
26	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	+	−		
35	*Hansenula subpelliculosa*	+	+	+	+	+	+	−				
50	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	+	−		
51	*Hansenula subpelliculosa*	+	+	+	+	+	+	+	+	−		
42	*Hansenula anomala*	+	+	+	+	+	−					
10	*Hansenula anomala* var. *ciferrii*	+	+	+	+	+	+	−				
9	*Hansenula suaveolens*	+	+	+	+	+	+	+	+	+	−	
30	*Hansenula minuta*	+	+	+	+	+	+	+	−			
43	*Hansenula minuta*	+	+	+	+	+	+	+	+	−		
28	*Pichia membranaefaciens*	+	+	+	+	+	+	−				
29	*Pichia membranaefaciens*	+	+	+	+	+	+	−				
48	*Pichia (Sacch.) pastori*	+	+	+	+	+	+	+	+	−		
40	*Debaryomyces nicotianae*	+	+	+	+	+	+	+	+	+		
57	*Sporobolomyces roseus*	+	+	−								
3	*Candida krusei*	+	+	+	+	+	+	+	+	+	+	−
15	*Candida krusei*	+	+	+	+	+	+	+	+	+	+	−
27	*Candida pulcherrima*	+	+	+	+	+	+	+	+	−		
34	*Candida Pulcherrima*	+	+	+	+	+	+	+	+	−		
17	*Candida mycoderma*	+	+	+	+	+	−					
56	*Candida mycoderma*	+	+	+	+	+	+	−				
7	*Candida parapsilosis*	+	+	+	+	+	+	+	−			
21	*Candida melinii*	+	+	+	+	+	+	−				
63	*Candida melibiosi*	+	+	+	+	+	+	+	+	+	−	
41	*Torulopsis stellata*	+	+	+	+	+	+	−				
55	*Torulopsis stellata*	+	+	+	+	+	−					
59	*Torulopsis stellata*	+	+	+	+	+	+	+	+	+	−	
62	*Torulopsis stellata*	+	+	+	+	−	−					
45	*Torulopsis magnoliae*	+	+	+	+	+	+	−				
36	*Torulopsis inconspicua*	+	+	−								
46	*Torulopsis bacillaris*	+	+	+	+	+	−					
44	*Rhodotorula minuta*	+	+	+	−							
49	*Rhodotorula mucilaginosa*	+	+	+	−	−						
61	*Oospora lactis*	+	+	+	+	+	−					
	Number growing	37	37	35	33	32	27	19	15	7	2	0

From our data and the volatility of formic acid, commercial practice would probably require about 2,000 ppm, which would impart a very objectionable odor.

Comparative evaluation. The data in the tables are the basis of Fig. 1, drawn by plotting the number of surviving cultures against the concentration of the preservative under test. These resistance curves, from their shape and their position with reference to the coordinate axes, appear to be nothing more than one-sided distribution curves. They are not typical of normal distribution, where the ordinate of the medium value delimits the area confined by the curve and the axis of abscissas.

In all cases the slope value attains its maximum at the beginning of the transection, and its minimum near the end. Thus, the majority of the cultures were halted in growth by small concentrations, with only a limited number withstanding higher concentrations. This is more evident with benzoic acid, where the slope value near the end of the curve transection approximates zero.

The formic acid curve, quite distant from those for the other chemicals, makes it difficult to include this substance among typical food preservatives.

The area confined by the axes of the coordinates and each inhibition curve, estimated with a planimeter, was, in sq cm, 71.50 for sulfur dioxide, 59.80 for salicylic acid, 71.60 for benzoic acid, 100.15 for sorbic acid, and 290.4 for formic acid. These values were considered to be a quantitative measure of the effectiveness of each in inhibiting the growth of spoilage yeasts in an acid medium since: 1) all the calculations were based on tests with a great number of yeast cultures of different species and genera; 2) conditions were kept constant (pH, soluble solids, prevention of loss, etc.); 3) the distance along the y-axis (the number of test cultures) was kept constant in test-

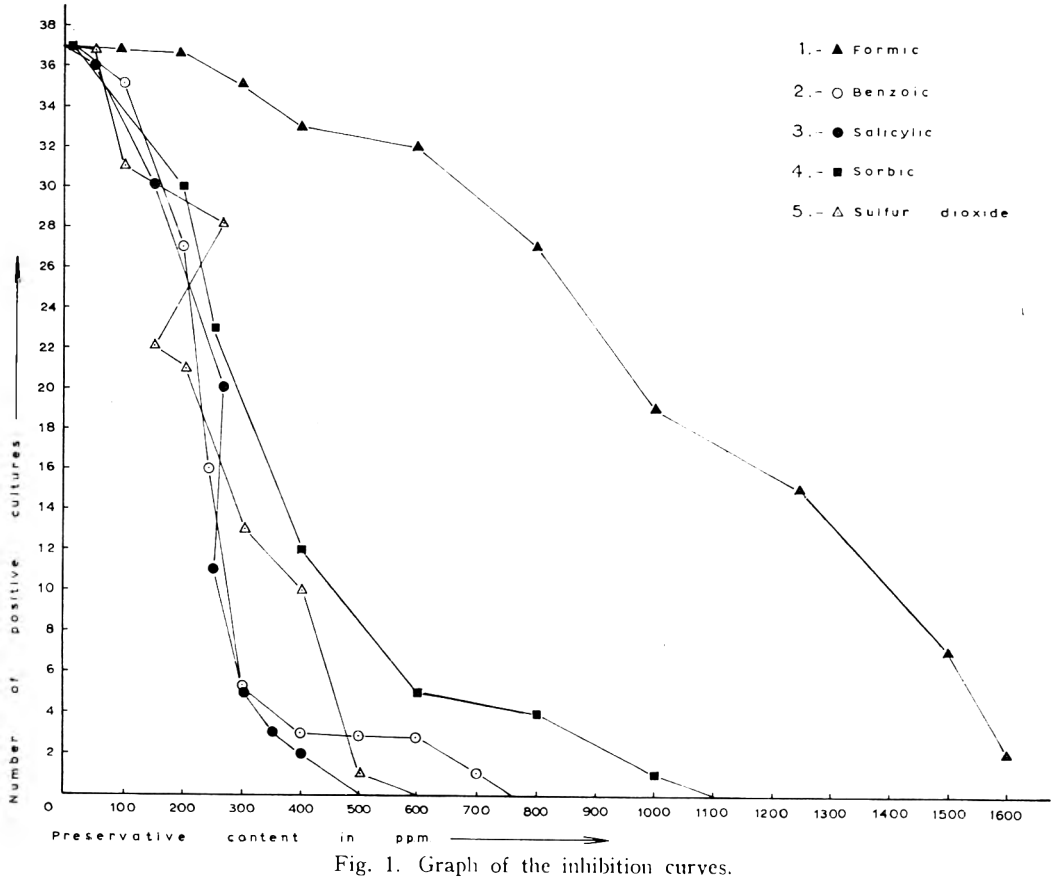

Fig. 1. Graph of the inhibition curves.

ing each preservative; 4) the variables that determine area—distance along the x-axis and the inhibition curve (positive test cultures in each concentration)—are functions of the inhibitory potency of each preservative. The milder the preservative, the higher are these two variables.

On the above basis, substances highly toxic to the cell (e.g., hydrogen cyanide) would give a point instead of an area. The rectangular coordinates of the two first points of the inhibition curve would be $A(0.37)$ and $B(dx.0)$, where 37 and dx respectively represent the number of the positive test cultures and the infinitesimal doses of the highly toxic substance. Consequently, the second point will coincide with the origin of the coordinate axes.

The area confined by the axes and the curve could have values from zero to infinity, ranging from great strength to no effect. In the latter case the inhibition curve would be a straight line parallel to the x-axis, showing the substance to be inert or a normal metabolite of the microbial cell. That would happen only when it had no effect on pH, osmotic pressure, oxidation-reduction potential, or other variables of the substrate.

On the basis of the above reasoning, the ranking of the test chemicals in effectiveness against spoilage yeasts was: salicylic acid; benzoic or sulfurous acid; sorbic acid; and formic acid.

Planimeter values represent absolute efficacy. Relative efficacy is calculated by assigning unity to a reference preservative and determining the ratios of each absolute value to that unity. These ratios were found to represent the amounts of each preservative that gave the same potency as the reference preservative.

Our comparisons, besides their immediate significance, suggest that this treatment of data offers a new method of comparing old preservatives and evaluating new ones.

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Toxin Production in Naturally Separated Liquid and Solid Components in Preparations of Heated Surface-Ripened Cheese Inoculated with Clostridium botulinum

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SUMMARY

Occasional solid-liquid separation of soft surface-ripened cheese packed into test tubes was examined for differences between the components with regard to growth and toxin production of *Clostridium botulinum*. Botulinum toxin was found in three tubes showing liquid-solid separation; in each case the toxin was present in the solid but absent from the liquid cheese portion. Two mechanisms were found to be responsible for the peculiar distribution of toxin in liquid-solid separated cheese: 1) the liquid portion possessed antimicrobial activity preventing the growth of *C. botulinum* whereas the solid component was antimicrobially inactive; 2) the particles in the solid cheese were able to adsorb botulinum toxin, thus preventing diffusion of preformed toxin into the liquid layer. Separation into a solid and liquid layer could be achieved deliberately by increase of the moisture content of the cheese preparation, or by mixing of thoroughly ripened cheese with relatively fresh cheese. However, in these tubes the toxin—when present—was distributed in essentially equal quantities in the adjacent solid and liquid components.

INTRODUCTION

This report describes some additional findings observed during a long-term investigation prompted by a rare occurrence of botulinum toxin in vacuum-packed cheese spread (Meyer and Eddie, 1951). This singular occurrence stimulated interest in a research program designed to study the many factors influencing growth and toxin production in cheese spreads experimentally inoculated with spores of *Clostridium botulinum* types A and B (Wagenaar and Dack, 1958). The experimental cheese preparations differed from commercial cheese spread in that no emulsifier was added, the moisture and salt content were varied to suit the experimental design, and the cheese was inoculated with heat-shocked spores of *C. botulinum* tubed in 30-g portions and stored under anaerobic conditions at 30°C.

When removed from storage and analyzed, many of the samples showed separation into a liquid and solid portion (Fig. 1). In earlier tests for botulinum toxin the entire sample was mixed thoroughly. Subsequently, however, the question arose as to whether the liquid and solid portions provided different conditions for microbial activity within a single sample. If this were so, there should be a difference in the amount of botulinum toxin in the liquid and solid portions. When tested, striking differences between the two components were found in the amount of botulinum toxin, thus forming the basis for this investigation.

MATERIALS AND METHODS

Cultural methods. A culture of type A *C. botulinum* strain 62A (from the collection of K. F. Meyer) was used for inoculation of experimental cheese preparations. The methods of preparing spores and maintaining stock cultures were described elsewhere (Grecz *et al.*, 1959a).

Cheese preparations. Cheese preparations were made as described by Wagenaar and Dack (1958) from type I cheese, a soft surface-ripened cheese the flora of which was made up of bacteria and yeasts. Approximately 10% water was added to the cheese to yield a NaCl-brine concentration

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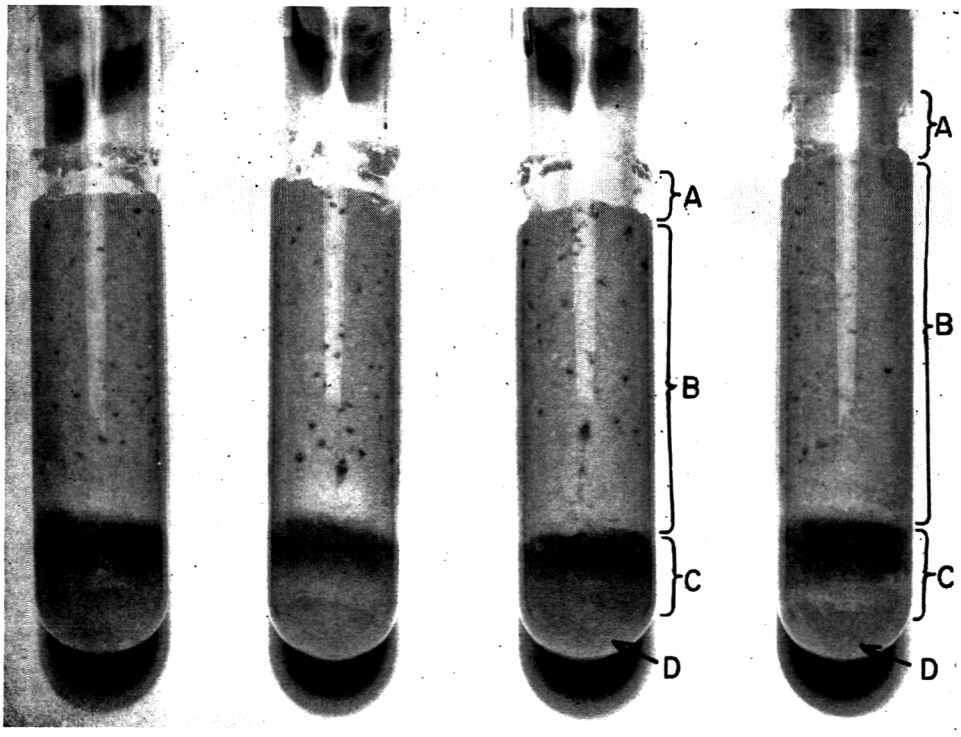

Fig. 1. Representative tubes showing separation of cheese into a liquid and solid portion. A) Marks left by rising cheese during repeated evacuation of anaerobic jar. B) Solid portion of cheese. Dark dots are pockets of liquid separated from cheese. C) Liquid portion. D) Fluffy sediment in the bottom of the tube.

favorable to growth of *C. botulinum* in the basic product. The cheese was heated at 70°C and stirred with a spoon and a motor-driven stirrer in order to obtain a homogeneous mixture. Two-hundred-gram portions were weighed into 400-ml beakers, and sufficient sodium chloride was added to some of the samples to yield a series having salt levels at 0.5% increments above the basic level of approximately 3%. The actual salt content of the samples considered in the present study is given in Table 2; these samples received no emulsifier, and the original pH of the cheese was not altered. However, they received 0.2% locust bean gum powder as stabilizer, a substance that is routinely added to many cheese products to prevent separation of brine water from the product. The addition of 0.2% locust bean gum had no effect on growth and toxin production of *C. botulinum* in experimental cheese preparations (Wagenaar and Dack, 1958).

To simulate commercial pasteurization the cheese was heated 10 min at 90°C and cooled, and sterile water was added to each beaker until its gross weight was the same as before the heat treatment. The samples were inoculated with heat-shocked spores of Type A *C. botulinum* 62A (100 spores/g) and mixed thoroughly. Heat shocking of spores (10 min at 85°C) was necessary to destroy any toxin and viable vegetative cells in the spore suspension as well as to activate the spores for germination. The inoculated cheese was weighed in 30-g portions into sterile 25 × 150-mm culture tubes and incubated anaerobically in desiccator jars at 30°C. Anaerobiosis was established by 25 g pyrogallic acid mixed with 25 g sodium carbonate and 100 ml H_2O, and evacuation of the atmosphere was done with Dack's anaerobic apparatus (Jordan and Burrows, 1945). Ten percent CO_2 was added to the jar before sealing. Samples were removed for toxin and pH analyses after 3, 7, 14, 30, and 60 days of incubation. Tubes showing liquid-solid separation were selected for study of the distribution of toxin in each component.

Separation of the components. *Liquid.* With a round wooden applicator, two holes were made in the solid cheese layer along opposite sides of the tube. A catheter was inserted into one of the holes, and the liquid was withdrawn with a 10-ml syringe. The second hole admitted air to replace the withdrawn liquid. Any contaminating solid cheese particles were removed by centrifugation.

In this manner, 2–5 ml of clear liquid could be collected from 30 g of cheese.

Butterfat. Only occasional tubes contained a distinct butterfat layer at the top of the cheese. When present, the butterfat was removed with a narrow stainless-steel spatula, cheese particles were scraped off, and the butterfat surface was rinsed with cold (2–4°C) buffer.

Cheese. The remaining solid cheese layer was uniformly mixed before determinations of toxin, pH, moisture, and salt.

Toxin assay. Samples were thoroughly triturated with two round wooden applicators in gelatin-phosphate composed of 0.2% gelatin, 0.73% $NaH_2PO_4 \cdot H_2O$, and 0.37% Na_2HPO_4, pH 6.8, after autoclaving for 20 min at 121°C (Naylor and Smith, 1946). The gelatin protein appears to minimize inactivation of toxin and to reduce nonspecific reactions in mice (Wentzel *et al.*, 1950; Boor *et al.*, 1955).

Samples of 0.5 and 1.0 ml of a 1:5 dilution of the cheese were injected intraperitoneally into duplicate 16–20-g mice. If death occurred within 4 days, the sample was re-examined with a test and a control mouse. In addition to the usual inoculum, the control mouse received 0.1 ml of specific botulinum antitoxin (Jensen-Salsbery Laboratories, Inc., Kansas City, Mo.).

The quantity of toxin was determined by intraperitoneal injection into duplicate mice of 0.4, 0.5 and 1.0 ml of the serial dilutions of the sample. The death of any one of the mice indicated a positive reaction when a similarly injected animal was protected with homologous antitoxin.

Assay of antibiotic activity. Aged surface-ripened cheese, particularly when stored over 8 weeks at 2–4°C, may contain an antibiotic principle related to *Brevibacterium linens* (Grecz *et al.*, 1959a, 1962). Antibiotic activity in cheese was assayed as follows. Samples of 0.25 ml of the clear liquid or 0.25 g of the cheese portion were filled into penicylinders (S & L Metal Products Corp., 25 Lafayette St., Brooklyn, N. Y.) placed on an agar sheet of uniform thickness seeded with 10^4 spores/ml of *C. botulinum* 62A. The assay plates were incubated at 30°C in an anaerobic incubator jar. To prevent excessive drying of the agar sheet, the atmosphere was kept saturated with moisture by keeping water in the bottom of the jar.

The size of the clear zone of growth inhibition around the penicylinder was taken as an index of antimicrobial activity.

Analytical methods. Moisture levels were determined by vacuum drying (Van Slyke and Price, 1952). The salt concentration was determined by titration with 0.1711*N* $AgNO_3$ solution using a 10% potassium chromate indicator as described in methods of analysis for the Butter Industry (American Butter Institute, 1937). The NaCl-brine concentration was calculated with the formula:

$$\% \text{ brine} = \frac{\% \text{ salt} \times 100}{\% \text{ moisture} + \% \text{ salt}}$$

The concept % brine was thought to represent the biologically effective sodium chloride concentration since it relates the sodium chloride concentration to the moisture content of the product. Thus, for example, a cheese containing 3% sodium chloride and 57% moisture would be said to have a 5% NaCl-brine. On the other hand, a cheese containing the same amount of sodium chloride, i.e., 3% but only 47% moisture, would have a NaCl-brine concentration as high as 6%.

Under experimental conditions, growth and toxin production by *C. botulinum* in type I cheese may occur at NaCl-brine concentration up to 8.0%; however, growth is best at 6.2% and lower. The usual brine concentration of type I cheese preparations falls within the range of 4.9 to 5.3% (Wagenaar and Dack, 1958; Grecz *et al.*, 1959b).

RESULTS AND DISCUSSION

Among 13,000 tubes of an experimental cheese preparation examined in this laboratory, a distinct liquid portion in the cheese was infrequent (approximately 2% of the tubes) and generally unpredictable. It seemed that, in the course of aging, cheese progressively acquired a more pronounced tendency to separate into liquid and solid phases. Separation was also promoted by increasing the moisture content of the cheese. Deliberate attempts to induce separation of liquid by adding aged cheese or by raising the moisture content of the cheese mixture met with uncertain success.

When the liquid and the solid portions of 24 tubes of cheese were separated and each portion analyzed, only three tubes contained detectable amounts of *C. botulinum* toxin. In each case essentially all of the toxin was present in the solid phase (Table 1, Group I).

When separation was experimentally induced either by addition of excessive amounts of water, e.g., Sample 4, Group II (Table 1), or by mixing various proportions of aged and fresh cheese, e.g., Samples 5 and 6, Group II (Table 1), there was either no toxin production or toxin was produced but was distributed among the portions in essentially equal quantities. Addition of excessive

Table 1. Distribution of botulinum toxin between liquid and solid components of cheese preparations.

	Sample no.	MLD of toxin in cheese phases		
		Liquid	Solid	Butterfat
Group I. Separation of liquid and solid *not* deliberately induced	1	<5	2,000	NT[a]
	2	<5	100	NT
	3	5	200	10
Group II. Separation of liquid and solid deliberately induced				
(a) by raising moisture to 70%	4	50,000	50,000	NT
(b) by mixing 14% of aged cheese (7 months) with fresh cheese (3 weeks)	5	20,000	50,000	NT
(c) by mixing 50% of aged cheese (7 months) with fresh cheese	6	100,000	100,000	200

[a] NT—not tested.
Specificity of botulinum toxin was ascertained by toxin-antitoxin neutralization tests.

amounts of water resulted in a milky-looking liquid portion with a gradual transition from the solid cheese to the liquid portion.

In two cases it was possible to remove an essentially pure butterfat layer from the top of the cheese (Sample 3 and 6, Table 1). The butterfat layer contained a relatively low level of toxin compared with that of the adjacent solid cheese layer, i.e., 0.2 and 5.0%.

Moisture and salt content. The liquid portion of samples showing natural separation had a higher moisture and salt content than the solid phase, but there was this interesting difference: The calculated NaCl-brine concentration was consistently higher in the solid component (Samples 1, 2 and 3, Group I, Table 2). The moisture and salt concentrations of samples in which the liquid-solid separation was experimentally induced behaved in a similar manner, although the NaCl-brine concentration varied irregularly (Samples 4, 5 and 6, Group II, Table 2).

pH changes. The differences in pH values of adjacent liquid-solid portions closely paralleled the differences in toxin levels of the respective portions (Table 3). The pH was lower in the solid component when levels of botulinum toxin were high. The decrease in pH in the solid containing the toxin was usually 0.20–0.35 pH units, although in one case (Sample 1, Table 3) the difference was as much as 1.03 pH unit.

These observations were in good agreement with earlier findings that *C. botulinum* activity in cheese was regularly accompanied by a decrease in pH (Grecz *et al.*, 1959a). The lower pH in the solid phase seemed to indicate that actual growth and toxin production by *C. botulinum* was restricted to the solid phase.

Table 2. Properties of the liquid and solid components of tubed cheese preparations.

	Sample no.	% moisture		% sodium chloride		% NaCl-brine concentration [a]	
		Solid	Liquid	Solid	Liquid	Solid	Liquid
Group I	1	58.05	82.54	4.6	5.6	7.34	6.35
	2	50.18	82.41	3.4	5.4	6.34	6.15
	3	52.26	81.92	4.1	5.7	7.20	6.50
Group II	4	69.21	83.04	4.4	5.1	5.98	5.82
	5	49.90	80.76	3.3	5.4	6.20	6.27
	6	55.76	82.68	4.1	6.2	6.68	6.98

[a] $\%\ \text{brine} = \dfrac{\%\ \text{salt} \times 100}{\%\ \text{moisture} + \%\ \text{salt}}$

Table 3. pH differences in the liquid and solid components of cheese preparations.

Group [a]	Sample	*C. botulinum* Inoculum (spores)	*C. botulinum* Toxin Solid	*C. botulinum* Toxin Liquid	pH Solid	pH Liquid	Differences in pH Solid-liquid	Differences in toxicity Solid-liquid
								MLD/g
I	1	+	+	−	5.82	6.85	−1.03	2,000
	2	+	+	−	5.85	6.15	−0.30	100
	3	+	+	−	6.65	7.00	−0.35	200
II	4	+	+	+	5.85	5.87	−0.02	0
	5	+	+	+	6.05	6.25	−0.20	30,000
	6	+	+	+	6.15	6.18	−0.03	0
III	7	+	−	−	6.26	6.32	−0.06	0
	8	+	−	−	5.90	5.90	0	0
	9	+	−	−	5.88	5.78	+0.10	0
IV	10	−	−	−	5.82	5.74	+0.08	0
	11	−	−	−	6.72	6.80	−0.08	0
	12	−	−	−	6.05	6.05	0	0

[a] Group I, toxic samples showing natural liquid-solid separation; Group II, toxic samples in which solid-liquid separation was experimentally induced; Group III, inoculated but non-toxic samples showing solid-liquid separation; Group IV, not inoculated, non-toxic samples showing solid-liquid separation.

Toxic samples containing equal amounts of toxin in the solid and liquid components (Samples 4 and 6, Table 3) also showed no essential differences in pH.

In addition to the samples that contained botulinum toxin, two groups (three samples each) of liquid-solid separated cheese containing no toxin are included in Table 3 for comparison (Groups III and IV). These samples showed essentially no differences in the pH values of adjacent liquid and solid portions.

Adsorption of toxin on cheese. Although the data suggested that botulinum toxin was formed in the solid component, it could not be explained why the toxin, once formed, did not diffuse into the liquid. Two possibilities were considered: 1) the toxin may have been "salted out" from the liquid portion, which contained high concentrations of salts and other water-soluble solutes, or 2) the toxin may have been adsorbed on the particles of cheese and thus retained in the solid component.

The former possibility could be eliminated by mixing highly concentrated (10,000 MLD) preformed botulinum toxin with the isolated liquid portion in any proportion. In these tests the liquid did not decrease the potency of the added botulinum toxin even after storage in the refrigerator overnight and repeated centrifugation. The results indicated that added toxin would not be precipitated from solution in the liquid cheese phase.

The possibility of adsorption of toxin on the cheese particles was tested in the following manner. One-gram quantities of cheese in which *C. botulinum* 62A had grown and produced 20,000 MLD/g toxin were suspended in 9 ml of sterile distilled water and in saline containing 5 or 10% NaCl. The cheese was triturated for 10 min with two wooden applicators. One ml of the homogeneous milky suspension was removed for determination of the initial level of toxin in the test solution. The remaining 9 ml were centrifuged for 15 min at 2500 rpm, yielding an apparently clear supernatant and a cheese pellet at the bottom of the tube. One ml from the top clear supernatant was taken for assay of the toxin remaining in the solution after centrifugation. The next 6 ml were removed with a syringe so as not to disturb the pellet, and discarded. The bottom 2 ml were mixed to produce a homogeneous suspension, and the amount of toxin in the cheese pellet was determined.

As shown in Table 4, the level of toxin in the cheese pellet was 20-fold higher when distilled water was used as a diluent, and 2-fold higher when 5 and 10% of NaCl solu-

Table 4. Distribution of botulinum toxin in a 1:10 suspension of toxic cheese preparations.[a]

H_2O-diluent (% NaCl)	Toxicity of 1:10 cheese suspension (MLD/ml)		
	Not centrifuged	Centrifuged	
		Supernatant	Cheese pellet
0	2,000	200	5,000
5	2,000	1,000	2,000
10	2,000	1,000	2,000

[a] The original cheese sample contained 20,000 MLD/g of botulinum toxin type A.

tions were used. This observation indicated that botulinum toxin formed in cheese had a tendency to be adsorbed on the cheese colloids. Such adsorption may have contributed to the localized accumulation of toxin in the solid portion of separated cheese.

Role of antibiotic in the liquid portion. The absence of toxin in the liquid portion as contrasted to the solid component of cheese samples of Group I (Table 1) was perplexing. The sodium chloride concentration, moisture content, and pH of the liquid appeared to be favorable for the growth of *C. botulinum*, and yet no toxin was found.

Two possibilities were considered in seeking to explain this situation: 1) botulinum toxin may have been formed in the liquid phase but subsequently removed into the solid by adsorption on the particles in the solid phase, and 2) the liquid phase may have contained substances inhibitory to growth and toxin production of *C. botulinum*.

The former possibility appeared to be precluded in view of the observation that high titers of botulinum toxin accumulated in the liquid of some of the samples in Group II (Table 1) where phase separation was deliberately induced.

Under ordinary conditions, liquid separation was noted only in well ripened cheese. This observation suggested that the antibiotic occurring in aged cheese (Grecz *et al.*, 1959a) may have accumulated in the liquid phase and prevented growth production of *C. botulinum*. Accumulation of the antibiotic in the liquid was in agreement with the high solubility of this antimicrobial agent in polar solvents, particularly in water (Grecz *et al.*, 1962).

The latter possibility was confirmed by assays of the antibiotic activity of liquid-solid separated cheese (Table 5). The presence of antibiotic activity in all cases appeared to preclude formation of botulinum toxin.

CONCLUSIONS

The possibility of island-like distribution of toxin was suspected in two reported food-poisoning cases involving botulinogenic cheese products of apparently uniform consistency (Anonymous, 1935; Meyer and Eddie, 1951). In neither case was definitive evidence available of island-like patches of toxin in the product. The present report for the first time demonstrates that uneven distribution of botulinum toxin may be achieved experimentally in a cheese product of non-uniform consistency.

The practical significance of uneven distribution of botulinum toxin in type I cheese is not yet fully clear, since the actual incidence of component separation in this product has not been studied. Furthermore, there

Table 5. Antimicrobial activity of cheese preparations showing solid-liquid separation.

	Component of cheese	Botulinum toxin	Clear zone of growth inhibition of *C. botulinum* 62A (mm)
Group I, Sample 1	Liquid	−	6.5
	Solid	+	0
Group II, Sample 5	Liquid	+	0
	Solid	+	0
Group III, Sample 7	Liquid	−	8.0
	Solid	−	1.5

is some doubt whether cheese that has separated into its component liquid and solid parts would be eaten by people. Nevertheless, the observation that botulinum toxin may be present in some parts of a food substrate while absent from other parts is of basic importance.

In connection with the subject raised in the present report, several further experimental studies suggest themselves. Thus, it would be important to know whether *C. botulinum* produced toxin in the cheese before or after separation into the solid and liquid components, i.e., whether component separation actually favored toxin production. Furthermore, under circumstances different from those described, the possibility of botulinum toxin synthesis in the liquid rather than in the solid portion should be seriously considered. The answers to these questions may have important public health implications.

Adsorption of botulinum toxin to colloidal cheese particles was considered only briefly in the present study. A further investigation, particularly quantitative measurements of adsorption in different food substrates and the effect of such adsorption on the stability of toxin, would be of interest.

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The Effect of Papain Preparations on Beef Skeletal Muscle Proteins[a]

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SUMMARY

The results reported here represent a preliminary study of the action of papain on the proteins of beef skeletal muscle. Suspensions of protein preparations which had been isolated from beef semitendinosus muscle were incubated with both crystalline papain and a commercial enzyme preparation. Analyses of ultrafiltrates of the enzyme digests indicated that all of the skeletal muscle protein fractions studied were affected by papain to some extent. Under the experimental conditions employed, the intracellular proteins were affected less by the commercial "meat tenderizer" than by crystalline papain. However, the effect of the two enzyme preparations on the connective tissue fractions was comparable. Papain appeared to affect mucoprotein and collagen more than the other skeletal muscle proteins. Incubation with papain markedly lowered the viscosity of the mucoprotein preparation. Collagen suspensions were converted to thick gels by the action of papain. These observations and the results of ultrafiltrate analyses suggest that the tenderizing effect of papain may be due at least in part to the breakdown of connective tissue.

INTRODUCTION

One of the more recent developments in meat tenderization is the application of proteolytic enzyme preparations to meat cuts before cooking. Still more recent is a patented process that involves ante-mortem injection of a proteolytic enzyme into the animal. Both treatments increase the tenderness of the meat. Although the biochemical changes associated with this desirable organoleptic effect have not been elucidated, there are indications that proteolytic changes occur (Robinson and Goeser, 1962). However, it has not been demonstrated conclusively whether all or only specific skeletal muscle proteins are attacked, or to what degree.

This study was initiated to obtain more information on the action of papain on the proteins of beef skeletal muscle.

EXPERIMENTAL METHODS

Isolation of muscle proteins. Semitendinosus muscle was obtained from two-year-old Hereford steers fed a control diet. Fat was trimmed from the muscles before grinding. The intracellular proteins were solubilized by extraction with phosphate buffer (pH 7.5) in 0.4*M* KCl [total ionic strength ($\Gamma/2$) = 0.55]. The protein extract was removed by filtration (McIntosh, 1961), leaving a residue of stromal protein, or connective tissue. The protein extract was fractionated to give the following components: actomyosin, myosin, globulin X, and myogen.

Actomyosin. The extract was diluted to an ionic strength of 0.225 (Weinberg and Rose, 1960). The precipitate that formed was obtained by centrifugation, suspended in water, and precipitated in three volumes ethanol. After standing overnight, the alcohol precipitate was obtained by centrifugation and dried over calcium chloride in a desiccator.

Myosin. The supernatant from the preceding treatment was further diluted to ionic strength 0.05 (Weinberg and Rose, 1960). The precipitate was obtained by centrifugation and purified by alcohol precipitation as described for actomyosin.

Globulin X. The resultant supernatant was dialyzed against distilled water for 24 hours in the cold. The precipitate that formed was obtained

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by centrifugation and purified as described for actomyosin.

Myogen. The remaining supernatant was precipitated in three volumes ethanol, obtained by centrifugation, and dried.

Connective tissue components. The connective-tissue components were isolated by various procedures from the stromal residue. Collagen was isolated by alkaline extraction (McIntosh, unpublished data) of stromal residue. Elastin was isolated manually with a forceps from acetone-treated stromal residue, dried, and partially pulverized. To remove traces of collagen and mucopolysaccharide, the elastin was purified essentially as described by Partridge *et al.* (1955). Mucoprotein was isolated from acetone-treated pulverized stromal residue by mild salt extraction (McIntosh, unpublished data).

Enzyme preparations. Crystalline papain (2×) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, which had been prepared as described by Kimmel and Smith (1954). These investigators reported a specific activity of 1.06 for this preparation after two recrystallizations, using α-benzoyl-L-argininamide as substrate. The commercial enzyme preparation (Adolph's Meat Tenderizer) was obtained on the retail market. This product contains "vegetable enzyme of the papaya melon" and is labeled "unseasoned—without spices."

Measurement of enzyme action. A sintered-glass ultrafilter has the ability to retain large protein molecules. This property has been utilized by a number of investigators (Ogston and Stanier, 1950; and others) as a tool for obtaining purified high-molecular-weight materials, free from small proteins. It has also been used as a criterion for protein degradation (Muir, 1958). Since it would be expected that proteolysis of skeletal muscle proteins would be accompanied by the formation of smaller fragments, it seemed that increased ultrafilterability of skeletal muscle fractions should serve as a valid measure of papain action.

Chemical determinations. Nitrogen was determined by micro-Kjeldahl. Hydroxyproline was determined colorimetrically, essentially as described by Neuman and Logan (1950). Hexosamine was determined colorimetrically as described by McIntosh (1961).

Viscosity. Viscosity measurements were made by measuring flow time of mucoprotein solutions in 0.02*M* phosphate buffer, pH 7, in an Ostwald pipette, at 25°C.

RESULTS AND DISCUSSION

Table 1 summarizes the results of a typical experiment in which 0.4% suspensions of the intracellular proteins were incubated with papain 0.02*M* phosphate buffer, pH 7, which contained 0.005*M* cysteine and 0.001*M* Versene (Kimmel and Smith, 1954). The papain concentration was 200 μg per ml. Also, suitable controls were run. The suspensions were incubated with benzoic acid at 45°C (Muir, 1958) for 20 hr. The results indicated that all of the intracellular proteins were affected to some extent, since 8–10% of the total nitrogen became ultrafilterable. The hexosamine-containing proteins, globulin X and myogen, showed over 30% of the original hexosamine as ultrafilterable hexosamine. Using suitable controls, suspensions of the same proteins were incubated with 0.5% concentration of the commercial enzyme preparation. There was a similar, though lower, increase in ultrafilterable nitrogen.

Table 1. Effect of papain preparations on ultrafilterable nitrogen of muscle proteins.

Muscle protein	Crystalline papain		Commercial enzyme preparation
	Nitrogen (% total)	Hexosamine (% total)	Nitrogen (% total)
Actomyosin	8.3		1.5
Myosin	10.2		1.0
Globulin X	8.8	33.0	.....
Myogen	8.3	38.0	1.0

The data in Table 2 summarize the results of incubating connective-tissue protein suspensions with papain (200 μg per ml). The ultrafiltrates of collagen and elastin suspensions were analyzed for both nitro-

Table 2. Effect of crystalline papain on ultrafilterable nitrogen, hexosamine, and hydroxyproline of connective-tissue constituents.

Connective tissue constituent	Nitrogen (% total)	Hydroxyproline (% total)	Hexosamine (% total)
Collagen			
Skeletal muscle prep.	5.7	43.0	
Commercial prep.[a]	13.8	11.4	
Elastin			
Skeletal muscle prep.	10.7	15.1	
Commercial prep.[b]	2.5	2.5	
Mucoprotein			
Skeletal muscle prep.			22.0

[a] Obtained from bovine Achilles tendon.
[b] Obtained from bovine ligamentum nuchae.

gen and hydroxyproline, since hydroxyproline is a unique constituent of these proteins. Since hexosamine is present in mucoprotein, the mucoprotein ultrafiltrates were analyzed for aminosugar content. The data indicate that papain attacks all three connective-tissue components. Results were similar with the commercial papain preparation (Table 3).

Table 3. Effect of a commercial papain preparation on ultrafilterable nitrogen and hydroxyproline of connective-tissue constituents.

Connective tissue constituent	Nitrogen (% total)	Hydroxyproline (% total)
Collagen		
Skeletal muscle prep.	15.1	22.4
Commercial prep.[a]	14.6	25.0
Elastin		
Commercial prep.[b]	1.0	3.3
Mucoprotein		
Skeletal muscle prep.	10.0	

[a] Obtained from bovine Achilles tendon.
[b] Obtained from bovine ligamentum nuchae.

During incubation with papain, the collagen suspensions became gelatinous. The other protein suspensions did not show gross visible changes. However, incubation of mucoprotein with crystalline papain resulted in a product that exhibited a 31% loss in viscosity when compared with a control sample incubated without papain. Incubation of mucoprotein with commercial papain under similar conditions resulted in a 26% loss in viscosity. Lessened viscosity is a recognized criterion for mucopolysaccharide degradation (Kent and Whitehouse, 1955) and is probably due to depolymerization (Kent and Whitehouse, 1955; p. 12). Thus, these observations serve to substantiate the appearance of nitrogen and hexosamine in the mucoprotein ultrafiltrate following enzyme treatment (Tables 2 and 3).

The results indicate that, using ultrafilterability as a criterion for proteolysis, all of the skeletal muscle protein fractions studied were acted upon by papain. These observations are in keeping with the concept that papain is a proteinase with a broad range of specificity. At the enzyme levels used here, the commercial preparation was not so effective on the intracellular proteins as was crystalline papain. The effect of the two preparations on the connective tissue constituents was comparable, however. Collagen and mucoprotein appeared similarly affected by the two preparations, whereas the commercial elastin preparation showed a lesser response.

Mucoprotein and collagen exhibited more over-all degradation by the papain preparations than the other skeletal muscle proteins. These results suggest that the tenderizing effect of papain may be attributed at least in part to the breakdown of connective tissue. However, ultrafilterability reflects only the more drastic changes. Other, more subtle (but still important) changes may occur in some of these proteins that also play an important role in the tenderization process. Thus, it seems likely that the intracellular proteins have also undergone significant alteration as a result of papain action, even though the degree of ultrafilterability was less pronounced.

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Effect of Linolenic, Linoleic, and Oleic Acids on Measuring Protein Extractability from Cod Skeletal Muscle with the Solubility Test

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SUMMARY

It has been assumed that the protein-solubility test is a measure of the amount of protein denatured *in situ* during frozen storage of fish muscle. This study shows, however, that the quantity of protein extracted from fresh cod muscle is reduced if C_{18} fatty acids are added to the extracting medium. The possibility is discussed that insolubilization occurs during extraction as a result of interaction between protein and fatty acid.

INTRODUCTION

Extractability in chilled 5% sodium chloride solution has been used by a number of investigators as a criterion for denaturation of fish-muscle proteins during frozen storage (Reay, 1933; Dyer *et al.*, 1950; Husaini and Alm, 1955; Love, 1958; Moorjani *et al.*, 1960; Sawant and Magar, 1961). Some of those investigators showed that for certain species of fish, the extent of denaturation as determined by extractability in salt solution parallels the development of toughness evaluated organoleptically (Husaini and Alm, 1955; Love, 1958; Moorjani *et al.*, 1960; Sawant and Magar, 1961). The assumption has been that denatured protein is not extracted under these conditions but that native protein is extracted. The protein that becomes inextractable is actomyosin (Dyer *et al.*, 1950).

Dyer and Fraser (1959), Bligh (1961), Olley and Lovern (1960), and Olley *et al.* (1962) found that the amount of non-esterified fatty acid that can be extracted from frozen stored cod muscle increases with progressive denaturation of the protein. When the solubility test is used to measure the amount of protein that can be extracted from frozen stored cod muscle, the non-esterified fatty acids present in the tissue as a result of degradation of lipid are dispersed in the homogenate along with the other constituents of the tissue. The reactivity of these fatty acids toward protein extracted by the salt solution will determine whether this soluble protein is extracted or insolubilized during the extraction process.

If the dispersed fatty acids are free to react with proteins during the extraction process, their presence in the solvent may be sufficient to cause denaturation of actomyosin during extraction. Insolubilization occurring during extraction would alter the amount of protein extracted in the solubility test, and the test would not correctly measure the amount of protein denatured *in situ*. Recent findings that small amounts of linoleic acid and linolenic acids rapidly insolubilize salt-extracted cod actomyosin (King *et al.*, 1962) suggest the possibility that protein and fatty acid interact during the extraction process.

The present study investigated the effect of C_{18} unsaturated fatty acids (linolenic, linoleic, and oleic) on the extractability of fresh cod muscle proteins. The study had two objectives: 1) to determine whether the presence of fatty acid affects protein extractability in the performance of the solubility test; and, if so, 2) to determine whether a reaction between fatty acid and protein is the cause of the inextractability.

FATTY ACID ADDED TO EXTRACTANT BEFORE EXTRACTION OF PROTEIN

In this, the first of two experiments, fatty acid was added to the extracting medium to show whether in the performance of the solubility test the presence of fatty acid affects protein extractability.

Materials. *Gadus morhua* (also called *G. callarias*, Cohen, 1959), caught not more than 24 hours previously, were obtained commercially. They had been gutted and stored in ice. The

midsections of the anterior portions of the fillets were used.

Linolenic, linoleic, and oleic acids obtained from Nutritional Biochemical Corp. were used immediately after the vials were opened. All inorganic reagents were analytical reagent grade.

The extractant contained 0.45*M* KCl + 0.0155*M* Na_2HPO_4 + 0.00338*M* KH_2PO_4 (Connell, 1958). The pH of 7.2 did not change after the addition of fish muscle and fatty acid in the quantities used. Although the extractant is not 5% with respect to its neutral salt content, its ion concentration falls within the range necessary to bring into solution any soluble protein present, including the fraction sensitive to denaturation (Ellis and Winchester, 1959).

Preparation of extracts. All of the preparative operations were carried out in a 4°C refrigerated room. Samples of cod muscle were blended with extractant in a Waring blender. A baffle technique (Dyer *et al.*, 1950) was used to reduce foaming. The blender was operated at reduced speed (about 7000 rpm) for six 10-sec periods, separated by 5-sec intervals to allow settling of undispersed material.

Addition of fatty acids to the extractant. Fifteen-gram samples of cod muscle were blended with 600 ml of extractant containing varying amounts (0.00 to 0.30 ml) of linolenic acid, measured volumetrically and added just before use.

Determination of extractable protein. Immediately after being blended, 75-ml samples of the suspensions were centrifuged for 20 min at 10,000 × G in a Spinco model L preparative ultracentrifuge with a type-21 rotor.

The protein nitrogen content of the centrifuged extracts was determined by a biuret method (Layne, 1957).

Fig. 1, curve 1, shows that the amount of protein extracted from fresh cod muscle decreased when increasing amounts of linolenic acid were added to the extractant. This result indicates that free fatty acids present in the extracting medium caused protein inextractability.

The amount of linolenic acid required to cause the observed insolubilization is probably much greater than would be necessary if the fatty acids were better dispersed. The effective concentration of the acid dispersed by the blending for 85 sec will be influenced by the size of the fatty acid micelle obtained, the solubility of the acid in the buffered salt solution, its degree of dissociation, and the fact that interaction with proteins is occurring during the dispersion process. C_{18} fatty acids would not be expected to dissolve and dissociate completely in pH 7.2 salt solution during the 85-sec blending process (Ralston, 1948). They would be expected to interact with the sarcoplasmic proteins in the muscle homogenate without resulting insolubilization (Putnam, 1948). This would reduce the amount of fatty acid available for interaction resulting in insolubilization.

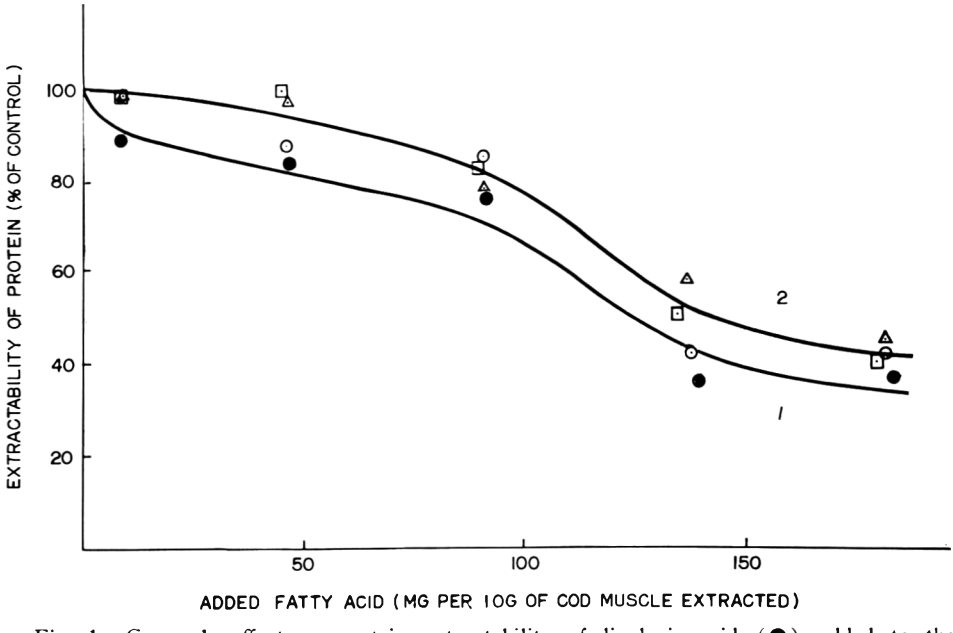

Fig. 1. Curve 1, effect on protein extractability of linolenic acid (●) added to the extractant. Curve 2, effect on protein extractability of linolenic (○), linoleic (△), oleic (□) acids added to muscle homogenates. Extractable protein in control samples containing no added fatty acid = 100%.

In applying the solubility test on frozen-stored cod muscle, the dispersion of fatty acid would be characteristic of that found naturally in biological material. Conceivably, the amounts found in such muscle, up to 300 mg/100 g muscle (Olley *et al.*, 1962), could insolubilize considerable protein. As stated previously, however, it is the reactivity of these fatty acids toward proteins that determines whether interaction takes place.

FATTY ACID ADDED AFTER HOMOGENATE FORMED

In this, the second of two experiments, fatty acid was added after the muscle had been homogenized with the extractant in order to determine whether the inextractability found in the first experiment was caused by a reaction between fatty acid and protein.

The same methods and material were used as in the first experiment except that the fatty acid was added to the muscle homogenate instead of to the extractant. Addition of fatty acid to the muscle homogenate was as follows:

A suspension was made by blending 20 g of cod muscle with 800 ml of extractant. This process was repeated three times. The suspensions were then pooled, mixed, and divided into 6 portions of 410 ml each. Varying amounts of linolenic acid (from 0.00 to 0.20 ml) were added to individual portions, which were again blended, this time for three 10-sec periods separated by 5-sec intervals. Other series were prepared and processed similarly after addition of linoleic or oleic acid.

Fig. 1, curve 2, shows that linolenic, linoleic, and oleic acids, homogenized into the muscle-extractant blend, insolubilized previously extracted protein.

DISCUSSION

These findings point out the need for a technique that unquestionably measures protein denatured *in situ*. They also indicate that more information is needed concerning the final forms taken by the fatty acids released as a result of degradation of lipid during the denaturation process. This information would throw light on the relation of lipid hydrolysis to loss of protein extractability. It would also help to resolve the question of whether the protein-solubility test is a true measure of protein denatured *in situ*.

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RESEARCH NOTE

A Chromatographic Comparison of the Polygalacturonases in Fungal Enzyme Mixtures

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In recent years, it has become clear that there are a number of distinct enzymes that act on pectic substances (Deuel and Stutz, 1958). In using commercial enzyme preparations, it would be of value to be able to determine readily what kinds of polygalacturonases are present. During investigations of polygalacturonases, we have collected a number of samples of commercial pectolytic enzyme mixtures, and have examined these chromatographically for their constituent enzymes. The chromatograms obtained show the presence of a variety of polygalacturonases in each commercial preparation and also striking differences between preparations.

The following enzyme preparations were kindly donated by the suppliers: Rohm and Haas Co., Pectinol 45 AP, Pectinol 46 AP (5.3× concentrate corresponding to Pectinol 10-M), Pectinol R-10 (14× concentrate), Cellulase 35 (1.3× concentrate), Lipase B; and Wallerstein Co., Pectinase 18. In addition, pectinase was purchased from the Nutritional Biochemicals Co. The products were partially purified by centrifuging out and discarding the water-insoluble matter, precipitating the colloid with two volumes of cold acetone, and ultrafiltration (McClendon and Somers, 1955). After this procedure the respective yields of the above enzymes were about 40, 35, 25, 45, 20, 4, and 7%. The polygalacturonase activities of the concentrated preparations were calculated from measurements on the original samples at pH 5.0 and fall in the neighborhood of 10 μmoles $\cdot$ min^{-1} $\cdot$ mg^{-1} except for Lipase B, which was about 0.2 μmole $\cdot$ min^{-1} $\cdot$ mg^{-1}. According to personal communications, the pectinols are derived from *Aspergillus niger*, and the Nutritional Biochemicals Co. pectinase is derived from *Rhizopus tritici*.

The chromatography was done by gradient pH elution from columns of cellulose phosphate (McClendon and Kreisher, 1963). The cup-plate assay of Dingle *et al.* (1953) was used to analyze the chromatographic fractions, using pectate as the substrate. Calibration with enzyme mixtures showed that the zone size was an approximately logarithmic function of the enzyme concentration, with a twofold difference in concentration, giving 2–3 mm difference in zone diameter after 24 hr, and 1–2 mm after 5 hr. This logarithmic response, as well as the difficulty of judging the position of the diffuse zone boundaries (especially at pH 3.5), made the assay rather imprecise. Citrate buffers (0.05*M*) were used at three different pH's in preparing the agar-pectate: pH 3.5, 4.3, and 6.5.

For convenience, the enzyme activity was plotted as a function of the pH of the effluent, the fractions being spaced about 0.05 pH units apart. The pH values were read off a graph constructed from measurements on every fifth tube.

Figs. 1–8 show the results. All preparations yielded curves with more than one peak, only one showing as few as two. These peaks have, for convenience, been numbered from I through VII according to the elution pH at which they were found.

Peak I was found only in Pectinol R-10, which was used by Albersheim *et al.* (1960)

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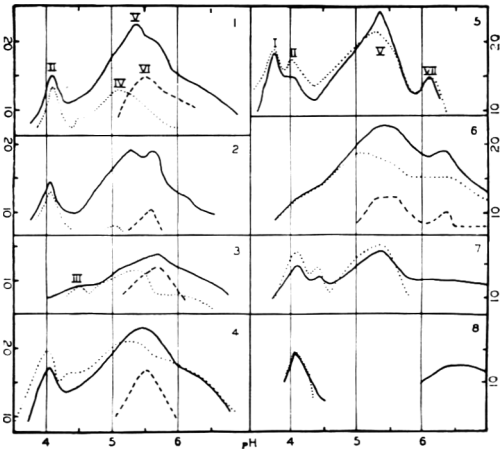

Figs. 1–8. Chromatograms of polygalacturonases. The vertical coordinate shows the zone diameter in mm (proportional to the logarithm of the enzyme concentration); the horizontal shows the pH of the eluted fractions. The solid line is the result of assay on pectate-agar at pH 4.3. The dotted line is the same at pH 3.5, and the dashed line at pH 6.5.

1) Nutritional Biochemicals "Pectinase," 30 mg, assayed for 22 hr.
2) Rohm and Haas "Pectinol 46 AP," 31 mg, assayed for 5 hr.
3) Rohm and Haas "Pectinol 45 AP," 32 mg, assayed for 5 hr at pH 4.3 and pH 6.5, assayed for 24 hr at pH 3.5.
4) Rohm and Haas "Pectinol 45 AP," 21 mg, "Pectinol 46 AP," 20 mg, assayed for 24 hr.
5) Rohm and Haas "Pectinol R-10," 36 mg, assayed for 24 hr. Assay at pH 6.5 was blank.
6) Rohm and Haas "Cellulase 35," 30 mg, assayed for 24 hr.
7) Wallerstein "Pectinase 18," 32 mg, assayed for 5 hr at pH 4.3 and 6.5, and for 24 hr at pH 3.5; pH 6.5 assay was blank.
8) Rohm and Haas "Lipase B," 102 mg, assayed for 22 hr; assay at pH 6.5 was blank.

and Albersheim and Killias (1962) as a source for pectin trans-eliminase. Since those authors stated that this enzyme does not act on pectate (used in our assay), the location of the eliminase was investigated. This enzyme was assayed with pectin-agar plates developed with 100% iso-propanol, and the peak position checked by spectrophotometric assay at 235 mμ. Only one peak was found in Pectinol R-10, and it corresponded closely to peak I on the agar-pectate plates. However, since the two assays yielded peaks that consistently differed in position by one or two fractions (the pectate activity coming off first), and since no evidence could be obtained with the ultraviolet assay for action of the eliminase on pectate, it was concluded that two enzymes were involved. Peak I was eluted at about pH 3.65, and the eliminase was eluted at about pH 3.75.

Peak II was eluted at pH 4.0–4.1 and is ascribed to exo-polygalacturonase on the basis of experiments on partially purified preparations from pectinase (McClendon and Kreisher, 1963). This peak appeared in all but Pectinol 45 AP and Cellulase 35. It was highly active on both the pH 3.5 and 4.3 assays, and inactive at pH 6.5. In the partially purified preparations the pH optimum was at pH 4.0.

Peak III, eluted at pH 4.4–4.5, is a minor component in Pectinol 45 AP (Fig. 3), Pectinase 18 (Fig. 7), and perhaps others, distinguished from II and IV by the elution position. When a mixture of 45 AP and 46 AP was run (Fig. 4), the peaks all appeared in the same places, indicating a lack of artifacts.

Peaks IV, V, and VI must be discussed together since they are the major polygalacturonases in all but one preparation and they also are eluted very close together. Peak VI can be readily distinguished since it is the only one of the major peaks that shows activity at pH 6.5. It is generally eluted between pH 5.5 and 5.7 in different preparations. In the Cellulase 35 (Fig. 6), it appears to be spread over a wider range, from 5.3 to 5.6, without a sharp peak. In N. B. Pectinase, Pectinol 46 AP, Pectinol 45 AP, and Cellulase 35 (Figs. 1, 2, 3, 4, 6), the peak of activity at an assay pH of 3.5 is not at the same place as that in the other two assays. This peak we are calling IV, at an elution pH of 5.0–5.4. In between IV and VI, in N. B. Pectinase and Pectinol 46 AP (Figs. 1, 2), there appears to be another peak of activity expressed at an assay pH of 4.3. We failed in an attempt to account for this peak as a sum of activities at pH 3.5 and 6.5, using suitable correction factors, necessitating the assumption of an independent enzyme or a synergistic effect of IV and VI. In Pectinol R-10 and Pectinase 18 (Figs. 5, 7) a peak appears at the elution position of V, without finding any VI. However, since V appear in Figs. 1 and 2 only in the pH 4.3 assay, and the peaks in Figs. 5 and 7 show at both pH 3.5 and

4.3, the latter may actually correspond to IV. Thus, the preparations tested contain at least two, and perhaps three, major polygalacturonases. Partially purified preparations from N. B. Pectinase containing peaks IV, V, and VI in varying amounts (but no I, II, III, or VII) reacted as endo-polygalacturonases with a pH optimum at pH 5.0 (McClendon and Kreisher, 1963).

Peak VII appeared as a separate peak in the weakest of the preparations tested, Lipase B. Other peaks eluted above pH 6 appeared as minor components on the tail of the major components in Pectinol R-10 and Cellulase 35 (Figs. 5, 6). It seems best to reserve judgment as to the uniqueness of Peak VII. In many instances not reported it has appeared that enzymes have been trapped in a matrix of insoluble or adsorbed matter of other sorts, not to be eluted until the latter is solubilized.

This demonstration of major qualitative differences between different commercial pectinase preparations should stimulate the use of chromatographic methods in the manufacture and use of these enzymes.

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Alteration of Post-Mortem Changes in Porcine Muscle by Preslaughter Heat Treatment and Diet Modification [a, b]

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SUMMARY

Subjecting animals to elevated environmental temperatures immediately before slaughter resulted in increased muscle temperature and a rapid rate of post-mortem glycolysis as indicated by a rapid pH decline and decreased color intensity. A high-fat high-protein low carbohydrate ration improved the water-binding, color, and texture characteristics of the muscle. The detrimental influence of sugar feeding was less marked than in previous experiments, but muscle from animals receiving the high sucrose ration had the lowest pH and (except for the heat-treated lot) the highest percent reflectance of all lots at 24 hr post-mortem. Phosphofructokinase activity was not affected by ante-mortem treatments and did not appear to be related to glycolytic rate or physical properties of the muscle at 24 hr post-mortem. The time course of rigor mortis was markedly accelerated by heat treatment.

INTRODUCTION

Glycogen content of porcine muscle can be elevated by feeding high levels of sucrose (Briskey *et al.*, 1959) or lowered by feeding a low carbohydrate ration (Briskey *et al.*, 1960). Callow (1937) found that the quantity of glycogen stored in muscle at the time of slaughter and the ultimate pH of the chilled muscle were influential in determining the color and juice-retaining properties of the muscle. More recently, Briskey and Wismer-Pedersen (1961) found that the rate of post-mortem pH decline was also very important in determining the ultimate physical characteristics of the muscle. An abrupt change to a cold environment prior to slaughter has been shown (Sayre *et al.*, 1961) to lower muscle glycogen levels at the time of slaughter and darken the color of the chilled muscle.

This investigation was made to study the effects of a readily available-carbohydrate and a low-carbohydrate ration as well as the effect of elevated environmental temperature on the rate of post-mortem glycolysis.

EXPERIMENTAL

Four pigs from each of the Hampshire, Chester White, and Poland China breeds were randomly assigned to each of four treatments giving 16 replicates per breed and 12 replicates per treatment. All animals received the experimental rations for a 10-day period prior to slaughter and were otherwise handled as described by Briskey *et al.* (1960). Lot I, the control, received a practical-type 14% protein basal ration; Lot II was fed a 14% protein, 50% sucrose ration; Lot III was given a 30% protein, 30% fat, low-carbohydrate ration (Briskey *et al.*, 1960); Lot IV was fed the same as Lot I, but was held in a controlled high-temperature chamber (Fig. 1) at 42–45°C for 1 hr immediately prior to slaughter.

A sample of the semitendinosus was removed immediately after exsanguination for measurement of elasticity and extensibility during the onset of rigor mortis. Samples were also taken from the longissimus dorsi for certain physical and enzymatic measurements. After evisceration the carcasses were allowed to cool at 3°C.

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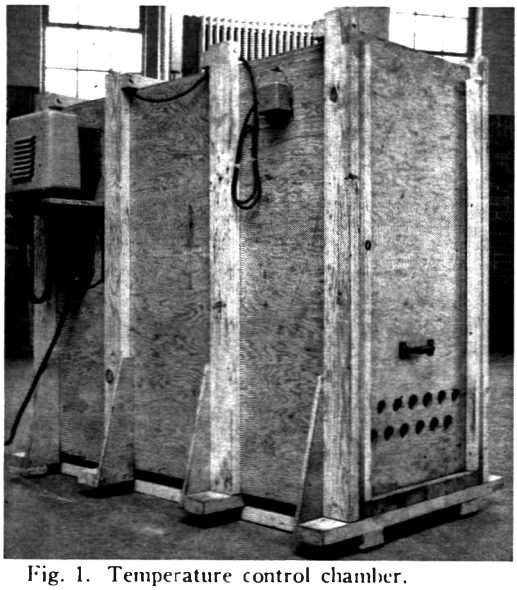

Fig. 1. Temperature control chamber.

Phosphofructokinase activity in the longissimus dorsi was determined according to the method of Ling *et al.* (1955). The fresh muscle was passed through a meat grinder in the cold room and a 20-g portion was homogenized for 1 min at 16,000 rpm with 60 ml of 0.03*M* KOH. Forty ml of 0.03*M* KOH were then mixed with the homogenate and allowed to stand for 1 hr in the cold room with occasional stirring. After the extraction period, 80 ml of water were added to make a 10% muscle homogenate, which was filtered through cheesecloth and a glass wool plug to obtain the final muscle extract. A 0.1-ml sample of the 10-fold diluted extract was used in the reaction mixture.

Buffer capacity of a 10% muscle homogenate was determined at 0 and 24 hr by adjusting the homogenate to pH 4.8 with 0.1*N* HCl followed by repeated additions of 0.5-ml portions of standard NaOH to a pH of 7.0 and noting the change in pH after each addition. Calculation of buffer capacity was based on the slope of a straight line through a plot of equivalents of NaOH versus pH between pH 5.2 and 6.5 and expressed as 10^{-5} equivalents per pH unit per gram of fresh tissue. pH values and percent reflectance were obtained at 0, 0.75, 2 and 24 hr as previously described for Sayre *et al.* (1963b). The temperature of the longissimus dorsi was determined at 0, 0.75, and 2 hr after death. Water-binding capacity as well as visual color and texture scores were determined at 24 hr post-mortem as previously described by Sayre *et al.* (1963b).

A strip of parallel fibers of 1 cm^2 cross section and 8 cm long was removed from the semitendinosus. The time course of rigor mortis was followed with the "rigorometer" of Briskey *et al.* (1962). The muscle strip was contained in a nitrogen-filled chamber at 37°C and a relative humidity of 100%. The times required for the delay, onset, and completion phases of rigor mortis were determined according to the terminology of Briskey *et al.* (1962).

RESULTS

Muscle phosphofructokinase activity was not affected by the treatments used in this experiment (Fig. 2). Also, there was no apparent association of this enzyme with glycolytic rate or ultimate pH.

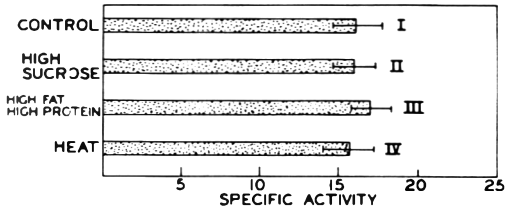

Fig. 2. Influence of treatment on phosphofructokinase activity in longissimus dorsi. |——| represents standard error of the mean.

Heat treatment of the animals in Lot IV elevated the temperature of the longissimus dorsi as shown in Table 1. The muscle temperature remained constant for more than 45 min after death and was not greatly decreased even at 2 hr post-mortem although the carcass was held at 3°C.

pH decline was much more rapid in Lot IV than in the other three lots (Fig. 3). However, at 2 hr, Lot IV was only slightly lower in pH than Lots II and III and after 24 hr the ultimate pH tended to be slightly higher in Lot IV than in the other lots. The pattern of pH decline was similar for Lots II and III, whereas in Lot I (control) the pH fell gradually and was ultimately the lowest of the four lots.

The rate of pH decline was reflected very closely in the rate of change in color intensity of the muscle (Fig. 4). Percent reflectance increased

Table 1. Effects of treatment on temperature (°C) of longissimus dorsi.

Time post-mortem	Treatment [a]			
	Lot I Control	Lot II High sucrose	Lot III High-fat high-protein	Lot IV Heat
0 hr	37.8	38.4	37.8	41.7
0.75 hr	37.2	38.4	37.8	41.7
2 hr	33.4	33.9	33.9	36.1

[a] Twelve pigs/treatment.

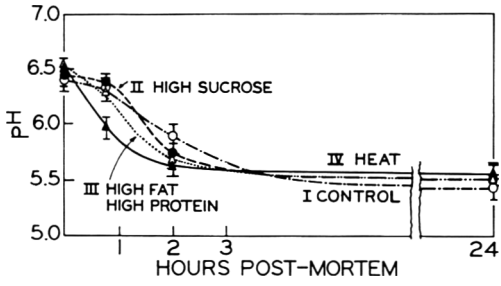

Fig. 3. Influence of treatment on rate of post-mortem pH decline in longissimus dorsi. |——| represents standard error of the mean.

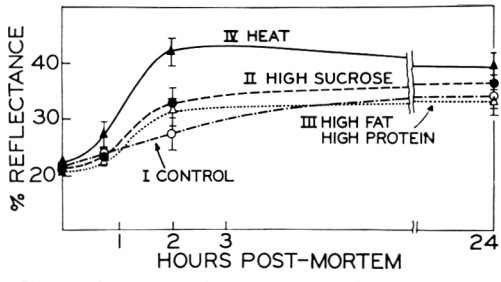

Fig. 4. Influence of treatment on the post-mortem rate of color change in longissimus dorsi. |——| represents standard error of the mean.

sharply during the first two hours post-mortem in Lot IV, and then declined slightly during the remainder of the 24-hr period. Conversely, the color in Lot I lightened slowly throughout the chilling period. Lots II and III were similar at 2 hr after death, but Lot II continued to lighten slightly whereas Lot III did not change appreciably in color after the 2-hr period.

Subjective color and texture scores presented in Table 2 were lowest in Lot IV, indicating a definite pale, soft condition of the muscle. Muscles from certain animals were extremely light in color and soft in texture. Color score, water-binding capacity, and texture score were superior in Lot III. Buffer capacity generally increased between 0 and 24 hr post-mortem; however, no marked differences were found between lots.

The time course of rigor mortis shown in Table 3 was similar for Lots I and III, although a somewhat longer onset phase occurred in Lot I. Lot II (high sucrose) had the longest delay phase, coupled with an onset phase shorter than in Lots I and III; thus, the total time required for completion of rigor was similar in the three lots. Heat treatment markedly shortened the time course of rigor mortis. The muscles of a few animals from these treatments exhibited no delay phase, but went directly into a short onset phase.

DISCUSSION

Phosphofructokinase catalyzes the conversion of fructose-6-phosphate to fructose-1,6-diphosphate. There is an accumulation of hexose monophosphates in resting muscle; however, muscular activity results in the rapid production of hexose diphosphate (Lardy, 1962). Mansour and Mansour (1962) found that phophofructokinase limits the rate of glycolysis in liver flukes by allowing a build-up of hexose monophosphates. They postulated that phosphofructokinase may exist in an active and inactive form. Thus, this enzyme may be implicated in the regulation of glycolytic rate.

The failure of preslaughter treatments to affect phosphofructokinase activity, as well as the lack of association between phosphofructokinase activity and rate of post-mortem

Table 2. Effect of treatment on some post-mortem characteristics of the longissimus dorsi.

	Treatment [a]							
	Lot I Control		Lot II High sucrose		Lot III High-fat high-protein		Lot IV Heat	
Characteristics	$\bar{X}$	$S_{\bar{x}}$	$\bar{X}$	$S_{\bar{x}}$	$\bar{X}$	$S_{\bar{x}}$	$\bar{X}$	$S_{\bar{x}}$
Expressible [b] water ratio 24 hr	3.2	±0.3	3.2	±0.2	2.8	±0.2	3.2	±0.3
Visual texture [c] score	2.7	±0.1	2.6	±0.1	2.8	±0.1	2.2	±0.3
Visual color [d] score	2.6	±0.2	2.6	±0.2	2.9	±0.2	2.2	±0.3
Buffer capacity,[e] 0 hr [f]	5.51	±0.24	4.65	±0.10	5.08	±0.31	5.00	±0.13
Buffer capacity,[e] 24 hr [f]	5.23	±0.20	5.37	±0.18	5.82	±0.32	5.58	±0.13

[a] Twelve pigs/treatment.
[b] Ratio of total area/muscle film area.
[c] Expressed on a four-point scale (1, very soft; 4, firm and dry).
[d] Expressed on a four-point scale (1, very light; 4, dark).
[e] Expressed as 10^{-5} equivalents/pH/g fresh tissue.
[f] Time post-mortem.

Table 3. Effects of treatment on the time course (min) of rigor mortis in the semitendinosus.

Rigor mortis [b]	Treatment [a]							
	Lot I Control		Lot II High sucrose		Lot III High-fat high-protein		Lot IV Heat	
	X	$S_{\bar{x}}$	X	$S_{\bar{x}}$	X	$S_{\bar{x}}$	X	$S_{\bar{x}}$
Delay phase [c]	71	±21	88	±13	75	±18	20	± 8
Onset phase [d]	125	± 9	74	±14	95	±17	49	± 9
Completion phase [e]	195	±44	161	±19	174	±24	65	±15

[a] 12 pigs/treatment.
[b] As described by Briskey *et al.* (1962).
[c] Period during which extensibility remained constant.
[d] Period of rapid decrease in extensibility.
[e] Total time to termination of the decrease in extensibility.

glycolysis, appear to discount this enzyme as a regulator of post-mortem glycolysis in porcine muscle. However, it must be kept in mind that the conditions of the enzyme assay may not have been the same as *in vivo* conditions. If active and inactive forms of the enzyme do exist, the relative proportion of these two forms may have been altered during extraction and assay procedures.

Sugar feeding resulted in a more rapid decline of pH and color intensity than in the control lot. High sucrose rations have been shown to extend the chain length of glycogen molecules (Sayre *et al.*, 1963a), which might result in slightly accelerated glycolysis. However, the water-binding capacity and subjective scores for texture and color were almost identical for animals fed the control rations and those fed the sucrose ration. This similarity can be explained by noting that the rate of pH and color change in Lot II was not greater than in Lot I until after 45 min post-mortem, and even then the glycolytic rate in Lot II was not exceptionally rapid. For maximal effect of pH on water-binding capacity, color, and texture, the low pH must be reached at an early stage before the temperature of the muscle has been greatly lowered.

The rate and amount of pH decline was not greatly different for muscle from animals receiving the high sucrose ration and those receiving the high-fat high-protein ration. However, as previously stated, this rate of pH decline was not exceptionally rapid. The rate of post-mortem color change was also similar for these two lots; however, the high-fat high-protein lot was darkest at each measurement. The muscle from the high-fat high-protein lot also had the highest water-binding capacity and exhibited the highest color and texture scores of all lots. These observations are in agreement with those of Briskey *et al.* (1960) showing that tendencies for decreased levels of muscle glycogen, increased water-binding properties, and higher muscle color scores result from feeding this high-fat high-protein ration.

Subjecting pigs to elevated environmental temperatures immediately prior to slaughter resulted in accelerated post-mortem glycolysis and generally inferior physical properties of the chilled muscle. This is particularly true with regard to water-binding capacities and subjective color and texture scores. Initial pH was approximately the same in the heat-treated lot as in the other three lots. This would indicate that lactic acid had not accumulated in the muscle at the time of slaughter as a result of the heat treatment. The greatly accelerated rate of pH decline in the heat-treated lot was probably the result of the elevated muscle temperature. This would be in agreement with results of Bate-Smith and Bendall (1949) and Wismer-Pedersen and Briskey (1961), who found that glycolytic rate was markedly influenced by temperature.

High environmental temperature did not have the same effect on all breeds used in this experiment even though the muscle temperature was the same in all breeds. Although the muscle of Hampshire and Poland China pigs became pale and watery, the muscle of Chester White pigs remained dark and dry ($P<0.05$). The pH of Chester White muscle did not fall below 5.8 at

24 hr post-mortem, and the percent reflectance remained low.

The above findings indicate that muscle glycogen as well as glycolytic intermediates were metabolically depleted prior to death in the Chester White pigs whereas these acid-forming substrates remained in the muscle of the other two breeds. Ludvigsen (1954) postulated that in some cases pale, soft, watery muscle in pigs results from the inability of the animal to adapt to stress conditions that confront it. He found that treatment of animals with adrenal cortical steroids prior to exercise allowed a greater amount of peripheral circulation, which could allow more extensive aerobic glycolysis and removal of lactic acid. If the Chester White breed possessed the inherent ability to adjust to heat by increasing peripheral circulation, then aerobic glycolysis might be increased in the muscle, allowing depletion of glycogen stores and resulting in reduced post-mortem anaerobic glycolysis.

The longissimus dorsi of some animals receiving the heat treatment was severely affected. In these cases the post-rigor muscle was white and dry, exhibiting a very loose, open structure that gave the appearance of having been cooked. An interesting phenomenon was noted in removing the entire longissimus dorsi muscle from the chilled carcass. After the connective tissue sheath covering the muscle was severed, it was found that there was no further attachment to the vertebra and the muscle could be easily pulled from the carcass. Apparently, the combination of high temperature under conditions of low pH resulted in breakdown of connective tissue attachments to the bone, allowing release of the muscle.

Differences observed in the time course of rigor mortis can be partially explained on the basis of work by Bate-Smith and Bendall (1949) and Lawrie (1953). They stated that the rate of glycolysis was governed by the rate of breakdown and resynthesis of ATP (adenosinetriphosphate). Following death, ATP levels were maintained by CP (creatine phosphate) breakdown and glycolysis until the CP had been depleted. Anaerobic glycolysis alone was insufficient to maintain the original level of ATP, so the concentration gradually dropped. When the ATP concentration reached 30–35% of its original level, the muscle went into the onset phase of rigor until the remainder of the ATP was utilized.

The relatively short delay and long onset phase noted in Lot I could be the result of the slow rate of glycolysis indicated by other observations previously mentioned. Assuming that factors other than ATP breakdown may influence glycolytic rate, a slow rate of glycolysis would allow the ATP level to fall rapidly, thus permitting the start of the onset phase. However, the continued slow resynthesis of ATP would prolong the onset phase until glycolysis ceased because of depletion of glycogen or pH inhibition. Conversely, the somewhat more rapid glycolysis in Lots II and III corresponds with the prolonged delay phase (noted particularly in Lot II) and the shortened onset phase. A rapid rate of glycolysis could maintain ATP at a higher level, thus prolonging the delay phase, but would also deplete the glycogen stores more rapidly, permitting a more rapid fall in ATP level, and consequently a shorter onset phase.

The accelerated rigor mortis in Lot IV is reflected in the very rapid rate of glycolysis that apparently results from high muscle temperature. The high muscle temperature, although instigating a rapid glycolytic rate, cannot account for the maintenance of the glycolytic rate in the muscle strips, since all the muscles were held at a constant 37°C throughout the extensibility measurements. According to Bate-Smith and Bendall (1949), the glycolytic rate is pH-dependent and decreases from physiological pH to about pH 6.5; from this value to about pH 5.6–5.7 the rate increases. If the high initial muscle temperature could cause a pH drop to pH 6.2–6.3 before cooling, the glycolytic rate might be maintained at a high level. However, this does not seem to be the complete answer for the rapid glycolysis in the muscle strips, since the muscles were removed from the carcass immedately after death and small strips were removed immediately for extensibility measurements. Thus, the effect of temperature after death was minimal. It was also apparent that rapid glycolysis and low pH were not nec-

essarily associated with inferior buffer capacity in the muscle. Additional factors controlling the rate of post-mortem glycolysis and rigor mortis, as affected by ante-mortem treatment, remain to be clarified.

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An Examination of the Free Amino Acids of the Common Onion (Allium cepa)[a]

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SUMMARY

Free amino acids in four onion cultivars were studied by two-dimensional ascending paper chromatography with the following solvents: 1) phenol-water, 8:2 (v/v), 2) *n*-butanol–acetic acid–water, 4:1:5 (v/v) (upper layer); and 1) *sec* butanol–*tert* butanol–methylethyl ketone–water, 4:4:8:5 (v/v), 2) n-butanol–acetic acid–water, 4:1:5 (v/v) (upper layer).

The amino compounds identified in the extracts were: glutathione, aspartic acid, cysteine, cystine, glutamic acid, serine, canavanine, asparagine, glycine, arginine, lysine, threonine, tyrosine, methionine sulfoxide, alanine, dihydroalliine, *S*-methyl cysteine, tryptophan, methionine, valine, phenylalanine, and mixed leucines. Suspected as present was α-L-glutamyl-*S*-[β-carboxy-*n*-propyl]-L-cysteinyl glycine.

Densitometry and colorimetry were used to determine the relative approximate amounts of alanine, aspartic acid, arginine, glutamic acid, glycine, leucines, lysine, methionine sulfoxide, phenylalanine, serine, *S*-methyl cysteine, threonine, tyrosine, and valine. The more abundant amino acids were: arginine, glutamic acid, phenylalanine, leucines, tyrosine, lysine, and methionine sulfoxide.

INTRODUCTION

The amino acid composition of many plants has been thoroughly investigated (Joslyn and Stepka, 1949; Rockland, 1959), but a survey of the recent literature indicates that virtually no data are available concerning the total composition of the free amino acids of *Allium cepa* L. Recently published papers on the pungent properties of onions (Niegish and Stahl, 1956) state that at least a portion of their flavor is related to the amino acid composition (Schwimmer and Weston, 1961).

It has been reported that *S*-propyl-cysteine sulfoxide (dihydroalliine) and *S*-allyl-cysteine (desoxoalliine) were present in the "cation fraction" of onion extract (Renis and Henze, 1958). Spare and Virtanen (1961) found that the crystalline precursor of the lachrymatory factor in the onion is an *S*-alkenyl-cysteine sulfoxide identified as *S*-propenyl-L-cysteine sulfoxide. Recently, a novel sulfur compound, cycloalliine, was isolated and characterized by Virtanen and Matikkala (1959a); those authors (Virtanen and Matikkala, 1960, 1962) also reported the presence of nine new peptides in onion. They characterized them as follows: 1. γ-L-glutamyl-L-valine, 2. γ-L-glutamyl-L-isoleucine, 3. γ-L-glutamyl-leucine, 4. γ-L-glutamyl-*S*-propenyl-L-cysteine sulfoxide, 5. ethyl ester of γ-glutamyl peptide no. 9 (possible artifact), 6. γ-L-glutamyl-L-methionine, 7. γ-L-glutamyl-*S*-methyl cysteine, 8. γ-L-glutamyl-L-phenylalanine, and 9. γ-L-glutamyl-*S*-[β-carboxy-*n*-propyl]-L-cysteinyl glycine.

Virtanen and Matikkala (1959b) and Carson and Wong (1961) have isolated both *S*-methyl-L-cysteine sulfoxide and *S*-propyl-L-cysteine sulfoxide from Finnish and American onions.

The present investigation reports data on free amino acids of four cultivars (varieties)

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of the common onion. Its prime purpose is to explore methods and to determine the uniformity of amino acid occurrence within this one species (*Allium cepa*). This study was undertaken as part of a long-term investigation of the taxonomy of the genus *Allium*.

EXPERIMENTAL

Samples. Mature onion bulbs of four cultivars of the common onion were studied: 'Southport White Globe'; 'Australian Flat White'; 'Asgrow Y-53'; and 'Asgrow W-45'.

'Southport White Globe' (SPWG) is a long-established cultivar and one of the most important dry-bulb onions of the northern United States. It has hard, white flesh and a pungent flavor.

'Australian Flat White' (AFW) is a small-bulbed, white, mild, soft-fleshed onion not well adapted to storage.

'Asgrow Y-53' and 'Asgrow W-45' are hybrid onions of recent introduction. Y-53 has large yellow bulbs and W-45 white bulbs of medium size. Both cultivars are of medium pungency and keeping quality.

Preparation of extracts. The techniques employed by Renis and Henze (1958) were followed with some modification.

Samples of 160–200 g of bulbs of each cultivar were cut into thin slices and boiled in 200 ml of distilled water for 3 min with constant stirring. Each sample was transferred to a Waring blender and homogenized for 2 min. The resulting paste was filtered with vacuum through cheesecloth on a Buchner funnel and rinsed three times with 50 ml distilled water. The filtrate was measured, and two volumes of absolute ethanol were added with continuous stirring. The liquid was refiltered, and the filter funnel rinsed three times with absolute ethanol. The combined filtrate and washings were evaporated to dryness in a rotary flash evaporator under reduced pressure (30 mm Hg) at 30°C. The residue was redissolved in distilled water and made up to volume such that 1 ml of the resulting solution represented the extract from 1 g of onion bulb (fresh-weight basis).

The amino acid fraction was separated on a cation-exchange resin (Amberlite IR-120 [H⁺]). The resin column was pretreated with 50 ml 2*N* HCl and washed with 80 ml distilled water. Then 50 ml of the extract were acidified with concentrated HCl to pH 2 and passed through the Amberlite column at about 0.5 ml per minute. A ninhydrin test (0.2% ninhydrin in *n*-butanol) was run to make certain that all of the amino acids were retained by the Amberlite. The column was treated with distilled water (approx. 200 ml) until the effluent showed a negative reaction for carbohydrates with the anthrone test (0.2% anthrone in concentrated H_2SO_4).

The amino acids were eluted from the column with 200 ml of 1*N* ammonium hydroxide. To be sure that no amino acids remained on the column, another ninhydrin test was run on the final portion of eluate. The total eluate was evaporated to dryness in a rotary flash evaporator (30 mm Hg; 30°C), and the residue was redissolved in distilled water and made up to 10 ml, so that 1 ml represented 5 g of fresh onion tissue.

This solution (solution A) was stored at 4°C under a thin layer of toluene.

Chromatography. Ascending one- and two-dimensional paper chromatograms were prepared from 1 mg/ml solutions of known amino acids. Whatman no. 1 filter paper of two sizes, 394×394 cm (15½×15½ inches) and 286×286 cm (11¼×11¼ inches), was used in covered glass cylindrical jars 457 cm high and 140 cm in diameter (18×5½ inches).

Buffered paper, which, according to McFarren (1951), should improve separation of some amino acids, did not improve our results with onions; and prewashed paper showed no advantages over unwashed paper (Thompson *et al.*, 1959). Resolution was not as good for one- as for two-dimensional chromatograms.

Samples of 0.125–60 µl were applied by a calibrated micropipette and dried from below by a blast of warm, dry air; the diameter of the spot in all cases was smaller than 6 mm.

Since the pH of the sample affects the resolution on the chromatogram, a pH of 11, achieved by addition of 1*N* ammonium hydroxide, was selected after considerable trial (McFarren, 1951).

Some 30 solvent systems were evaluated, and results were best with phenol-water, 8:2 (v/v) in the first dimension, and *n*-butanol–acetic acid–water, 4:1:5 (v/v) (organic layer), in the second dimension.

The chromatograms were run at 24.4–26.6°C in the first direction with 100 ml of solvent for 18–20 hr, by which time the solvent front had traveled about 30 cm from the origin. Chromatograms were dried 24 hr at room temperature and run 7–8 hr in the second direction with 100 ml of solvent at 24.4–26.6°C. The solvent front reached 25 cm.

For color development the following reagents were used: ninhydrin in acetone, 0.20%; ninhydrin in *n*-butanol, 0.20%; ninhydrin in *n*-butanol, 0.20% + 5% acetic acid (for buffered paper) (McFarren, 1951); isatin in acetone, 0.25%; isatin in acetone, 0.20% + 4% acetic acid (for buffered paper); platinic iodide (Block *et al.*, 1958); and sodium azide with iodine (Chargaff *et al.*, 1948).

The sequence most frequently used was: platinic iodide, ninhydrin, and isatin.

Identification of amino acids. The following criteria were used for identification: a) R_f of knowns on one-dimensional chromatogram; b) color of particular spots; c) special spray reagents for individual amino acids; and d) increased intensity of a spot of a known amino acid when co-chromatographed with the extract (Joslyn and Stepka, 1949).

Quantitative estimation of amino acids. The maximum-color-density method (Block *et al.*, 1958) with a Photovolt densitometer was used in quantitative determination of the following amino acids: aspartic acid, glutamic acid, threonine, alanine, tyrosine, *S*-methyl-cysteine, phenylalanine, and "leucines" (leucine and isoleucine overlap on the chromatogram) (Snell and Snell, 1954).

The standard curves of known amino acids were obtained by running two-dimensional chromatograms in duplicate. Standard solutions in amounts of 1–20 μl were chromatographed, and the concentration was plotted against optical density. Straight lines were obtained for all the amino acids except *S*-methyl-cysteine and isoleucine. These showed decreased optical density at higher concentrations.

Unknowns were run in duplicate under identical conditions on two-dimensional chromatograms. The amount of sample varied from 10 to 20 μl. Optical density was measured with a 540-mμ filter, and a spotless zone set at 100% was used as a blank.

In all chromatograms a large and concentrated spot appeared, ranging from $R_f = 0.45$ to $R_f = 0.85$ (phenol-water, 8:2, v/v) (Fig. 1). From R_f values obtained with small amounts of samples, lysine and arginine were suspected to be present in this area. The presence of arginine was confirmed by the Sakaguchi reaction. The presence of methionine sulfoxide was partially confirmed by the platinic iodide reaction.

To investigate other components, several two-dimensional chromatograms were run under identical conditions with 30–40 μl of onion extract. One was used as a marker and treated with ninhydrin. The others were cut following the contour obtained from the marker, and the papers containing the unknowns were placed in a test tube and eluted with distilled water, and the eluate was filtered, evaporated under vacuum, and redissolved in 1 ml of distilled water. This solution (solution B) was used to run additional chromatograms with different solvents.

Resolution of solution B was best with *sec*-butanol–*tert*-butanol–methylethyl ketone–water, 4:4:8:5 (v/v) in the first dimension, and *n*-butanol-acetic acid-water, 4:1:5 (v/v) (upper layer), in the second dimension. With this solvent system, it was possible to detect the presence of arginine, lysine, and, tentatively, methionine sulfoxide, and to establish the absence of histidine.

Quantitative determination of arginine. The Sakaguchi reaction was used for determining arginine (Snell and Snell, 1954): 50 μl of solution B were pipetted into a Klett-Summerson colorimeter tube, and 2 drops of 15% NaOH plus 2 drops of 3% α-naphthol solution were added. The solution was agitated for 1 min. Then 5 drops of 0.3*N* NaOCl were added, followed by agitation for 1 min. Finally, 3 ml of water were added. After 5 min of refrigeration, the color developed was measured. The same procedure was carried out with standard solutions of arginine. A 540-mμ filter was used.

Estimation of the sum of lysine and methionine sulfoxide. The sum of lysine and methionine sulfoxide was determined by difference between the total amount of amino acids in solution B and the amount of arginine.

The following steps were carried out:

a) Separate colorimetric measurements were made of color developed by aliquots of standard solutions of arginine, lysine and ninhydrin (Block *et al.*, 1958).

b) Colorimetric measurement was made of color developed by a mixture of aliquots of standard solutions of the same amino acids with ninhydrin (Block *et al.*, 1958).

c) Colorimetric measurement was made of color developed by aliquots of solution B for each sample of onion.

d) With these data, it is possible to calculate the percentage due to each amino acid according to the color developed with ninhydrin by the mixture of standards.

Estimation of other amino acids. As has been pointed out (Snell and Snell, 1954), each amino acid develops a different degree of color per mole of amino acid when reacted with ninhydrin reagent. Based on densitometric readings, estimates were made of the amounts of serine, glycine, and valine.

RESULTS AND DISCUSSION

Table 1 gives average R_f values of 47 known amino acids and one peptide. These values were obtained from replicate chromatograms (at least three chromatograms for each amino acid). A few values differ from those presented by Lederer and Lederer (1957) (somewhat lower values were found for arginine, asparagine, canavanine, cystine, hydroxyproline, and norleucine). The ones obtained in the system *n*-butanol-acetic acid-water, 4:1:5 (v/v), could not be fully compared, because the literature pro-

Table 1. R_f values of some amino acids.

Amino acid	Solvent Phenol-water, 8:2 (v/v)			Solvent *n*-butanol-acetic acid-water, 4:1:5 (upper layer) (v/v)		
	Kuon and Bernhard	Lederer	Renis & Henze	Kuon and Bernhard	Lederer	Renis & Henze
α-Alanine	0.59	0.585		0.20	0.33	
β-Alanine	0.65	0.654		0.27	0.32	
Alliine	0.76		0.77	0.31		
Allo-cystathionine	0.20			0.08		
Allo-hydrolysine	0.12			0.07		
α-Aminobutyric acid	0.68	0.69		0.30		
δ-Aminobutyric acid	0.76	0.751		0.33	0.39	
α-Amino isobutyric acid	0.73			0.35		
β-Amino isobutyric acid	0.74	0.757		0.35		
Arginine	0.52	0.56		0.05		
Asparagine	0.38	0.442		0.07		
Aspartic acid	0.20	0.179		0.16		
Canavanine	0.41	0.509		0.06		
Carnosine				0.08		
Cysteic acid	0.06	0.069	0.13	0.09		
Cysteine	0.20		0.16	0.07		
Cystine	0.18	0.30	0.18	0.06		
Djenkolic acid	0.22			0.10		
Dihydro-alliine	0.79		0.79	0.36		
Glutamic acid	0.33	0.311		0.21		
Glutamine	0.57			0.08		
Glutathione	0.12	0.11	0.44	0.10		
Glycine	0.39	0.385		0.14		
Histidine	0.65	0.643		0.09		
Homocysteine thiolactone	0.33		0.45	0.16		
Hydroxyproline	0.66	0.72		0.20		
Isoleucine	0.82	0.84		0.58		
Leucine	0.85	0.84		0.58	0.68	
Lysine	0.51			0.03		
Mesolanthionine	0.11			0.05		
Mesohomocystine	0.20			0.20		
Methionine	0.79	0.80	0.75	0.48		
Methionine sulfone	0.59	0.60	0.81	0.20		
Methionine sulfoxide	0.78	0.79	0.78	0.19		
Methylhistidine	0.76			0.09		
Norleucine	0.78	0.88		0.56		
Norvaline	0.75	0.76		0.43		
Phenylalanine	0.83	0.836		0.49		
Proline	0.84	0.86		0.27		
Serine	0.35	0.36		0.13		
S-Methyl cysteine	0.72			0.34		
S-Methyl cysteine sulfoxide	0.58			0.18		
Thiolhistidine	0.22			0.16		
Threonine	0.49	0.492		0.20		
Tryptophan	0.77	0.802		0.53		
Tyrosine	0.59	0.665		0.31		
Valine	0.79	0.777		0.47	0.53	

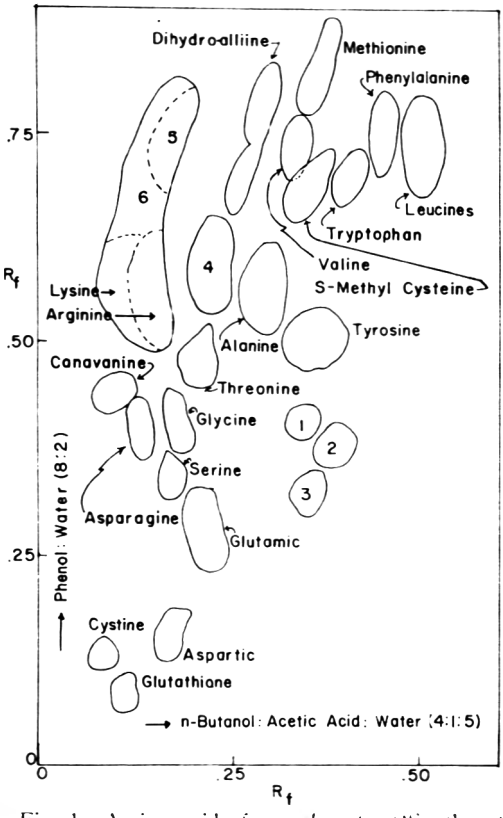

Fig. 1. Amino acids from *A. cepa* ('Southport White Globe'). A tracing of a two-dimensional chromatogram. Outlined areas were ninhydrin-positive.

vides very few R_f values for amino acids employing this solvent system.

Fig. 1 is a composite tracing of a typical two-dimensional chromatogram of the free amino acids obtained from Southport White Globe onion bulbs. For clarity, methionine and valine are indicated as being well-defined in this drawing, but Table 1 indicates that they have essentially the same R_f values, and in actual practice they do overlap considerably.

The sulfur-containing compound in spot 3 (Fig. 1) is suspected to be the peptide γ-L-glutamyl-*S*-[β-carboxy-*n*-propyl]-L-cysteinyl glycine (GCPCG), found by Virtanen and Matikkala (1960). Those authors reported that this compound had R_f values of 0.31 (butanol–acetic acid–water, 4:1:5) and 0.33 (phenol-water, 8:2), which agrees well with the R_f values for spot 3 (Fig. 1).

Spots 4 and 5 (Fig. 1) are also sulfur-containing compounds. Spot 4 has been tentatively identified as *S*-methyl-l-cysteine sulfoxide on the basis of R_f values reported by Shannon (1961), and spot 5 as methionine sulfoxide on the basis of the authors' experimental R_f values and spot tests (Table 1).

The identities of spots 1, 2, and 6 (Fig. 1) are at present unknown. Spots 1 and 2 were found only in sample SPWG. They reacted weakly with ninhydrin reagent and with platinic iodide reagent, indicating that they too contain some sulfur compound. Spot 6 reacts with ninhydrin reagent but not with platinic iodide reagent. It is present in all four samples.

It was consistently observed that dihydroalliine (*S*-propyl cysteine sulfoxide) gave two elongated and partially joined spots (Fig. 1) when chromatographed under the conditions described herein. Lysine always appears as an elongated spot adjacent to arginine.

Desoxoalliine (*S*-allyl cysteine), reported by Renis and Henze (1958), was not detected in this study; dihydroalliine (*S*-propyl cysteine sulfoxide), found in onion bulbs by Renis and Henze (1958), was detected in all four samples examined. The spot corresponding to the peptide GCPCG (Virtanen and Matikkala, 1960) was also detected in all four bulb samples.

The sodium nitroprusside test was positive for cystine in the SPWG sample and positive for both cystine and cysteine in samples Y-53, W-45, and AFW, indicating a possible oxidation of cysteine to cystine in sample SPWG.

Specific tests for proline and histidine failed to indicate their presence in any of the onion extracts.

Cysteine appears to be present in a larger amount and cystine in a lesser amount in Y-53 than in SPWG. Weak spots were noted for glutathione, tryptophan, valine, tyrosine, and methionine. Methionine sulfoxide appears to be present in relatively large amounts, indicating a partial oxidation of methionine in this sample.

Chromatograms of sample AFW revealed multiple small spots in the glutathione region, possibly due to the presence of cysteic acid.

For all onion samples (SPWG, Y-53,

Table 2. Estimated amino acid content (mg/100 g onion[a]) of Allium cepa.

Amino acid	Method of estimation	SPWG	Y-53	AFW	W-45
Alanine	Densitometry	1.13	0.93	1.85	1.35
Aspartic acid	Densitometry	1.05	1.60	1.93	0.63
Arginine	Colorimetry	16.33	20.68	16.41	25.07
Glutamic acid	Densitometry	3.65	2.95	4.83	3.13
Glycine	Molar deg. col.[b]	0.33	0.46	0.43	0.32
Leucines	Densitometry	3.50	3.93	3.25	3.30
Lysine	Colorimetry	9.61	9.44	12.95	10.50
Methionine sulfoxide	Colorimetry	1.57	1.49	2.05	1.63
Phenylalanine	Densitometry	3.50	2.40	3.50	2.75
Serine	Mol. deg. col.[b]	0.51	0.56	0.45	0.44
S-Methyl cysteine	Densitometry	0.93	1.15	1.30	1.93
Threonine	Densitometry	0.95	0.93	1.10	1.43
Tyrosine	Densitometry	3.20	1.93	4.30	2.80
Valine	Molar deg. col.[b]	1.00	0.62	0.92	1.07
Total		47.26	49.07	55.27	56.35

[a] Fresh-weight basis.
[b] Molar degree of color development.

W-45, AFW) the large spot on the upper left of the chromatograms (Fig. 1) (which includes lysine, arginine, methionine sulfoxide, and an unknown) showed a very similar configuration. In general, both the qualitative and quantitative aspects of each of the four samples are quite alike.

Table 2 shows the relative amounts of some of the amino acids in the four onion cultivars. The values are only approximate, however; several authors (Snell and Snell, 1954) have indicated that the three methods employed in this study have accuracies of only 60–80%.

From the results, it appears that taxonomic differentiation based on free amino acid composition does not appear to be feasible with the varieties examined. There is sufficient evidence (Shannon, 1961) that at least a number of species can be differentiated on the basis of amino acid content. This study has demonstrated that for the amino acids examined, the pattern within *A. cepa* is quite uniform from cultivar to cultivar.

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Autoxidation of Fish Oils. II. Changes in the Carbonyl Distribution of Autoxidizing Salmon Oils[a,b]

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SUMMARY

The carbonyl compounds of autoxidizing salmon oil were qualitatively and quantitatively analyzed at predetermined levels of oxidation. The 2-thiobarbituric acid (TBA) number, peroxide values, and total carbonyls were determined in addition to the individual volatile and Girard-T-isolable carbonyls. The C_1-C_{12} alkanals, C_6-C_{12} alk-2-enals, and the C_8-C_{10} alk-2,4-dienals composed the major part of the monocarbonyl fractions. The shorter-chain carbonyls were predominant, with methanal and propanal in the highest concentration. It appears that the Girard T reagent, as employed in the investigation, degrades the precursors of the volatile carbonyl fraction.

The hydroperoxides were readily destroyed in unpolymerized oil by the Girard T reaction conditions, but as polymerization proceeded the yield of hydroperoxides increased and that of the Girard-isolable monocarbonyls and malonaldehyde decreased. Polymerization of the salmon oil stabilized a large portion of the measurable hydroperoxides toward the degradative effects of the Girard and TBA reactions.

Carbonyl compounds have been implicated in the flavor deterioration of lipids (Lea and Swobodi, 1953) and in the rusting or browning reaction of fishery products (Nonaka, 1957). Work on the chemical definition of carbonyls in autoxidizing salmon oil has been carried out by Yu *et al.* (1961), and a wealth of information has been accumulated for other lipids in recent years (Day and Lillard, 1960; Lillard and Day, 1961; Gaddis *et al.*, 1960, 1961a; Forss *et al.*, 1960a,b,c).

The carbonyl compounds have been classified as volatile, or free, and nonvolatile, or bound (Gaddis *et al.*, 1960). The volatile fraction has been defined for many lipids, and it is usually less than 3% of the total measurable carbonyls (Lillard and Day, 1961; Gaddis *et al.*, 1960). The volatile monocarbonyls consist largely of alkanals, alk-2-enals, alk-2,4-dienals, and traces of vinyl ketones (Stark and Forss, 1962), and if the lipid is thermally oxidized, methyl ketones may occur (Crossley *et al.*, 1962). The remainder of the volatile carbonyl fraction has been reported as dicarbonyls in salmon oil (Yu *et al.*, 1961) and in lard (Gaddis *et al.*, 1960), but chemical definition of the mixture has not been reported.

Gaddis *et al.* (1960) distinguished between volatile and free carbonyls by finding that a portion of the free carbonyls were not accountable in the volatile fraction. The free carbonyls were measured by the Pool and Klose (1951) procedure. Recent work indicates, however, that their interpretation may be incorrect because of inherent errors in the original Pool and Klose method. Keith and Day (in press) modified the procedure to enable measurement of the major monocarbonyl classes, thus eliminating the quantitative error in the original method. They found that the volatile monocarbonyl fraction in milk fat was comparable to the free monocarbonyl fraction measured by the modified Pool and Klose procedure.

The nonvolatile (bound) carbonyl fraction has not been chemically elucidated, but it may be defined as that portion of the

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total measurable carbonyls that is not distillable or detectable by the Pool and Klose procedure. The fraction appears to be largely derived from the degradation of precursors by the reagent used in measuring it. Gaddis *et al.* (1960) attempted to define the fraction more clearly by using the Girard T reagent. They employed a procedure that caused degradation of the carbonyl precursors, and the carbonyls were isolated as the water-soluble hydrazones. They found that the volatile portion of the Girard-T-isolable compounds was at least superficially similar to the free volatile carbonyls, but they also found nonvolatile monocarbonyls and dicarbonyls.

Both the volatile and bound carbonyl fractions of salmon oil are of practical significance. The volatile fraction appears to be largely responsible for the rancid odor (Yu *et al.*, 1961), and, from available evidence, it is logical to assume that the "so-called" nonvolatile fraction, which exists as carbonyl precursors, is the origin of the volatile fraction. Both fractions undoubtedly are implicated in the rusting of frozen salmon and the browning of fish meal. Since the extent to which the salmon oil or the oil-containing products are oxidized varies so widely, the work reported herein was designed to study changes in the carbonyl composition of the oil at different stages of oxidation. An attempt was made to extend the qualitative identification of the volatile carbonyl fraction reported on previously by Yu *et al.* (1961) and to characterize the nonvolatile fractions. Attention was also directed to ascertaining the effect of oxidation on both the total and relative quantities of the various carbonyl compounds in the two fractions.

METHODS

Solvents. Hexane, chloroform, benzene, and ethylene chloride were purified by the procedure of Schwartz and Parks (1961). Absolute ethanol was refluxed for 3 hr with 2,4-dinitrophenyl-(DNP)hydrazine and trichloracetic acid, followed by distillation. Equilibrated hexane was prepared by shaking hexane with excess acetonitrile, and the acetonitrile layer removed.

Column-chromatography packing. Analytical-grade Celite was dried 18 hr at 160°C. Celite 545 was dried 24 hr at 160°C. Seasorb 43 (Fisher Scientific) was activated for 48 hr at 400°C. The iodine adsorption number of the Seasorb was 58. The iodine adsorption number of the Seasorb was determined by weighing to the nearest 0.01 g a 4-g sample of Seasorb 43. One hundred ml of 0.0500*N* iodine in carbon tetrachloride was added, and the sample was shaken for 15 min. After settling for 5 min, 25 ml of the clear solution was pipetted into a flask containing 50 ml of 0.03*N* KI in 75% ethanol. The solution was titrated with standard 0.05*N* sodium thiosulfate (detailed procedure available from Food Machinery Corporation, New York, New York). The silicic acid–Celite 545 mixture was prepared by using five parts of 100-mesh silicic acid with one part Celite, and the mixture was dried 12 hr at 200°C.

Oxidation of salmon oil. Fresh salmon oil, edible grade, was oxidized by maintaining it under positive oxygen pressure in a sealed bottle at room temperature. The oil was shaken daily and analyzed periodically for peroxide value and 2-thiobarbituric acid (TBA) number. The fresh oil and four samples taken at predetermined levels of oxidation were subjected to carbonyl analysis.

Peroxide value. Peroxides, determined by the AOCS (1959) method, are expressed as mg of peroxide per kg of oil.

Thiobarbituric acid number. The procedure of Sinnhuber and Yu (1958) was used to measure the mg of malonaldehyde per kg of oil.

Total carbonyl values. Total saturated and unsaturated carbonyls were determined by the method of Henick *et al.* (1954).

Isolation of volatile carbonyl compounds. The volatile carbonyls were isolated by means of the reduced-pressure distillation apparatus of Day and Lillard (1960) except that 100-g samples were used and 100 ml of distillate were collected. The distillate was reacted with an equal volume of 5*N* HCl, saturated with DNP-hydrazine. The DNP-hydrazones of the monocarbonyls were isolated by extraction with hexane. The extracts were combined and the hexane removed under reduced pressure. The bis-DNP-hydrazones of the dicarbonyls were subsequently isolated from the reaction mixture by benzene extraction.

Isolation of water-soluble Girard T carbonyl derivatives. The Girard T reagent was used to isolate the carbonyls from salmon oil. Reaction conditions were selected to give the maximum yield of carbonyl derivatives. The procedure was essentially as outlined by Gaddis *et al.* (1960) except the reflux time was increased to 3 hr. One hundred grams of oil, 10 g of Girard T reagent, 2 g of Dowex 50 cation-exchange resin, and 200 ml of carbonyl-free ethanol were refluxed 3 hr. The mixture was cooled, 2 L of distilled

water were added, and the oil was removed by extraction with petroleum ether. Sodium chloride was added during the extraction step to help break the emulsion and to facilitate removal of final traces of oil. The water-soluble hydrazones were converted to DNP-hydrazones by reacting equal volumes of the aqueous solution and 5*N* HCl saturated with DNP-hydrazine. The reaction was allowed to proceed 12 hr at room temperature, after which the DNP-hydrazones were isolated by extraction with chloroform. Upon removal of the chloroform, the DNP-hydrazones of monocarbonyls were separated from bis-DNP-hydrazones by exhaustive extraction with hexane. The procedure as outlined gave an average yield of 57% of the estimated total carbonyls in unpolymerized salmon oil.

Quantitative analysis of DNP-hydrazones. The DNP-hydrazones were separated into classes with the Seasorb-Celite-ethylene chloride procedure of Schwartz *et al.* (1960). The quantity of DNP-hydrazones represented in each class was determined by measuring the absorbance of chloroform solutions at 358, 373, and 390 mμ for alkanals, alk-2-enals, and alk-2,4-dienals, respectively, and calculating the millimoles of carbonyl using molar absorptivity indices of 22,500, 27,500, and 37,500 for the respective classes.

Each of the DNP-hydrazone mixtures obtained from the class separation was chromatographed by the column-partition method of Corbin *et al.* (1960). The column was modified in that no water was used during preparation. The eluate from the column was collected in 10-ml fractions, and the absorbance of each fraction was measured at the wavelength of maximum absorption for the class of DNP-hydrazones being separated. The data, when plotted in the manner described by Corbin *et al.* (1960), depicted the typical chromatograms in which each peak represented a specific member of a DNP-hydrazone class. The area under each peak was determined with a planimeter, and these values were used to calculate the percentage of each compound in each carbonyl class. Since the total carbonyl concentration of each class had been determined in a previous step, the amount of each carbonyl was easily calculated from the percentage composition data.

Quantitative analysis of bis-DNP-hydrazones. Excess DNP-hydrazine present in the crude bis-DNP-hydrazone mixtures was removed by the cation-exchange procedure of Schwartz *et al.* (1962). The bis-DNP-hydrazones were separated on the silicic acid-Celite column of Wolfrom and Arsenault (1960). All of the chromatographic bands gave positive tests for the bis-DNP-hydrazones of vicinal dicarbonyls. The absorbance of each fraction, in chloroform, was measured at 393 mμ, and the moles of carbonyl were calculated using a molar absorptivity value of 4.3×10^4.

Qualitative analysis of DNP-hydrazones. Evidence to establish the identification of DNP-hydrazones was based on the following criteria. Class separation of the DNP-hydrazones as described by Schwartz *et al.* (1960) and confirmed in our laboratory was effective in separating the various hydrazone classes of the monocarbonyls. The chain length of compounds in each class was ascertained as described by Corbin *et al.* (1960) through preparation of reference chromatograms from authentic DNP-hydrazones. The chromatographically resolved compounds were checked by determining absorption maxima, and chain lengths were confirmed by comparing chromatographic behavior with authentic derivatives on paper chromatograms. Melting-point analyses were conducted on questionable derivatives.

RESULTS AND DISCUSSION

Table 1 shows gross changes in the salmon oil during autoxidation. The oil was checked periodically for the TBA number and peroxide values. Aliquots were taken for detailed analyses at the points indicated in the table.

The quantity of Girard-isolable monocarbonyls reached a maximum during the oxidation period and the subsequent decrease in yield was accompanied by visible evidence of polymerization of the oil. The percent Girard-isolable monocarbonyls of the total estimated carbonyls decrease from 89% in Sampling 3 to less than 4% in Sampling 5. Peroxide values for Samplings 1 and 5, after treatment of the oil by the Girard procedure, were 0 and 153.5, respectively. This means that in advanced stages of oxidation, polymerization appears to stabilize a substantial portion of the hydroperoxides towards the degradative effects of the Girard reaction. The decrease in yield of Girard-isolable monocarbonyls with the concomitant stabilization of hydroperoxides also suggests that hydroperoxide degradation effected by the Girard reaction accounts for a large portion of the carbonyls isolated by this procedure. This is true also for the Girard-isolable dicarbonyls even though the quantity in this fraction increased as oxidation proceeded. The maximum yield (3% of the total carbonyls) was obtained for Sampling 3.

Table 1. Gross changes in salmon oil during oxidation.

	Sample no.				
	1	2	3	4	5
Days' storage	0	16	31	66	163
TBA no.	8.35	350	1102	2024	1472
Peroxide value	4.0	62.6	171	224	358
Total carbonyls [a]	9.5	52.6	110.8	312.3	569.8
Volatile monocarbonyls [a]	0.096	0.176	0.562	1.463	2.057
Girard-isolable [a] monocarbonyls	4.456	3.820	98.670	77.888	19.209
Volatile dicarbonyls [a]	0.039	0.047	0.289	0.308	0.890
Girard-isolable [a] dicarbonyls	0.194	1.114	3.309	5.972	8.250

[a] m.M/kg of oil.

The above conclusions may be questioned because the quantity of Girard-isolable monocarbonyls is lower in Sampling 2 than in Sampling 1 while the peroxide value is much higher in Sampling 2. A possible cause for this discrepancy may be the poor recovery of the monocarbonyls at one of the several steps in the isolation procedure. The data in Table 3 show that methanal and propanal are the compounds accounting for the decrease in yield. These compounds, along with ethanal, are the most difficult to recover because of their limited solubility in the solvents employed.

The decrease in TBA number, while not simultaneous with the decrease in Girard-isolable monocarbonyl fraction, also appeared to be related to stabilization of hydroperoxides. Previous investigators (Day, 1960; Day and Lillard, 1960; Sinnhuber *et al.*, 1958; Patton and Kurtz, 1955) have reported that only a small percentage of the total measurable malonaldehyde exists free in oxidizing lipids, and Day (1960) has suggested that the remaining malonaldehyde results from degradation of hydroperoxides during the analysis.

Tarladgis and Watts (1960) observed that malonaldehyde production and the rate of oxygen absorption reached a maximum simultaneously in oxidizing fatty acids and that the rancid-odor intensity decreased concomitantly with the TBA number. In the present study, no attempt was made to evaluate odor intensity quantitatively. However, it appeared to increase throughout the oxidation period. The observed increase in volatile carbonyls (Table 1) and the reported high correlation between flavor intensity of oxidizing fat and volatile monocarbonyls (Lillard and Day, 1961) lends credence to this point.

Table 2 shows qualitative and quantitative composition of the volatile monocarbonyl fractions of the salmon oil. In earlier work, Yu *et al.* (1961) obtained evidence for tentative identification of a number of methyl ketones. The ketones were not found in the present work except as contributed by contaminated solvents. It is believed that the methyl ketones found by Yu *et al.* (1961) came from the same source. Compounds found in this investigation that were not reported by Yu *et al.* (1961) were the C_1, C_{10}, C_{11}, C_{12} alkanals; C_{12} alk-2-enal; C_6, C_8, C_9 alk-2,4-dienals.

Substantial amounts of volatile monocarbonyls were found in the fresh oil, and the total quantities continued to increase during autoxidation. Of possible significance was the observed coincidence of the TBA number maximum and the peak concentration for a large number of the alkanals. In fact, only hexanal continued to increase during oxidation, and if quantitative data were available for the C_1, C_2, C_3, and C_{12} alkanals of Sampling 4 (Table 2) it is probable that the total alkanal value would show a maximum at that point.

The volatile alkanal concentration increased at an early stage whereas no substantial increase was apparent in the unsaturated carbonyls until after the 16th day of oxidation. The large increase in the

Table 2. Volatile monocarbonyl compounds from autoxidizing salmon oil (m*M*/kg).

	Sample no.				
	1	2	3	4	5
Alkanals					
n-Dodecanal	.0029	.0017	.....	.....	.0126
n-Undecanal	.0023	.0022	.0058	.0709	.0194
n-Decanal	.0022	.0018	.0102	.0705	.0675
n-Nonanal	.....	.0019	.0058	.1292	.0606
n-Octanal	.0015	.0016	.0087	.1212	.0720
n-Heptanal	.0020	.0023	.0109	.0758	.0755
n-Hexanal	.0024	.0043	.0310	.2234	.2549
n-Pentanal	.0025	.0097	.0358	.1794	.0269
n-Butanal	.....	.0036	.0375	.0698	.0274
n-Propanal	.0079	.0421	.1287	.....	.1875
n-Ethanal	.0168	.0062	.0621	.....	.1600
n-Methanal	.0111	.0720	.1335	.....	.0514
Total	.0516	.1494	.4700	.9400	1.0517
Alk-2-enals					
Dodec-2-enal	.0008	.0006	.0010	.0031	.0407
Undec-2-enal	.0011	.0006	.0008	.0041	.0101
Dec-2-enal	.0016	.0015	.0018	.0139	.0275
Non-2-enal	.0010	.0010	.0025	.0291	.0407
Oct-2-enal	.0012	.0013	.0019	.0272	.0473
Hept-2-enal	.0023	.0022	.0025	.0595	.0891
Hex-2-enal	.0024	.0030	.0205	.0724	.2463
Pent-2-enal	.0054	.0034	.0262	.0451	.2386
But-2-enal	.....	.....	.0146	.....	.....
Total	.0159	.0135	.0717	.2543	.7412
Alk-2,4-dienals					
Dec-2,4-dienal	.0022	.0010	.0008	.0198	.0080
Non-2,4-dienal	.0012	.0004	.....	.0120	.0627
Oct-2,4-dienal	.0025	.0011	.0023	.0225	.0458
Hept-2,4-dienal	.0227	.0102	.0143	.1704	.1005
Hex-2,4-dienal	.....	.0009	.0029	.0441	.0471
Total	.0285	.0135	.0204	.2688	.2641

alkanals is accountable primarily by methanal and propanal, both of which could arise from the oxidation of terminal unsaturated fatty acids and the fatty acids with a double bond between the third and fourth carbons from the methyl end of the chain. These acids are very common in menhaden oils (Stoffel and Ahrens, 1960), and it is conceivable they would occur in salmon oil.

Table 3 shows qualitative and quantitative data for the Girard-isolable monocarbonyls. The qualitative composition of the various classes is comparable to that of the fraction isolated by distillation (Table 2). The Girard procedure was not as sensitive or consistent as the distillation procedure in detecting the compounds occurring at lower concentrations in the oil. For some unknown reason, some of the compounds were not detectable at consecutive sampling periods. This is especially noticeable for Sampling 4, in which the longer-chain members of each class were conspicuously absent. A recheck of the sample failed to reveal the missing compounds. To check this point further, a second sample of fresh salmon oil was oxidized to a comparable TBA number (2125) and the analysis of the Girard monocarbonyls again did not reveal the missing compounds.

When the individual monocarbonyls in the volatile fraction (Table 2) are compared with those of the Girard-isolable fraction (Table 3), in practically all cases the rela-

Table 3. Monocarbonyl compounds from autoxidizing salmon oil isolated with the Girard T reagent (mM/kg).

	Sample no.				
	1	2	3	4	5
Alkanals					
n-Dodecanal	.....	.....	.....	.....	.2883
n-Undecanal	.....	.....	.....	.....	.4580
n-Decanal	.....	.....	.3619	.....	1.2045
n-Nonanal	.....	.....	.4891	.....	.8991
n-Octanal	.....	.....	.4206	.....	1.0603
n-Heptanal	.....	.0328	.8706	.4669	1.0008
n-Hexanal	.1253	.1669	3.8345	1.7714	2.7226
n-Pentanal	.1473	.3086	6.5442	1.7714	.7548
n-Butanal	.....	.....	10.7700	11.9812	.6106
n-Propanal	1.8208	1.2328	30.5690	24.8687	2.4767
n-Ethanal	.2598	.7253	11.8360	9.4270	.6785
n-Methanal	2.0433	1.0537	32.1440	18.3666	2.0696
Total	4.3960	3.5200	97.8200	68.6600	14.2240
Alk-2-enals					
Dodec-2-enal	.....	.0065	.0056	.....	.0063
Undec-2-enal	.....	.0045	.0048	.....	.0208
Dec-2-enal	.....	.0076	.0286	.....	.0444
Non-2-enal	.....	.0056	.....	.....	.0679
Oct-2-enal	.....	.0140	.0413	.0755	.1160
Hept-2-enal	.....	.0118	.1255	.3186	.4804
Hex-2-enal	.....	.0101	.3440	1.7440	.4947
Pent-2-enal	.....	.0109	.0564	2.2247	.5808
But-2-enal	.....	.....	.0330	.2953	.0798
Total		.0710	.6391	4.6580	1.8912
Alk-2,4-dienals					
Dec-2,4-dienal	.0101	.0206	.0047	.....	.1234
Non-2,4-dienal	.....	.....	.0152	.1352	.1898
Oct-2,4-dienal	.0101	.0422	.0116	.5407	.1234
Hept-2,4-dienal	.0403	.1669	.1625	2.8585	2.2965
Hex-2,4-dienal	.....	.....	.0174	1.0356	.3606
Total	.0605	.2297	.2113	4.5700	3.0936

tive amounts in the carbonyl classes are comparable for both fractions. Apparently the conditions of the Girard reaction cause degradation of the volatile carbonyl precursors in the oil.

A portion of the ethanal shown in Table 3 may have resulted from dehydrogenation of the ethanol used in the Girard reaction, in the manner described by Gaddis *et al.* (1961b). The amount could not have been significant, however, because the ethanal concentration closely followed the trend of the other compounds in the mixture during oxidation of the oil.

Methanal and propanal formed a large percentage of the alkanal fraction of the Girard carbonyls, and, again, the most feasible origin of these compounds would be as mentioned for the volatile fractions. An additional mechanism that may help account for methanal has been described by Bell *et al.* (1951), in which decomposition of the primary alkoxy radical could give rise to methanal and the alkyl radical:

$$R - CH_2O\bullet \rightarrow R\bullet + CH_2O$$

Relatively large amounts of methanal were also reported in oxidizing milk fats by Lillard and Day (1961). The decrease in methanal and propanal may be associated with the destruction of the fatty acid precursors. Similarly, the drop in the alk-2-enals and alk-2,4-dienals in advanced stages of oxidation is undoubtedly related to oxi-

Table 4. Volatile dicarbonyl compounds from autoxidizing salmon oil (m*M*/kg).

Fraction	Sample no.				
	1	2	3	4	5
1	.0019	.0150	.0742	.0213	.2211
2	.0345	.0065	.0550	.1455	.1982
3	.0027	.0098	.0631	.0923	.0734
4	.....	.0164	.0966	.0491	.3968

Table 5. Dicarbonyl compounds from autoxidizing salmon oil isolated with Girard T reagent (m*M*/kg).

Fraction	Sample no.				
	1	2	3	4	5
1	.0542	.4307	.6341	.7991	4.7725
2	.0511	.2193	1.000	1.2045	1.9545
3	.0890	.4636	1.059	1.3229	1.5225
4	.....	.....	.6159	1.7729	.....
5	.....	.....	.....	.8727	.....

dative destruction of the aldehydes and the polyunsaturated fatty acids.

The quantitative data for the bis-DNP-hydrazones are presented in Tables 4 and 5. Positive identification of these fractions is not complete. Recent work in our laboratories has indicated that the Wolfrom and Arsenault (1960) procedure does not give complete separation of mixtures of dicarbonyl derivatives such as isolated from oxidizing fats and oils. The percentage of volatile dicarbonyls in the volatile carbonyl fraction ranged from 17 to 35%. There was not a consistent trend in changes of the relative amounts of individual fractions in either the volatile or Girard-isolable dicarbonyls during oxidation of the salmon oil.

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Role of Individual Phospholipids as Antioxidants[a]

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SUMMARY

Synthetic lecithin (phosphatidyl choline) and cephalin (phosphatidyl ethanolamine) are inactive as antioxidants for menhaden oil at 50°, but when present with added ethoxyquin are very effective synergists. Phosphatidyl inositide also has no antioxidant effect but is a much weaker synergist with ethoxyquin. Purified fish "lecithin" and "cephalin" fractions and a commercial soybean phosphatide preparation act like the synthetic phospholipids. It is concluded that most of the synergistic effects may be attributed to the nitrogenous moeities of lecithin and cephalin.

Bollman patented vegetable "lecithin" as an antioxidant in 1923. In an effort to determine which of the several individual components carried the antioxidant effect, Olcott and Mattill (1936a) fractionated crude soybean "lecithin" and reported that the "cephalin" fraction was responsible. They later (1936b) found that "cephalin" was a synergist rather than a primary antioxidant, and attributed this effect to the acidic phosphate group (Jukes, 1934). Those observations were made with preparations that are now recognized to have been very crude mixtures. Recent developments in methods of phospholipid synthesis and separation have made it possible to obtain relatively pure samples of lecithin (phosphatidyl choline), phosphatidyl ethanolamine, and phosphatidyl inositide; and also to obtain mixtures of natural phospholipids in which the phosphatidyl choline plus choline plasmalogen has been separated from phosphatidyl ethanolamine plus phosphatidyl serine and their plasmalogens. Several such synthetic phospholipids and separated natural phospholipid fractions have now been investigated for antioxidant activity, both alone and as synergists for ethoxyquin (1,2-dihydro-6-ethoxy-2, 2, 4-trimethylquinoline). In brief, the results suggest that the phospholipids possess little, if any, primary antioxidant action and that the greater part of their activity as synergists resides in the nitrogen-containing moeity rather than in the phosphate group (*cf.* Olcott and Kuta, 1959).

EXPERIMENTAL

Materials and methods. A number of phospholipid preparations were used. The results were usually consistent although occasional runs were not duplicable. Conclusions in this paper are based on observations with the following: synthetic dipalmitoyl phosphatidyl choline (lecithin) and dipalmitoyl phosphatidyl ethanolamine (cephalin) (California Corporation for Biochemical Research, Sigma Chemical Co.), dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl ethanolamine (courtesy of E. Baer), preparations of tuna "lecithin" and "cephalin" fractions obtained by silicic acid chromatography (Shuster *et al.*, 1962), wheat germ phosphatidyl inositide sodium salt (Morelec-Coulon and Faure, 1957, 1958) for which we are indebted to M. Faure, and a commercial preparation of soybean phosphatides ("Alcolec," American Lecithin Corporation).

The menhaden oil used as substrate in most of the tests had been subjected to molecular distillation. We are indebted to M. Stansby for this material. Samples were dissolved in petroleum ether and passed over a column of silicic acid (Mallinckrodt AR, 100-mesh) to remove traces of peroxides, then held in solution at 0°F under nitrogen until used. Antioxidant activity was estimated by the weight-gain method previously described (Olcott and Einset, 1958). Aliquots equivalent to 200±5 mg were pipetted into 10-ml beakers. Antioxidants and synergists were similarly added in solvent solutions. The beakers were placed in constant-temperature draft ovens and left uncovered for one day to permit rapid removal of solvents, then kept covered by watch glasses. Once each day the beakers were removed from the ovens, allowed to cool, weighed, and examined for odor. The end of the induction

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period was usually easily recognized by a sharp gain in weight coinciding with the development of rancidity.

RESULTS AND DISCUSSION

None of the phospholipids tested alone gave measurable increases in induction period by the method used in amounts up to 50μM per g (3.5–4.2%). In combination with ethoxyquin, however, remarkable protection was afforded even with lower concentrations, particularly by the lecithin and cephalin preparations. The effects observed with the phosphatidyl inositide were less striking. Data for some typical runs are given in Table 1.

These results indicate that phospholipids do not possess appreciable antioxidant activity in a purified substrate devoid of natural or added antioxidants. This is consistent with previous observations with lecithin preparations by Olcott and Mattill (1936a), Hilditch and Paul (1939), and Urakami and Kameyama (1960). However, Desnuelle (1952) reported that highly purified soybean phosphatides were effective in sunflower seed esters. Tocopherols may have been present in the esters. The possibility exists that residual tocopherols may account for some of the reported antioxidant effects of crude phosphatide preparations.

The small but easily measurable synergistic effect of phosphatidyl inositide with ethoxyquin appears to be readily explainable in terms of the acidic phosphate group (Morelec-Coulon and Faure, 1957, 1958). This conclusion is in accord with the conclusion (Olcott and Mattill, 1936b, Privett and Quackenbush, 1954a) that phosphoric acid and its esters are acid synergists.

We have finally to account for the much greater synergistic effectiveness of both synthetic and natural lecithins and cephalins

Table 1. Effect of phospholipids as synergists for ethoxyquin in purified menhaden oil at 50°C.

Antioxidant	Synergist[a] (μM)	Induction period (days)		
None	None	1,1[b]		
	10 synthetic dipalmitoyl lecithin	1,1		
	10 synthetic dipalmitoyl cephalin	1,1		
	10 tuna "lecithin"	1,1		
	10 tuna "cephalin"	1,1		
	10 phosphatidyl inositide	1,1		
	10 soybean phosphatides	1,1		
0.2μM EQ[c]	None	2,2		
	2 synthetic dipalmitoyl lecithin	39,43		
	5 synthetic dipalmitoyl lecithin	43,62		
	2 phosphatidyl inositide	3,4		
	5 phosphatidyl inositide	3,9		
	2 soybean phosphatides	50,55		
	5 soybean phosphatides	63,69		
		Run 1	*Run 2*	*Run 3*
0.5μM EQ[c]	None	1,1	4,5	8,7
	5 synthetic dipalmitoyl lecithin	23,23	>75,>75[d]	45,46
	5 tuna "lecithin"	25,32	....	....
	5 synthetic dipalmitoyl cephalin	13,14	....	23,28
	5 tuna "cephalin"	16,24	....	....
	5 phosphatidyl inositide	....	7,7	....
	10 phosphatidyl inositide	....	6,9	
	5 soybean phosphatides		>75,>75[e]	

[a] See text for description of preparations. Amounts are those added to 200 mg oil. 10μM of phospholipid is equivalent to 3.5–4.2% by weight.

[b] The method does not distinguish between samples with induction periods of less than one day.

[c] EQ = ethoxyquin.

[d] These runs were discontinued at 75 days and found to have peroxide values of 5 and 7.

[e] These runs were discontinued at 75 and 77 days and found to have peroxide values of 10 and 7.

as compared to phosphatidyl inositide. Since both contain basic moeities and it has been shown that simple aliphatic amines are effective synergists (Olcott and Kuta, 1959), it appears reasonable to assign the marked additive effect to the basic groups. In general, phosphatidyl choline appeared to be more effective than phosphatidyl ethanolamine, but our observations lacked sufficient duplicability to make a final judgment. With aliphatic amines, the tertiary and secondary amines were usually superior to primary amines as synergists for ethoxyquin (Olcott, 1962).

There remains the problem of accounting for the synergistic phenomena. Some investigators have suggested that synergists are effective by virtue of removing pro-oxygenic heavy metals from the scene of the reaction (Evans, 1935; Frankel *et al.*, 1959). It is not believed that heavy metals mediate the observations recorded in this paper, since the addition of small amounts of metal salts to the substrate-antioxidant mix did not appreciably modify the synergistic effect (data not shown). However, more detailed work will be required to rule out this possibility.

Synergists may also be effective by other reactions (Golumbic, 1946; Calkins, 1947; Privett and Quackenbush, 1954b; Privett, 1961; Uri, 1961), but evidence is as yet insufficient to determine which of those suggested is the actual mechanism.

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Use of Capillary Gas Chromatography with a Time-of-Flight Mass Spectrometer

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SUMMARY

The feasibility and advantages of using a capillary chromatographic column directly with a time-of-flight mass spectrometer were demonstrated in the analysis of several flavor extracts. The high resolving power of this column proved to be indispensable in cases where chromatographic fractions previously unresolved showed similar mass spectra. Other limitations of this combined technique are covered.

In recent years considerable attention has been given to the direct introduction into a rapid-scan mass spectrometer of effluents emerging from a gas chromatographic column (Ebert, 1961; Gohlke, 1959; Lindeman and Annis, 1960). This technique is of value when it is not possible or convenient to collect the effluent and perform the analysis by means of a conventional inlet system, for example, when the unknown chemical is unstable and polymerizes or oxidizes before the operator can perform the mass analysis. Frequently, the effluent contains only a few micrograms of material, and subsequent transfer to the mass spectrometer may be difficult or inconvenient. Often these small amounts of material are collected with very low efficiency, primarily because of aerosol effects, and direct introduction becomes desirable.

Previous work has been done with packed chromatographic columns by diverting approximately 1% of the total effluent into the mass spectrometer. With capillary chromatographs the total gas flow is normally about 1% of that used in packed column chromatographs, so it seemed logical to consider the possibility of introducing the total effluent into the mass spectrometer. This simple extension of the existing technique is very desirable in analyzing complicated mixtures that can be fractionated satisfactorily only with a capillary chromatograph. Normally, the capillary column can be expected to have 10–100 times the theoretical plates obtainable with a packed column, and consequently will give separations not achieved by the other. This resolution is particularly necessary when the unresolved components have a similar mass spectral pattern, such as occurs with meta- and para-xylenes.

The apparatus used in this work is shown schematically in Fig. 1. The chromatographic equipment is operated in a conventional manner except that the exit pressure is reduced to vacuum. To compensate for this, the gauge pressure at the inlet is reduced by 15 lb, and in this manner retention times are close to those observed with the same equipment operating under normal atmospheric outlet pressures. The auxiliary vacuum is used for preliminary evacuation of the chromatograph exit. Occasionally it is used to reduce by a factor of two to four the amount of effluent entering the mass

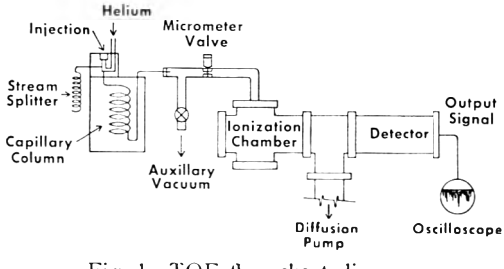

Fig. 1. TOF flow-chart diagram.

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spectrometer. In operation, the micrometer valve is wide open.

The helium carrier gas from the chromatograph enters directly into the ionization chamber of a Bendix Time-of-Flight Mass Spectrometer. The mass spectrometer vacuum system maintains a pressure of about 10^{-5} Torr (estimated from an ion gauge calibrated for dry air). The presence of an organic compound in the effluent is determined by observing the mass spectral output on the oscilloscope. When an unknown appears, the mass spectrum is recorded on a Minneapolis-Honeywell Visacorder at a scan rate of m/e 24–200 in 8 seconds.

In this preliminary work, a simultaneous chromatographic detector has not been used. Consequently, without a chromatographic record, it is not possible to compare directly chromatograms obtained under vacuum with those obtained under normal operating conditions. However, reconstructed chromatograms have been obtained by plotting the intensity of the base peak recorded on the mass spectrum versus retention time. Although this does not give an exact measure of the amount of material, it enables the placement of the observed compounds, and hence gives a general estimation of the chromatographic efficiency. Results have indicated this method to he as good as or better than the use of a similar column operating with a conventional ionization detector at atmospheric pressure. This observation is in accordance with currently accepted views on column operating efficiency (Giddings, 1961).

Fig. 2 shows a typical chromatogram from a packed column. The sample was a small fraction separated from an orange juice condensate oil. The column was a 1-in. preparatory column, but the separation efficiency was essentially the same as can be obtained on a ⅛-in. column. The substrate used for this separation was neopentyglycolsuccinate; it is considered to be favorable for compounds of the type present. The chromatogram shows the presence of six compounds, which were identified primarily by matching the relative retention times of classically known compounds.

Fig. 3 shows a capillary chromatogram of the same sample. In spite of the fact that

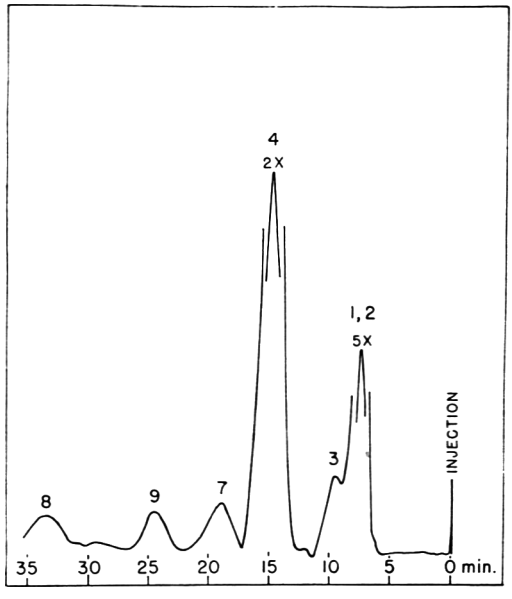

Fig. 2. Packed column chromatogram of orange condensate oil on 7-ft neopentylglycolsuccinate at 110°C (peaks identified in Fig. 3).

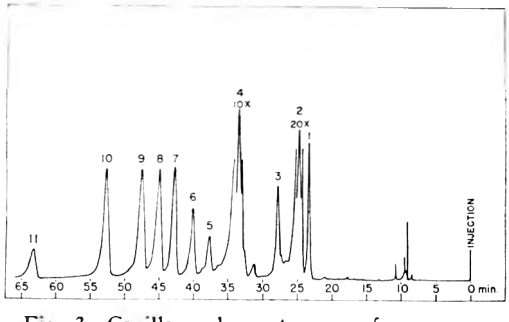

Fig. 3. Capillary chromatogram of orange condensate oil on 250-ft DOW 710 silicone oil at 97°C.

1. α-thujene
2. α-pinene
3. camphene
4. myrcene
5. α-phellandrene
6. α-terpinene
7. limonene
8. octanal + $C_{10}H_{16}$ terpene
9. p-cymene
10. γ-terpinene
11. unidentified

the Dow 610 silicone oil gives poorer separation than the neopentylglycolsuccinate when used in the packed column, the mixture is now resolved on the capillary column to show twelve compounds. Because the majority of these are $C_{10}H_{16}$ terpenes, it is essential that they be separated for mass spectral identification. For example, the mass spectra of α-thujene and α-pinene are similar enough that a 1–5% impurity of α-thujene in α-pinene would go completely

undetected. The pattern for camphene differs considerably in the intensity of the base peak at m/e 93 (22% of the total ionization for α-pinene, 13% for camphene), but this difference is spread rather evenly throughout the other peaks so that 2% camphene in α-pinene might be interpreted as a small error in the base peak intensity. Similarly, chromatographic peaks 5 and 6 in Fig. 3 would not have been identified as small components of the overwhelming myrcene fraction. Clearly, separation of these types of compounds is necessary.

Another example that emphasizes the need for the utmost separating power in the chromatographic equipment is given by results obtained on a tiny sample of extract from peas. After a laborious series of chemical and extractive separations performed on 5000 lb of fresh peas, about 3–4 μl of a residual oil was obtained, in addition to other fractions. This mixture gave 22 clear chromatographic peaks on a packed column. On a capillary column, 29 peaks were observed, but this was not run with an optimum temperature program. With a lower temperature program, the effluent from the capillary column was introduced into the mass spectrometer, and 39 separate compounds were observed.

The class identity of these 39 compounds is shown in Table 1. Eighteen alkyl benzenes were observed. It is again emphasized that, because many of these have similar mass spectral patterns, the identification of one in the presence of an isomer is difficult, even in relatively large amounts.

Table 1. Compounds observed in extractant from blanched fresh peas.

	Number of compounds
 C_2	3
 C_3	6
 C_4	9
Aliphatic hydrocarbons above C_7 (also possibly ketones)	12
Others: terpenes, indenes, furans, etc.	9
Total	39

Although the presence of several aliphatic hydrocarbons was determined, the mass spectra of the many isomers of C_9H_{20}, $C_{10}H_{22}$, etc., are so similar that positive identification of such small quantities is almost impossible. It is of value, however, to get an estimate of how complex the mixture of this class of compound might be, and again the combined capillary chromatography and mass spectrometry offer the most practical solution. Identification of some of the other compounds in this pea extract is still tentative and awaits further study.

Presented as a final example of the advantages of this technique are some of the data obtained on an apple juice extract. Fig. 4 is a synthetic chromatogram obtained by plotting the intensity of the strongest mass spectral peak versus the retention time. Several of the peaks have been identified and, as would be expected, correspond to compounds established by classical identification methods. In addition, a pentenal and hexenal were tentatively identified. These compounds might have been missed in classical work, because of the long, tedious ex-

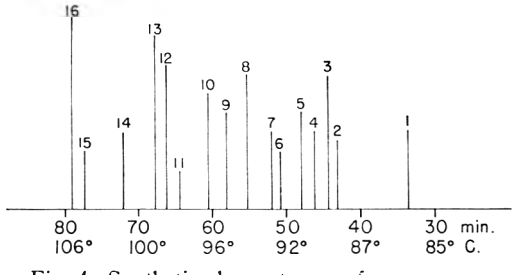

Fig. 4. Synthetic chromatogram from mass spectral intensities. Extract of apples chromatographed on DOW 710 silicone oil.

1. butyl acetate
2. C_8–C_9 aliphatic hydrocarbon
3. amyl acetate
4. pentenal
5. hexenal
6. C_9–C_{10} aliphatic hydrocarbon
7. hexyl acetate
8. C_9–C_{10} aliphatic hydrocarbon
9. C_9–C_{10} aliphatic hydrocarbon
10. C_9–C_{10} aliphatic hydrocarbon
11. C_9–C_{10} aliphatic hydrocarbon
12. C_{10}–C_{12} aliphatic hydrocarbon
13. C_{10}–C_{12} aliphatic hydrocarbon
14. C_{11}–C_{13} aliphatic hydrocarbon
15. possible octanal
16. possible pentenyl acetate

tractive procedures in which such labile compounds are decomposed. In the present work, since only a few microliters were required, a quick extraction was performed on 1 L of apple juice with a low-boiling solvent that was easily evaporated. The total time from beginning to final analysis was only a few hours, and thus, the possibility of error due to decomposition or oxidation is considerably reduced.

Chromatographic peaks 15 and 16 have not yet been identified. A preliminary study indicates that these are uncatalogued compounds, possibly aldehydes, unsaturated esters, or unsaturated alcohols in the molecular-weight range of 114–128. If this work had been performed with a packed column, these two peaks would most likely have been one. It is often difficult to identify uncatalogued compounds of moderate complexity from the mass spectrum of their mixture, and in this case it would not have been easy to tell that two materials were present.

In conclusion, it is appropriate to note that this technique is only an extension of work pioneered and established by other workers (Ebert, 1961; Gohlke, 1959; Lindeman and Annis, 1960). Because it makes possible separations and mass analysis not attained from previously described techniques, it is desirable to emphasize the simplicity with which capillary chromatography can be coupled to a rapid-scan mass spectrometer.

ACKNOWLEDGMENTS

We wish to acknowledge the contribution and encouragement of many other members of the Western Regional Laboratory staff, in particular Dr. J. W. Ralls and Mr. R. M. Seifert, who permitted us to use their work on pea extractant as an example.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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Analysis of the Flavor and Aroma Constituents of Florida Orange Juices by Gas Chromatography [a]

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SUMMARY

The flavor of orange juices was studied objectively through analyses of recovered volatile materials. Organic extracts of freshly recovered volatiles from juices of established varieties were shown with programmed-temperature gas chromatography (PTGC) and thermal conductivity detection to contain 40–50 components. Aroma profiles of the different juices were obtained with a programmed-temperature flame ionization gas chromatograph.

Comparative evaluations were conducted of the flavor and aroma patterns of three varieties of Florida oranges: Hamlin, Pineapple, and Valencia. Preliminary investigations revealed no significant qualitative differences among varieties in gross analyses obtained with thermal conductivity and the ionization detection system using PTGC. Quantitative differences appeared responsible for the flavor differences noted among varieties.

Analyses showed some compositional differences among varieties in control juices, peel-oil-free juices, their respective juice essences, and peel oils. The presence of certain chemical constituents in the juice was directly related to the peel oil. No significant qualitative differences existed in similarly prepared samples from different varieties. Some specific chemical identification and the methods used are outlined.

Characterization of the flavor of orange juices has been dependent largely upon subjective methods employing taste panels. Although the ultimate criterion of acceptability lies in flavor appeal as determined by taste, feel, and smell, there is a definite need for analytical methods for determining chemical constituents contributing to the flavor and aroma. Consequently, chemical analysis has been the approach used by many investigators.

Emphasized in most of the published work has been the flavor contribution of the volatile constituents of orange peel oil, normally as cold-pressed oil. Poore (1932) was among the first to analyze the volatile constituents of California orange oil extensively. Guenther and Grimm (1938) showed the presence of citral in California Valencia oil. The citral content of Florida Valencia orange oil was studied in detail by Nelson and Mottern (1934). Blair *et al.* (1952) referred to flavorful components that give orange juice its distinctive character as having their origin in the peel oil. Kefford (1955) related the characteristic aroma and flavor of citrus fruits to the aldehydes and esters present in the peel oils.

Kirchner and Miller (1957), working with California Valencia orange juice, identified some 25–30 volatile components. Hall and Wilson (1925), in earlier work, identified volatile constituents of the juice as alcohols, carbonyls, acids, and esters.

With the advent of gas chromatography, techniques for the separation and isolation of volatile components have steadily developed. Stanley *et al.* (1961) conducted work based upon the major aldehydes in cold-pressed oils of California lemon, orange, and grapefruit. Work by Bernhard (1960, 1961) and Clark and Bernhard (1960) carried out principally on California lemon and orange oils revealed a large number of

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constituents in the terpene and terpenoid fractions. More recently, Kesterton and Hendrickson (1962) studied the composition of Florida Valencia orange oil as related to fruit maturity and presented evidence for the identification of 27 components in the terpeneless fraction.

The application of gas chromatography to analysis of the volatile flavor components and their identification was reported by Wolford and Attaway (1961). Attaway *et al.* (1962a) reported on isolation and identification of some of the volatile carbonyl components from orange essence. Analysis of chemical components in organic extracts of concentrated commercially recovered orange essences by Wolford *et al.* (1962), using the techniques of PTGC and two column phases, showed tentative identification and peak assignments for 43 components.

The present paper presents a basic approach to recovery, separation, isolation, and identification of the volatile flavor components in freshly extracted orange juices. The experiments were aimed at extending the knowledge of flavor composition in known commercial varieties of Florida oranges. Some specific aims were: 1) to examine the compositional differences in control juice simulating commercially extracted juice, peel-oil-free prepared juice, and peel oil of Hamlin, Pineapple, and Valencia oranges; 2) to determine the contribution to the flavor by volatile components inherently present in the juice and/or peel oil; 3) to define any qualitative or relative quantitative changes in flavor components with maturation; and 4) to compare analyses of the fresh aroma of the juice of each variety.

EXPERIMENTAL

Used for analyses of organic extracts was an F & M Scientific Model 502 dual-column linear temperature-programmed gas chromatograph. Direct vapor-injection analyses were carried out on a Loenco Model 15F flame ionization gas chromatograph modified for column temperature programming by using an F & M circulating air oven with the programmer and flow control facilities of an F & M Model 300. Infrared analyses were conducted on a Beckman Model IR-4.

The dual-column oven was fitted with matched columns (12-ft × ¼-in. aluminum tubing). The columns were packed with Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P. Some analyses were also carried out with unmatched columns consisting of one of the above and a 12-ft × ¼-in. DEGS (diethylene glycol succinate), 30% w/w on 60–80-mesh Chromosorb P. The use of unmatched columns permitted successive liquid injections for two-dimensional analyses aimed at peak assignments and tentative identification of components (Wolford *et al.*, 1962). The direct vapor-injection analyses were made on the modified flame ionization instrument using a 12-ft × ¼-in. aluminum column packed with Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P. The attendant operating parameters for analyses are indicated with the figures.

Preparation of samples. An effort was made to process the juice of each variety coincident with its use by the industry in producing frozen concentrated orange juice. Absolute adherence to the plan was not always possible, but the timing was considered near enough for the aims of this work. Each of the three varieties, obtained from the Florida Citrus Experiment Station groves, was picked during the 1961–62 season for processing and recovery of volatile flavor components at two stages of maturity that were roughly 4–6 weeks apart. Each lot of fruit was divided into two equal portions of about 25 boxes each. One portion was processed as control fruit, receiving only a normal germicidal wash. The fruit was then extracted with a rotary juice extractor and the juice passed through a screw-type finisher fitted with a 0.020-in. screen. The juice was stored briefly in a low-vacuum discharge tank prior to stripping the volatile components. In each case, the other portion was used to prepare a peel-oil-free juice according to the procedure of Blair *et al.* (1952). The fruit, washed as above, was first passed through a Fraser-Brace Grater, described by Kesterson and Hendrickson (1953), to rasp the peel and wash off the major portion of the oil. All split fruit and fruit having any flavedo (the cuticular portion of the peel) remaining were discarded. The rasped fruit was then dipped in 1% $KMnO_4$ solution, rinsed in water, dipped in 1% $NaHSO_3$ solution, and again thoroughly rinsed with water prior to extraction of the juice. The juices were extracted and finished in the manner used for the control juices. Peel oil analyses, determined by the Clevenger procedure, showed that all of the peel-oil-free juices contained extremely low or only trace amounts of recoverable oil. Amounts of juice between 80 and 120 gal were used in the recovery of volatile constituents from the six runs made. Samples of the freshly extracted juices were analyzed immediately for aroma, and additional

quantities were quick-frozen in 6-oz cans for further study.

Recovery of volatile materials. A pilot-plant recovery unit was used for removal of a portion of water and volatile constituents from the single-strength orange juice. The principle involved heating and flashing of the juice and condensing the vapors. The sequence of flow through the recovery unit was as follows: the juice entered a fin-tube heat exchanger that discharged at 125°F into a vacuum chamber (29 in. Hg). The level of juice in the vacuum chamber was controlled for removal of about 4% of the liquid as vapor. The vapor passed into a refrigerated fin-tube condenser and the condensate was discharged at 40°F. The condensed portion of the juice volatiles plus water was collected in traps submerged in dry ice and alcohol. The separate collecting traps were allowed to thaw, the total volume of condensate measured, and the aqueous samples were stored at 32°F prior to extraction.

The isolation of volatile components from the condensates was carried out by the methylene chloride extraction procedure outlined by Wolford *et al.* (1962).

Samples of peel oil were recovered by carefully shaving off very thin slices of the flavedo and extracting with methylene chloride. The resulting extract was concentrated in a rotary vacuum evaporator on a 40°C water bath.

Aroma analysis. Preliminary investigations into analysis of the aroma of orange juices and/or their respective recovered essences were conducted as follows: A 500-ml Erlenmeyer flask, previously flushed with nitrogen gas, was filled with fresh juice to within about 1¼ in. from the top, a plastic stirring bar was introduced, and the top was sealed with heavy aluminum foil immediately after a nitrogen sweep of the headspace. The aluminum foil was held in place by a tight band of masking tape. An additional piece of tape was placed on top of the foil cover to minimize the size of the hole produced by a syringe needle. The sealed flask was partially immersed in a beaker of water to provide a uniform temperature (28–30°C), and the juice was kept in gentle circulation by a magnetic stirrer. After *ca.* 15 min of stirring, a 4-ml sample of the headspace vapors was removed by a syringe and injected directly into the gas chromatograph.

Identification of volatile components. The methods employed for tentative identification consisted of enrichment procedures using known compounds and retention temperature coincidence for peak assignment (Wolford *et al.*, 1962). The results contained therein served in the assignment of peaks in the present article. Infrared analyses were carried out on condensed, eluted components using Irtran cells or liquid transfer to capillary cells. The direct application of confirmatory tests served to characterize certain peaks according to their chemical classification. The principal tests employed were the nitrochromic acid test of Walsh and Merritt (1960) for the detection of alcohols, and the bubbler technique for carbonyls (Attaway *et al.*, 1962a) using 2,4-dinitrophenylhydrazine. Some preliminary work conducted on a subtractive-type method for selective partitioning between polar and non-polar immiscible phases described by Suffis and Dean (1962) was applied to the esters and alcohols. Positive identification of some alcohols was achieved through their urethan derivatives by the method of Attaway *et al.* (1962b).

RESULTS AND DISCUSSION

The six pilot-plant runs made for the recovery of volatile flavor components produced vapor condensate yields varying from 3.5 to 4.6% of the juice used. These six samples of condensate vapor were found by analysis to be representative of the chemical spectrum found in earlier work on commercially recovered orange essences (Wolford *et al.*, 1962).

Previously published results (Wolford *et al.*, 1961, 1962) have shown the merits of PTGC in analysis of the complex mixture of volatile flavor components in citrus juices. Figs. 1, 2, and 3 represent such separations of organic extracts of volatile components of the control juice, peel-oil-free juice, and peel oil. The total peak count in each figure is numbered from the combined chromatograms shown for that variety.

The amount of peel oil removed by the Fraser-Brace grater ranged from 89 to 98%, depending on the physical condition of the peel, namely, turgid or spongy. Table 1 shows the Brix/acid ratio and percentage

Table 1. Date of picking, Brix/acid ratio, and percentage oil for control and peel-oil-free juices of Hamlin, Pineapple, and Valencia oranges.

Variety	Picking date	Brix/acid ratio	Recoverable oil (%)	
			Control	Peel-oil-free
Hamlin	11/28/61	10.7	0.020	0.001
	2/ 1/62	12.9	0.003	Trace
Pineapple	12/ 5/61	8.7	0.018	0.002
	2/ 1/62	10.7	0.010	Trace
Valencia	3/21/62	9.3	0.024	0.001
	5/ 1/62	12.5	0.025	Trace

recoverable oil found in the control and peel-oil-free juices of each variety.

The chromatograms in Fig. 1 show 53 resolved and indicated component peaks for Hamlin orange juice volatiles consisting of substances from the juice and peel oil. Basically, it may be considered that the components shown in chromatogram 1-C plus those in 1-B give the chromatogram of volatile components in Fig. 1-A. Also, Fig. 1-B provides indication of some components that are inherently present in the juice.

Some of the qualitative observations are contained in the ensuing discussion. The component peaks in the peel oil were identified as α-pinene (15), β-myrcene (20), *d*-limonene (22), *n*-octanal (25), *n*-nonanal (29), *n*-decanal (34), linaloöl (35), and *n*-octanol (36). There was little indication of

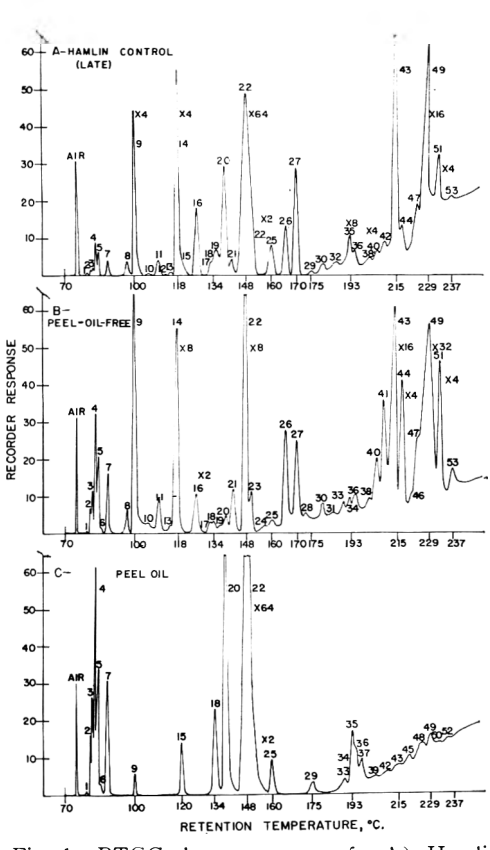

Fig. 1. PTGC chromatograms of: A) Hamlin (late) control juice volatiles; B) peel-oil-free juice volatiles; C) peel oil. Sample size, 6 μl; carrier gas (helium), 50 psi, 60 ml/min; stationary phase, Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P; program temperature rate, 4.0°/min from 70 to 245°C.

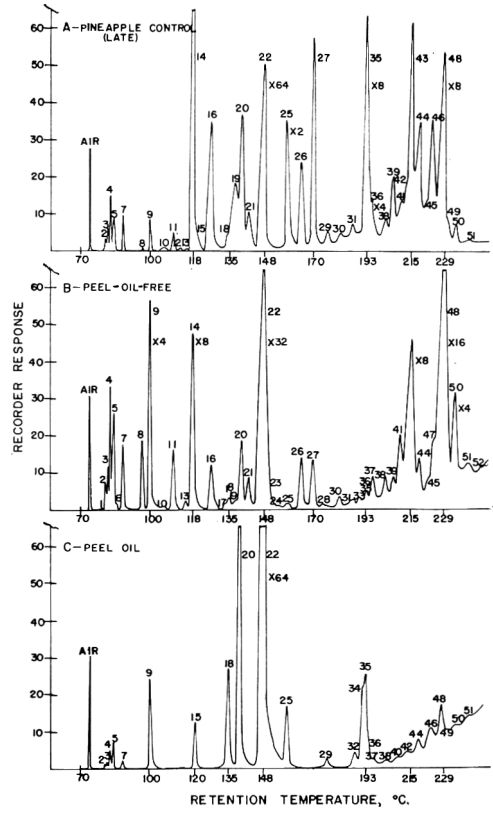

Fig. 2. PTGC chromatograms of: A) Pineapple (late) control juice volatiles; B) peel-oil-free juice volatiles; C) peel oil. Sample size, 6 μl; carrier gas (helium), 50 psi, 60 ml/min; stationary phase, Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P; program temperature rate, 4.0°/min from 70 to 245°C.

the presence of citral in the Hamlin peel oil. However, deterpenation of the oil would enhance the concentration of the oxygenated components and thus reveal citral (Bernhard, 1961; Kesterson *et al.*, 1962). The presence of *l*-carvone was indicated in the peel oil. Low concentrations of all terpene carbonyls and alcohols were shown because of the dilution by the large terpene hydrocarbon fraction.

The following major components with their peak numbers were shown to be present in the juice (Fig. 1-B): methanol (9), ethanol (9), *n*-hexanal (16), an unidentified component (21), 1-hexanol (26), and 3-hexenol (27). Those components shown in the retention temperature range between 193 and 237°C are in relatively high concentration and will probably require further

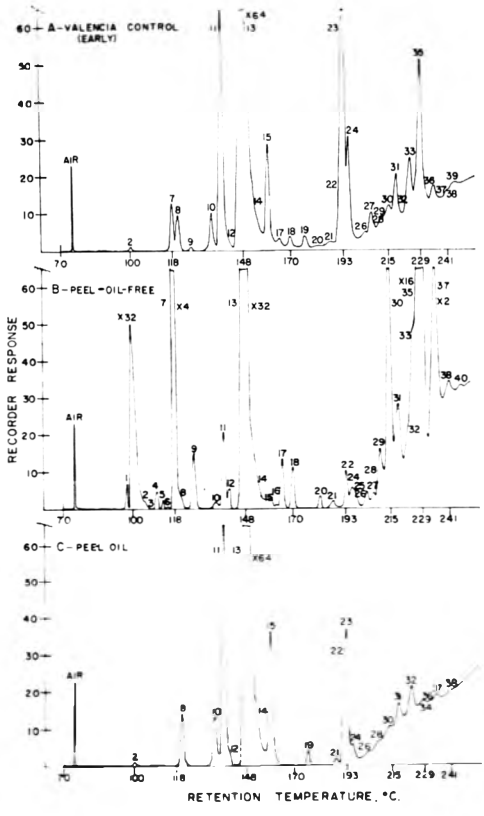

Fig. 3. PTGC chromatograms of : A) Valencia (early) control juice volatiles; B) peel-oil-free juice volatiles; C) Peel oil. Sample size, 6 μl; carrier gas (helium), 50 psi, 60 ml/min; stationary phase, Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P; program temperature rate, 4.0°/min from 70 to 245°C.

study of the oxygenated fraction of the oil for specific identifications. However, peaks 44, 47, and 51 have been respectively assigned as the citral isomers, neral and geranial, previously identified (Attaway *et al.*, 1962a), and geraniol. The last has thus far received only tentative assignment by retention temperature coincidence employing enrichment techniques on two columns, Carbowax 20M and DEGS (Wolford *et al.*, 1962). The two major component peaks, 43 and 49 at 215° and 229°C, were studied by infrared analysis of condensed eluted vapors from each. Component 43 was shown to be predominantly an ester. Infrared interpretation showed the alcohol portion of the ester to be a primary or tertiary alcohol. With the nitrochromic acid test (Walsh *et al.*, 1960) for primary and secondary alcohols, a positive test was obtained in 30 sec. With the possibility of cleavage of the ester occurring under the acidic conditions employed, some known esters were checked. Although discoloration of the reagent occurred, the time element was about 20 min. These results indicated the possibility that peak 43 is a mixture of a free primary or secondary alcohol with possibly a tertiary alcohol ester. It is suspected that three or more unresolved components are responsible for peak 49. Comparisons by retention temperature and enrichment techniques have, thus far, shown only *l*-carvone present. Infrared analysis also showed the possibility of an olefin. Ester and carbonyl absorption were weak, but not absent.

The material in peak 14, Fig. 1-A and B, was collected by condensation of eluted vapors. Comparison of its infrared spectrum with a series of low-boiling esters, synthesized for the analyses, revealed that component to be ethyl butyrate. To our knowledge this ester has never been reported in orange juice, and therefore stands as a newly identified constituent of Florida orange juices. The component, peak 27, has now been positively identified as 3-hexenol through its 3-hexen-1-yl *o*-nitrophenylurethan (Attaway *et al.*, 1962b) by paper chromatographic comparison with the R_f value of the known urethan and by retention temperature assignment on two columns by PTGC.

Much of the previous description concerning Fig. 1-A,B,C is applicable to the volatile flavor components of Pineapple orange juice shown in Fig. 2-A,B,C. Qualitatively, Fig. 2 is superimposable on Fig. 1 for Hamlin oranges. The total component peak count, a somewhat arbitrary value dependent upon the GC column efficiency, was nearly identical on a cumulative basis for the two varieties. Small quantitative differences in a few major components can affect the chromatographic resolution of those components in a very low concentration.

In Fig. 2-C, linalool, peak 35, appears to be in slightly higher concentration in the Pineapple orange peel oil. A higher concentration of this tertiary alcohol is also evidenced in the volatiles from the control Pineapple orange juices, as is evident when

Fig. 2-A is compared with Fig. 1-A, where both peaks received the same attenuation. Citral *b* and *a* (neral and geranial) were shown to have retention temperatures respectively coinciding with peaks 44 and 46.

It should be pointed out that the designation "late" in Figs. 1 and 2 refers to the last picking date for the Hamlin and Pineapple orange in the general categories of early-season and mid-season fruit, respectively. For Valencia (late-season) oranges, only the early and mid (middle) picking dates were selected. Valencia juice from oranges to be picked later in the season is yet to be studied.

A comparison of Fig. 3 with the two preceding sets of chromatograms would at first indicate some qualitative and quantitative differences in the Valencia orange juice. Examination of Fig. 3-A,C, clearly shows the influence on qualitative and relative quantitative results obtained in the presence of larger amounts of peel oil. However, in Fig. 3-B, where a large portion of the peel oil has been removed, the resolution of volatile flavor components is closely comparable to those in the Hamlin and Pineapple peel-oil-free juices.

The Valencia orange juices had the highest percentage of recoverable oil of the three varieties studied. The major components, β-myrcene (11), *d*-limonene (13), *n*-octanal (15), linaloöl (23), *n*-octanol (24), and possibly *l*-carvone (35) in Fig. 3-A, were mostly contributed by the oil from the peel. The large dilution factor produced by the oil suppressed the resolution of components in low concentration, and consequently the resulting total peak count.

Thus far it has been shown that Florida orange juices from these three varieties of fruit are nearly qualitatively identical. Any differences in flavor and/or aroma may then be attributed to relative quantitative differences between components. Even these differences did not appear until the later-maturity juices were studied. Figs. 4-A, B, C show chromatograms representing the three varieties for specific comparison based upon retention temperature correlation of individual components. The solid-line chromatograms in A and B are identical to those in Figs. 1-A and 2-A for control juices of those

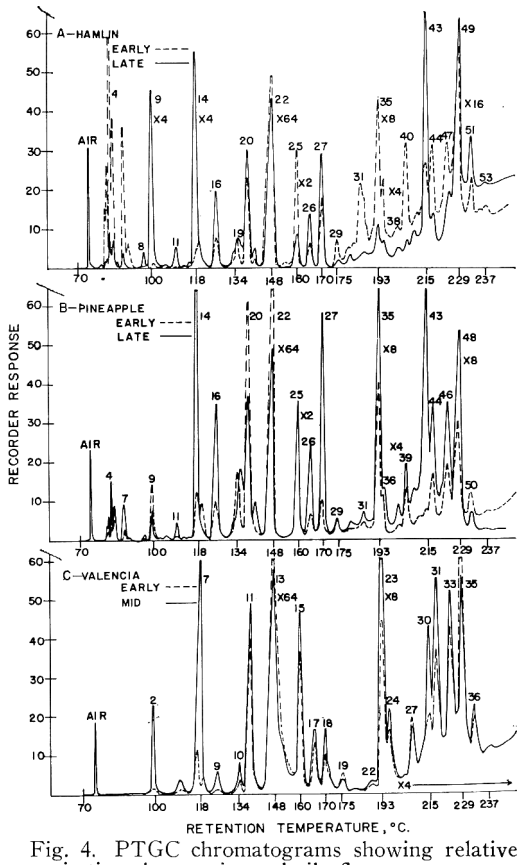

Fig. 4. PTGC chromatograms showing relative quantitative changes in volatile flavor components of: A) Hamlin (early and late) juice; B) Pineapple (early and late) orange juice; C) Valencia (early and mid) orange juice. Sample size, 6 μl; carrier gas (helium), 50 psi, 60 ml/min; stationary phase, Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P; detector current, 200 ma; program temperature rate, 4.0°/min from 70 to 245°C.

varieties at the later maturity levels. In Fig. 4-C the solid-line chromatogram represents the middle (mid) maturity Valencia control juice.

Some preliminary conclusions may be drawn from these results. In lieu of strict quantitative results, certain trends in the quantitative changes in some components are indicated.

In the late Hamlin juice increases in the concentration of ethyl butyrate (14), *n*-hexanal (16), 1-hexanol (26), 3-hexenol (27), peaks 43, 49, geraniol (51), and attendant reductions in the amounts of acetaldehyde (4), *n*-octanal (25), linaloöl (35), 1-octanol (36), and neral (47) were observed compared to the early-maturity Hamlin juice. In the late Pineapple orange juice,

increases over the early-maturity juice were indicated in the concentration of acetaldehyde (4), ethyl butyrate (14), *n*-hexanal (16), 1-hexanol (26), 3-hexenol (27), linaloöl (35), peaks 43, 48, and the citral isomers (44 and 46). There appeared to be some reductions in the terpene hydrocarbons, especially, β-myrcene (20), and *d*-limonene (22). However, reference to Table 1 shows a greater reduction in recoverable peel oil with increasing maturity in Hamlin juice. Also, *n*-octanal contributed by the oil did not appear to change in concentration in Pineapple orange juice. In the mid Valencia juice, an increase in concentration of ethyl butyrate (7), slight increases in *n*-hexanal (9), and *n*-octanal (15), small increases in 1-hexanol (17) and 3-hexenol (18), and increases in linaloöl (23) and 1-octanol (24) were seen compared to early Valencia juice. The change in the methanol and ethanol contents of the three juices was difficult to determine since some methylene chloride used in the extractions was always in peaks 9 in Figs. 4-A,B and peak 2 in Fig. 4-C. Analyses on DEGS effected separation of methanol from ethanol but little significance could be placed on their relative concentrations, because of methylene chloride in the ethanol peaks.

The following components in particular appear to provide some characterization for each of the three varieties: ethyl butyrate, *n*-hexanal, *n*-octanal, 3-hexenol, linaloöl, and possibly some of the higher-boiling terpene carbonyls, alcohols, and esters.

The retention temperatures determined for various components in this study matched those reported in the recent publication (Wolford *et al.*, 1962). Therefore, direct reference to the identifications given therein provided confirmation of component identities in the three varieties.

Table 2 shows the chemical components given peak assignments based on retention temperatures and peak coincidence in each of the three varieties studied. The components in parentheses either have received study on only one column or did not show peak assignment agreement by analysis on two columns. All other peak assignments received positive identification or stand on tentative identification.

Table 2. Component peak assignments on chromatograms of Hamlin, Pineapple, and Valencia orange juice volatile flavor components. Reference to Figs. 1, 2, 3, and 4.

Compound	Hamlin	Pineapple	Valencia
Acetaldehyde	5	5	....
Acetone	7	7	....
(Ethyl formate)	7	7	....
(Ethyl acetate)	8	8	....
Methanol	9	9	2
Ethanol	9	9	2
(Methyl isovalerate)	13	13	6
(1-Propanol)	13	13	6
Ethyl butyrate	14	14	7
(α-Pinene)	15	15	8
n-Hexanal	16	16	9
β-Myrcene	20	20	11
Δ^3-Carene	20	20	11
α-Terpinene	21	21	12
d-Limonene	22	22	13
(*n*-Amyl alcohol)	22	22	13
2-Hexanal	22	22	13
γ-Terpinene	24	24	14
n-Octanal	25	25	15
2-Hexenal	25	25	15
Terpinolene	25	25	15
(*p*-Cymene)	25	25	15
n-Hexanol	26	26	17
3-Hexenol	27	27	18
(2-Octenal)	27	27	18
n-Nonanal	29	29	19
(Methyl heptenol)	30	30	20
Ethyl *n*-caprylate	30	30	20
2-Nonanol	32	32	22
n-Decanal	34	34	22
Linaloöl	35	35	23
n-Octanol	36	36	24
(*n*-Undecanal)	40	39	26
(Terpinen-4-ol)	41	40	27
(*n*-Octyl butyrate)	42	42	29
1-Nonanol	42	42	29
(*n*-Octyl isovalerate)	43	43	30
Neral	44	44	31
α-Terpineol	44	44	31
Terpinyl acetate	46	45	32
Geranial	47	46	33
(Methyl-*n*-methyl anthranilate)	47	46	33
Citronellol	47	46	33
l-Carvone	49	48	35
Nerol	50	50	36
Geraniol	51	50	36
(*trans*-Carveol)	53	51	40

It can readily be seen that a number of multiple peak assignments exist, pending absolute identification. Studies on two or

more columns, chemical methods for subtractive analysis, and paper chromatography (Wolford *et al.*, 1962; Attaway *et al.*, 1962a) have been used to solve the multiplicity of component peak assignments.

Little reference has been made to the series of low-boiling components eluted between 70 and 100°C. Of these components, acetaldehyde has been identified (Attaway *et al.*, 1962a). The others have been tested for acetone, ethyl formate, and ethyl acetate, but lack agreement in peak assignment on two columns for tentative identification. The presence of this group of low-boiling components in Hamlin and Pineapple orange juices but not in the Valencia juice could be due to recovery techniques or the solvent extraction procedure used.

To obtain additional knowledge of the highly volatile materials, analysis of the fresh aroma of juices and recovered volatile flavor components was initiated. Fig. 5 shows an aroma profile of fresh Valencia orange juice of fruit from the mid picking date. The procedure employed was outlined in the experimental methods. A 4-ml sample of the vapor, contained in a sealed headspace above fresh juice at room temperature, showed 35 flavor components through *n*-octanol. Extending the range of compounds in analyses of the aroma of citrus juices is the aim of work now in progress. Some of the flavor components are listed in Table 3, with their peak assignments in Fig. 5. Most of the components have been previously identified. Enrichment techniques, using only the vapor above the known chemicals, were conducted to indicate peak assignments. Under the conditions employed it was found that a nonlinear temperature-programmed column provided the degree of resolution shown.

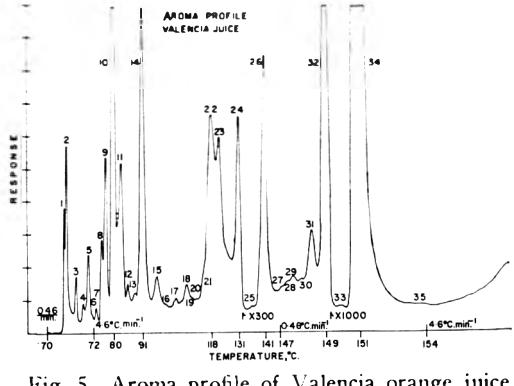

Fig. 5. Aroma profile of Valencia orange juice, mid picking date. Sample size, 4 ml aqueous vapor; carrier gas (helium), 50 psi, 30 ml/min; hydrogen, 30 psi, 28 ml/min; air, 25 psi, 275 ml/min; detector temperature, 90°C; splitter closed; non-linear-programmed column temperature rate as indicated; stationary phase, Carbowax 20M, 20% w/w on 40–60-mesh Chromosorb P.

The aroma chromatograms, using the ionization detection methods, have shown the following results: 1) The apparent aroma of orange juice or recovered juice volatiles can be shown qualitatively, and the potentialities of semi-quantitative determination are good. A satisfactory chromatogram of volatile components from a limited headspace over 1 L of orange juice can be obtained at temperatures as low as 10°C, indicating adequate vapor pressures of components through *d*-limonene and including *n*-octanal in some samples. As would be expected, increasing the temperature of the juice increases the over-all concentration of the components in the headspace. 2) The aroma chromatograms of recovered dilute essences from Hamlin, Pineapple, and Valencia orange juices, although not presented here, are directly comparable with the aroma analyses of the fresh juices themselves. Except for the slightly higher concentrations in the recovered volatile essences, no chemical changes resulting from the recovery procedure were indicated. 3) Distinct qualitative similarities were shown in the aroma chromatograms of the three varieties of Florida orange juices studied. Some rela-

Table 3. Component peak assignments in aroma profile of Florida Valencia orange juice.

Compound	Peak no.
Acetaldehyde	11
(Ethyl formate)	12
Acetone	15
Methanol	22
Ethanol	22
Ethyl butyrate	26
α-Pinene	27
n-Hexanal	29
β-Myrcene	32
d-Limonene	34
2-Hexenal	34
n-Octanal	35

tive quantitative differences throughout the spectrum might be used to differentiate between the varieties.

The results have established some fundamental points for studies presently in progress. Although some of the results are quite preliminary, it is believed some contribution has been made to knowledge of the flavor and aroma constituents of orange juices from an objective viewpoint.

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A Technique for the Study of Lipid-Soluble Food Flavor Volatiles [a,b]

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SUMMARY

A low-temperature high-vacuum distillation technique utilizing a molecular still is described. The flavor volatiles are distilled into liquid N_2 traps, transferred to a stainless-steel helical trap of special design, and then blown into a gas chromatograph. Identification of the flavor volatiles is based on relative specific retention volume and collection of the fractions for analysis by techniques such as mass spectrometry. Results are given for application of the described techniques to study of the lipid-soluble flavor volatiles of Cheddar cheese.

Because of their elusive character, minute concentration, and often unstable nature, food flavor volatiles present the analyst with a problem of considerable magnitude in isolation and identification of unaltered flavor components. This problem has been attacked in several ways. The most popular techniques have been solvent extraction and atmospheric or vacuum distillation. Solvent extraction has the disadvantage that one is usually faced with the problem of evaporating relatively large quantities of solvent while retaining the volatile flavor components. However, judicious choice of a low-boiling solvent and carefully controlled evaporation can help to minimize the loss of flavor volatiles. Distillation, if one assumes efficient trapping of the flavor volatiles, offers the possibility of collecting most of the several classes of flavor volatiles without adding a solvent that must later be removed.

Several distillation techniques for the isolation of flavor volatiles from foods have recently been published. Nawar and Fagerson (1960) developed a recycling gas-liquid extraction apparatus. In their technique the sweeping gas is continuously recycled and pushes the flavor volatiles into a refrigerated trap. Chang (1961) described a system for the isolation of flavor volatiles from fats and oils. His method entails countercurrent contact of the oil with steam in an Oldershaw column, and liquid-liquid extraction of the condensed steam with ether. Lea and Swoboda (1962) described a simple vacuum distillation procedure that quantitatively recovers added aldehydes from fats. The method has considerable promise for the collection of aldehydes from autoxidizing fats and oils. De Bruyn and Schogt (1961) described a high-vacuum apparatus for isolating the volatiles from fats and oils. Their system is versatile, relatively simple, and capable of excellent recoveries. The present publication presents the details of a continuous high-vacuum technique, utilizing a molecular still, that has been developed for isolation of the flavor volatiles from Cheddar cheese fat.

EXPERIMENTAL

The general scheme followed for the analysis of Cheddar volatiles is given in Fig. 1. The cheese was packed by hand into 50-ml stainless-steel centrifuge tubes and centrifuged 15 min at 30,000 × G in a Servall SS-3 superspeed centrifuge. The cheese attained a maximum temperature of 43°C during centrifuging. The centrifugation technique was essentially that of McGugan and Howsam (1962). After centrifugation the supernatant liquefied fat was decanted off. Sensory evaluation of the decanted cheese fat and the curd residue indicated that the typical Cheddar aroma resided in the fat portion. The Cheddar flavor volatiles were effectively isolated by passing the fat through the distillation assembly pictured in Fig. 2. Sensory evaluation of the cheese fat

[a] Technical Paper 1617, Oregon Agricultural Experiment Station.

[b] This investigation was supported by PHS research grant EF-269 from the National Institutes of Health, Division of Environmental Engineering and Food Protection.

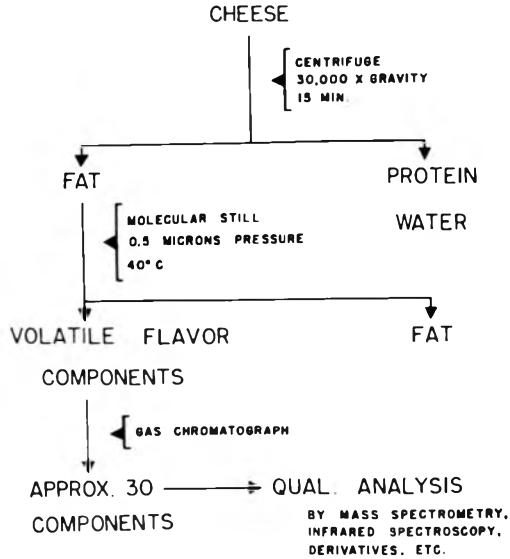

Fig. 1. Scheme for the isolation of Cheddar volatiles.

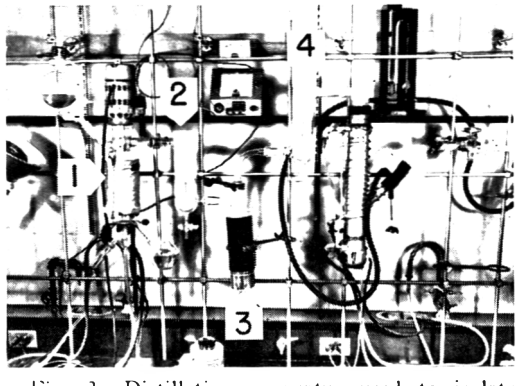

Fig. 2. Distillation apparatus used to isolate Cheddar volatiles. 1) Rota-Film molecular still; 2), 3), 4), liquid nitrogen traps; 2) and 4) are cold-finger traps; trap 3 contains 3-mm glass beads.

before and after distillation indicated that the molecular still effectively removed the Cheddar aroma. The feed flask of the Rota-Film molecular still (1, in Fig. 2) was warmed with an infrared lamp to keep the fat liquefied. The fat was usually passed through the molecular still at the rate of 3 ml/min. Although some of the Cheddar volatiles underwent molecular distillation and were deposited on the cold finger of the still, the more highly volatile components passed through the molecular still and were stopped by liquid nitrogen traps (2–4, in Fig. 2). In order to stop components of the highest volatility it was necessary to use a trap packed with glass heads and cooled with liquid nitrogen (trap 3, Fig. 2). The last liquid nitrogen trap (trap 4), located immediately before the oil diffusion pump, acted as a safety trap to prevent contamination of the Cheddar volatiles with diffusion-pump fluid. After a distillation was completed, the vacuum pumps were turned off and the vacuum was broken. The volatiles were in three locations: on the molecular-still cold finger and in the first two of the three liquid nitrogen traps. The contents of the liquid nitrogen traps can be analyzed separately, or the material from the first liquid-nitrogen trap can be transferred to the glass-bead trap. The transfer of cheese volatiles was accomplished by removing the liquid nitrogen from the first trap and replacing the liquid nitrogen with warm water while the system was evacuated and liquid nitrogen was retained in traps 3 and 4. After the volatiles were transferred to the glass-bead trap (trap 3), the vacuum pumps were turned off and the vacuum broken. The molecular still was disconnected from the rest of the system, and the volatiles on the cold finger of the molecular still were washed down with iso-pentane or other suitable solvents. Since the last liquid-nitrogen trap acts as a safety trap, its contents were discarded. Prior to gas chromatographic analysis, the flavor volatiles were transferred from the glass bead trap to a stainless-steel helical trap. A drawing of the helical trap is given in Fig. 3, and the transfer operation is shown in Fig. 4. As can be seen in Fig. 3, the upper part of the helical trap goes through an aluminum block. The block was heated to about 100°C by a 50-watt cartridge heater regulated with a rheostat. The heated block helps ensure that the volatiles will freeze out in the loops and not the arms of the trap. This is important since only the loops are immersed in heating medium when the volatiles are injected into the gas chromatograph. In transfer of the volatiles to the helical trap (Fig. 4), both traps were initially immersed in Dewar flasks containing liquid nitrogen while the system was evacuated to less than 1 mm Hg with a rotary vacuum pump attached to the helix. With the vacuum pump still running, the liquid nitrogen on the glass-bead trap was removed and this trap was allowed to warm up. Allowing the glass-bead trap to warm up gradually gave the best results. In the later stages of the transfer a beaker of warm water was placed on the glass-bead trap to hasten the transfer.

After completion of the transfer of the cheese flavor volatiles to the helical trap, the vacuum was broken and the trap fitted with a hypodermic needle at one end and appropriate Swagelok fittings on the other. Prior to gas chromatographic analysis the loops of the helix were removed from the liquid nitrogen and immersed in a Dewar flask containing ethanol and dry ice; this allowed the escape of CO_2 and liquid O_2. Injection of the

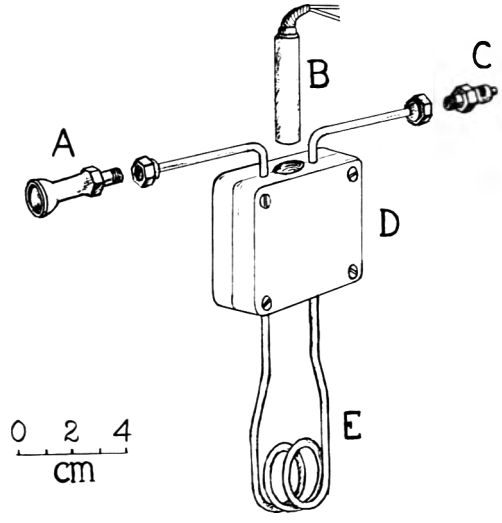

Fig. 3. Helical trap used to transfer flavor volatiles from the distillation apparatus to the gas chromatograph. A) Stainless steel 12/5 spherical joint silver-soldered to a Swagelok union for 1/8-inch OD tubing. B) 50-watt cartridge heater; heating is controlled by a Powerstat. C) Luer-Lok male adapter fitting from hypodermic syringe silver-soldered to a Swagelok union for 1/8-inch OD tubing. D) Aluminum block. E) 1/16-inch stainless-steel tubing; no packing is used.

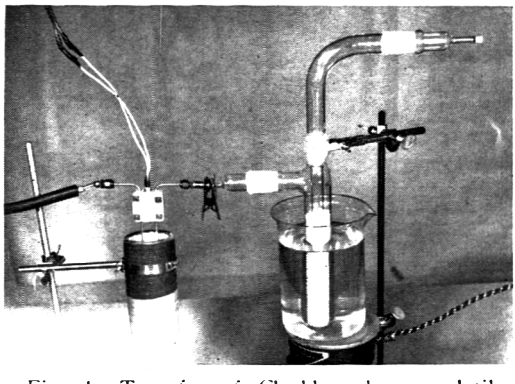

Fig. 4. Transfer of Cheddar cheese volatiles from the glass bead trap to the helical trap.

flavor volatiles from the helical trap into the Barber-Colman Model 20 gas chromatograph is shown in Fig. 5; the volatiles in the warmed helix were blown into the chromatograph with an external argon line. To avoid a split-peak artifact, the two carrier-gas toggle valves must be operated in a definite sequence. The external gas line, at the same pressure as the internal gas line and equipped with a toggle valve in the "off" position, was first attached to the Swagelok end of the helix while the helix was kept immersed in the dry ice-ethanol mixture. The hypodermic-needle

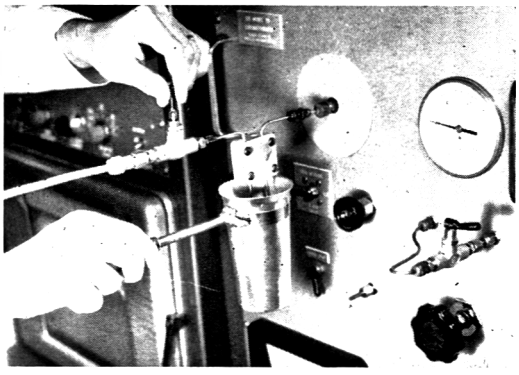

Fig. 5. Injection of flavor volatiles from the helical trap into the Barber-Colman Model 20 gas chromatograph.

end of the helix was inserted into the chromatograph septum, and the toggle valve of the external line was immediately opened. As quickly as possible the Dewar flask containing the dry ice-ethanol mixture was replaced by a container of heating medium (boiling water or heated oil) and the toggle valve controlling internal carrier gas flow was turned off. Injection times of 5–10 sec were usually adequate. After injection, the internal carrier gas flow was resumed, the valve on the external line closed, and the helical trap withdrawn. With practice, two people were able to perform these injection operations so that there was very little difference in retention times when known compounds were injected by syringe or helix. For example, diacetyl was injected onto a diethylene glycol succinate column at 72°C by both the syringe and the helix with less than a 1% difference in specific retention volume.

To aid in the conclusive identification of flavor constituents it was desirable to be able to trap the various chromatographic peaks for further study. In collecting for mass spectrometric analysis the trap described by Bazinet and Walsh (1960) has been modified. A glass-bead trap was added as a special precaution to avoid the possibility of contaminating the collected sample through the suck-back of atmospheric water and CO_2 while the trap was immersed in a Dewar flask containing liquid nitrogen. This modified trapping system is pictured in Fig. 6.

In transferring the flavor volatiles to the gas chromatograph, the 1/16-inch ID helix is usually sufficient. In certain cases, however, a larger-diameter helix would be desirable. In order to handle larger quantities of sample, a 1/8-inch ID stainless-steel helical trap was constructed. This helix permitted handling larger samples than was possible with the 1/16-inch ID helix, and larger samples were necessary with the relatively insensi-

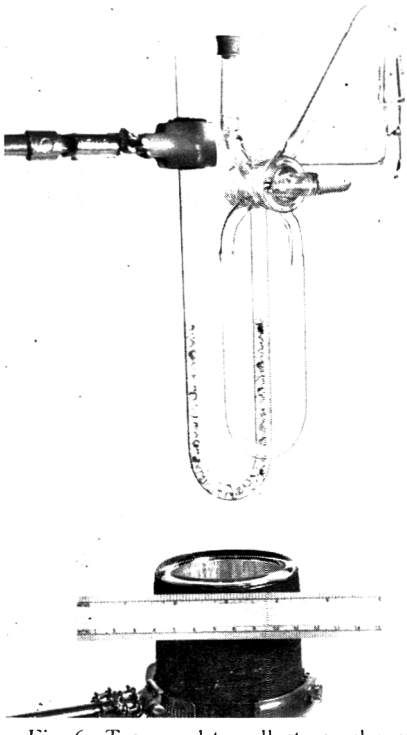

Fig. 6. Trap used to collect gas chromatographic fractions for mass spectrometric analyses. In actual use the loops of the trap are immersed in liquid nitrogen, and the hypodermic needle on the outlet of the gas chromatograph is inserted into the serum cap.

tive thermal conductivity detectors. Current work is directed toward use of the 1/16-inch ID helix in conjunction with a splitter on chromatographs utilizing β- and hydrogen-flame ionization detectors. In both cases the column effluent is split about 10:1 (collection/detection) prior to detection. The split effluent can be either examined for odor or collected.

RESULTS AND DISCUSSION

A gas chromatogram of the volatiles obtained from an 18-month-old raw-milk Cheddar cheese, utilizing the techniques described above, is presented as Fig. 7. Packed 1/16-inch ID columns were used in the Barber-Colman Model 20 gas chromatograph at 70°C. Both polar (diethylene glycol succinate) and non-polar (Apiezon L or M) stationary liquid phases were used. Comparison of the relative specific retention volumes of the unknowns with those of authentic compounds enabled tentative identification of over half of the chromatographic peaks. This information is presented as Table 1. To our knowledge the components tentatively identified as forming peaks 4, 7, 11, 15, 20, 23, 29, and ethyl butyrate of peak 22 have not been reported as being present in Cheddar flavor. The identifications are tentative, and analyses of the chromatographic fractions by mass spectrometry, infrared spectroscopy, and analysis of derivatives will be required for conclusive identification.

In some respects the helical trap described above resembles the collection and injection systems described by Day *et al.* (1957), Nawar *et al.* (1960), and Hornstein and Crowe (1962). The helical trap has the advantages, however, that it contains no valves, is versatile and easy to use, and permits the

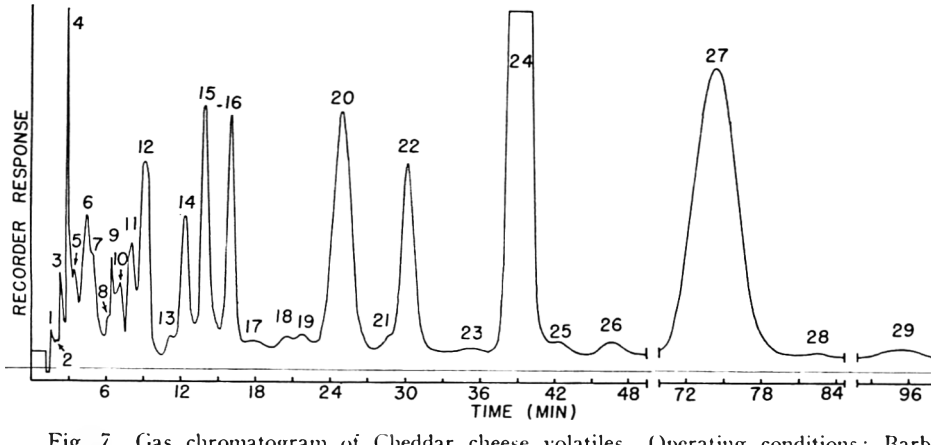

Fig. 7. Gas chromatogram of Cheddar cheese volatiles. Operating conditions: Barber-Colman Model 20, Sr^{90} β-ionization detector; 10.4-ft × 1/16-inch ID, column packed with 20% Apiezon L on 100/120-mesh Celite 545; column temperature 70°C; injector temperature 160°C; corrected flow rate 16 ml/min; cell current 1250 v.

Table 1. Relative specific retention values of gas chromatographic fractions from Cheddar cheese volatiles.[a]

Component	Peak no.[c]	Vg/Vg, diacetyl			
		Apiezon L and M [b]		DEGS [b]	
		Unknown	Known	Unknown	Known
Hydrogen sulfide	1	0.259	0.253	0.106	0.103
Acetaldehyde	3	0.372	0.364	0.222	0.210
Methyl mercaptan	4	0.467	0.466	0.156	0.158
Ethanol	5	0.560	0.541	0.646	0.610
Acetone	5	0.560	0.581	0.421	0.402
Ethyl mercaptan	7	0.807	0.788	0.194	0.188
Diacetyl	8	1.03	1.00	0.943	1.00
Butanone	10	1.17	1.14	0.572	0.578
Methyl propionate	11	1.34	1.34	0.572	0.544
3-Methyl butanal	13	1.85	1.82	0.509	0.527
n-Butanol	14	2.07	2.12	1.67	1.75
Ethyl propionate	15	2.32	2.39	0.646	0.647
Acetoin	17	2.94	2.98	1.67	1.74
Butyl mercaptan	20	4.15	3.95	0.421	0.426
Ethyl butyrate	22	5.03	5.02	0.943	0.920
2-Hexanone	22	5.03	5.05	1.36	1.38
Hexanal	23	5.88	5.88	1.25	1.25
Methyl hexanoate	28	12.4	12.6	2.14	2.19
Heptanal	29	13.7	13.7	2.14	2.10

[a] All measurements were made at 70°C.
[b] Liquid phases.
[c] Peak numbers refer to the chromatogram in Fig. 7; Apiezon L was the liquid phase used.

transfer of variable quantities of volatiles with good reproducibility. The unknown sample is introduced through the syringe injection port, which requires no alterations in normal operation of the chromatograph. Furthermore, the techniques employed allow efficient isolation and analysis of a wide range of compounds from variable quantities of fats and oils with little chance of oxidative or thermal decomposition. The ability to work with variable quantities of fats and oils enables isolation of sufficient sample for the desired analysis. Although designed primarily to isolate the volatile components of Cheddar cheese, the techniques described should be generally applicable to the collection and analyses of the volatiles from oils and liquefied fats.

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Gas Chromatography of Chicken and Turkey Volatiles: The Effect of Temperature, Oxygen, and Type of Tissue on Composition of the Volatile Fraction

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SUMMARY

Concentrated poultry volatiles were prepared by distilling water from chicken and turkey, extracting volatile components from the distillate with isopentane, and concentrating the dried isopentane extract. These volatiles were investigated by gas chromatography on an apparatus equipped with thermal conductivity detectors. Essentially all volatiles were heat-produced. Cooking in air, as contrasted to cooking in nitrogen, resulted in a much larger and more complex volatile fraction. Rancid chicken yielded a greater amount of volatiles than did fresh chicken, but qualitatively they were similar. The overall yield of volatile material was greater and of a more complex nature from skin and skin fat than from lean leg and breast muscle. Chromatograms of chicken and turkey volatiles indicated differences in their composition. It was not determined, however, whether the difference in composition was responsible for the different and distinctive flavor of chicken and turkey broths determined by sensory methods. *n*-Hexanal and *n*-2,4-decadienal were identified as two of the larger volatile fractions of fresh chicken and turkey and rancid chicken. Information on the nature of chicken and turkey volatiles can also be obtained by directly sampling vapors over the product and subjecting them to dual hydrogen flame chromatography.

INTRODUCTION

Analysis of chicken volatiles by methods using adsorption chromatography has proved to be difficult and time consuming (Pippen *et al.*, 1958; Pippen and Nonaka, 1960). Although gas chromatography has long promised to fulfill some of the requirements for a faster and more generally applicable method for investigating poultry volatiles, its use in this area has been limited (Lineweaver *et al.*, 1962).

In this study gas chromatography was used to investigate principally chicken volatiles and, to a lesser extent, turkey volatiles. Most results were obtained by chromatographing concentrated volatiles, prepared as shown in Fig. 1, on an apparatus equipped with thermal conductivity detectors. By this method, the general effects of some factors, such as temperature, raucidification, type of tissue, and oxygen availability during cooking on the yield and nature of chicken volatiles could be demonstrated. Fractions separated were identified to a

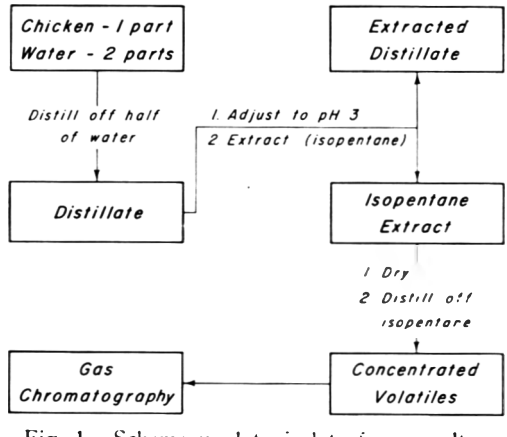

Fig. 1. Scheme used to isolate from poultry a dry concentrate of volatiles for gas chromatography in an apparatus equipped with thermal conductivity detectors.

[a] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

limited extent. By using the more sensitive dual hydrogen flame detector, chromatograms of vapors taken from directly over turkey and chicken distillates were obtained.

MATERIALS AND METHODS

Isopentane. Commercially available isopentane was redistilled through a column 1 m long packed with glass helices. On redistillation of several liters of the redistilled isopentane, only isopentane could be detected by gas chromatography in a residue of less than 0.5 ml.

Chickens. Stewing fowl were obtained on the day of processing from a commercial source. They were packaged in polyethylene, frozen at −30°F in a blast freezer, and stored at −10°F until used. The rancid chickens were commercially packed cut-up fryers that had been held 5 years at +10°F.

Turkeys. White fryer-roaster hens, weighing 3.7–4 kg, were processed to the ready-to-cook state and frozen in Cryovac bags at a commercial processing plant. They were stored at −10°F until used.

Isolation of volatiles for gas chromatography. Table 1 gives details pertaining to the isolation of volatiles for each experiment. The raw poultry was placed in a flask equipped with a stirrer and a side arm leading to a water-cooled condenser. The flask was usually charged with 1:2 w/w raw poultry and water. Heat was applied with a heating mantle. With the stirrer operating, the mixture was brought to a boil; then the distillate was collected until it was approximately equal to one-half the added water. The pH of the distillate was adjusted to 3.0 with 2*N* phosphoric acid and extracted four times with portions of isopentane equal to 10% of distillate volume. The isopentane extracts were combined and dried over anhydrous sodium sulfate. After the sodium sulfate was filtered off, about 95% of the isopentane was distilled off at atmospheric pressure through a 1-m column packed with glass helices. Most of the remaining isopentane was distilled off in a smaller column at a bath temperature of 55°C. The concentrate was transferred to a micro centrifuge tube with isopentane rinses. Excessive isopentane required for this transfer was evaporated directly from the tube by warming it at 35°C. The final, pale yellow concentrate was stored under nitrogen at −30°F until it was chromatographed.

When volatiles were isolated by distillation at lower temperature and pressure, the procedure was somewhat modified. A stirrer was not used. A capillary leak was used to promote smoother boiling. Additional traps were used—one cooled with ice and the other with dry ice-alcohol. After distillation, the total condensates were combined and worked up as usual.

Identification of *n*-2,4-decadienal and *n*-hexanal. The peaks emerging at approximately 4 and 70 minutes in chromatograms of fresh and rancid chicken and in fresh turkey were respectively

Table 1. Type and amount of poultry tissue used for each experiment and conditions under which cooking and/or distillation were carried out.

Experiment	Poultry (g) (uncooked state)	Water added (kg)	Cooking and/or distillation conditions: Hours	Cooking and/or distillation conditions: °C	Distillate (ml)
I. Low temperature	1945 [a]	3.0	6.0	23–28 [b]	1330
High temperature	1945 [a]	3.0	4.0	100	1330
II. Skin and skin fat	445 [c]	3.0	4.0	100	1700
Lean meat	1945 [a]	3.0	4.0	100	1330
III. Air	1500 [d]	3.0	4.5	100	1800
Nitrogen	1500 [d]	3.0	4.5	100	1800
IV. Fresh chicken	6542 [e]	8.0	10.0	100	4800
Rancid chicken	2722 [f]	6.0	6.3	100	3000
V. Turkey	7870 [g]	8.0	10.0	100	4500
Chicken	6542 [e]	8.0	10.0	100	4800
VI. Turkey	4704 [h]	4.7	3.0	90–95	110
Chicken	4188 [h]	4.2	3.0	90–95	155

[a] Leg and breast muscle, equivalent to 4 chickens, ground and mixed.
[b] Distilled at 21–26 mm Hg pressure.
[c] Skin and skin fat, equivalent to 4 chickens, ground and mixed.
[d] Leg and breast muscle from 3 chickens, ground.
[e] Bone, skin, and muscle from 6 chickens.
[f] Bone, skin, and muscle from 3 chickens.
[g] Bone, skin, and muscle from 3 turkeys.
[h] Leg and breast muscle, diced.

identified as *n*-hexanal and *n*-2,4-decadienal by trapping these peaks in a cold trap, reacting them with 2,4-dinitrophenylhydrazine, and characterizing the 2,4-dinitrophenylhydrazone derivatives. The melting points and the ultraviolet and infrared spectra of the recrystallized (EtOH) derivatives from the peaks at 4 and 70 minutes were respectively identical with those of authentic *n*-hexanal and *n*-2 *trans*, 4 *trans*-decadienal-2,4-dinitrophenylhydrazones (Forss and Hancock, 1956; Hoffmann and Keppler, 1960; Stitt *et al.*, 1960; Bailey and Nonaka, to be published). In addition, mixed melting points with the authentic derivatives showed no depression, and the authentic aldehydes showed retention times corresponding to these peaks.

Recovery of *n*-2,4-decadienal from chicken. Authentic *n*-2,4-decadienal (0.1 g), prepared as previously described (Pippen and Nonaka, 1958), was mixed with 1870 g of ground, raw chicken leg and breast muscle. Water (3:1) was added, and vacuum distillation was carried out until 1600 ml of condensate was obtained (3½ hr, 21–23 mm Hg, 21–24°C). The condensate was then extracted with isopentane and worked up as usual.

Gas chromatography. The detectors in the gas chromatography apparatus contained four thermal conductivity cells in matched pairs. The detector and injector systems were maintained at 200°C. The 8-ft × ¼-in. stainless-steel column was packed with 15% Tween 20 on 100–120-mesh firebrick and maintained at 150°C. Before runs, the helium flow rate was adjusted, if necessary, to reproduce the retention times of authentic compounds. The concentrates were adjusted, just before injection, to a volume of approximately 100 μl by addition or evaporation of isopentane. To give the chromatograms shown in experiments I through V, a third of this solution was injected.

In experiment VI, chromatography was carried out on an apparatus equipped with dual hydrogen flame detectors, as described by Buttery and Teranishi (1961). The 12-ft × ⅛-in. stainless-steel column was packed with 20% Apiezon M on firebrick. Nitrogen carrier gas was introduced under 15 psi, hydrogen under 18 psi, and air under 30 psi. The potential between electrodes at the burners was 600 v, and the recorder was operated at 2.5 mv full-scale sensitivity.

RESULTS AND DISCUSSION

Isolation of volatiles for gas chromatography. Arbitrary steps must be used to isolate a dry concentrate of volatiles required for use in a gas chromatography apparatus equipped with thermal conductivity detectors. The isolation steps used are shown in Fig. 1. Isopentane extraction removed most, but not all, of the odor in the distillate. Varying the pH of the distillate between 10 and 3, by addition of sodium hydroxide or phosphoric acid prior to isopentane extraction, had no effect on the nature of the concentrate, as determined by informal sensory observations and by gas chromatography. Before extraction, distillates were routinely adjusted to pH 3 to alleviate a tendency toward emulsion formation. During concentration of the isopentane extract, portions of the more volatile components, such as acetaldehyde and hydrogen sulfide were lost. The concentrates obtained from cooked poultry were highly odorous. Such concentrates lacked some characteristic aroma notes; nevertheless, at proper dilution in air, their odor remarkably resembled the original cooked poultry.

Experiment I. Effect of temperature on the volatile fraction. The difference in aroma between raw and cooked chicken is obvious. Therefore, it is of interest to determine how cooking affects the chemical composition of chicken volatiles. During low temperature distillation, the chicken retained its raw characteristics, whereas the chicken was thoroughly cooked during distillation at 100°C. Volatiles from uncooked chicken were very small and essentially odorless, whereas volatiles from cooked chicken were relatively large and highly odorous.

Gas chromatograms confirming these observations are shown in Fig. 2. In curves I and II, a large peak in the region of 2–3 minutes is due primarily to the solvent, isopentane. In the portions of the curves beyond 3 minutes, only 2 or 3 very minor peaks can be seen in curve I (low temperature distillation) whereas many substantial peaks are present in curve II (high temperature distillation).

n-2,4-Decadienal is one of the higher-boiling components observed in the volatile fraction obtained by distillation at 100°C. An experiment demonstrated that *n*-2,4-decadienal added to uncooked chicken could be recovered by low temperature distillation (Fig. 2, curve III). This demonstrates

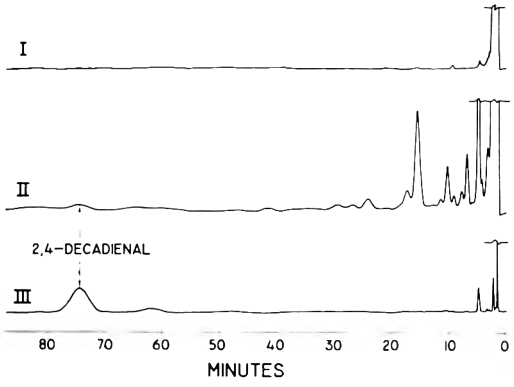

Fig. 2. Experiment I. Gas chromatograms showing the effect of temperature on yield and nature of the volatile fraction of chicken and the recovery of authentic *n*-2,4-decadienal after its addition to chicken. Curve I. Raw chicken distilled at 23–28°C, 21–26 mm Hg. Curve II. Chicken, initially raw, distilled at 100°C. Curve III. Raw chicken plus *n*-2,4-decadienal (added at a level of approximately 50 ppm) distilled at 21–24°C.

that higher-boiling volatiles, if present, can be distilled readily from uncooked chicken by low temperature, vacuum distillation.

Except for the *n*-2,4-decadienal peak in curve III, curves I and III should be alike. They are practically alike except for peaks at 2, 5, and 62 minutes which are present in curve III but scarcely discernible or absent in curve I. These peaks in curve III apparently represent decomposition products of authentic *n*-2,4-decadienal that arise during chromatography, for they appear even when chromatographically pure *n*-2,4-decadienal is rechromatographed. It is evident that relatively little solvent was present in the volatiles used for curve III; otherwise, the peak at 3 minutes would have been obscured by the solvent peak.

Thus it is clear that raw chicken yielded a negligible amount of volatiles, whereas cooked chicken gave a relatively large volatile fraction of considerable complexity. This demonstrates that the volatiles isolated from the cooked chicken were heat-produced.

Experiment II. Volatiles from lean meat and from skin and skin fat. There is general agreement among investigators that lean muscle tissue is one of the important sources of chicken flavor (Bouthilet, 1951; Kazeniac, 1961; Pippen *et al.*, 1954). Those same investigators found it difficult to establish whether fat or fatty tissue has any direct effect on chicken flavor. Pippen *et al.* (1954) indicated that chicken fat contributes to the aroma of chicken broth. Therefore it was of interest to determine what differences could be observed in the yield and composition of the volatiles of lean and fatty tissues.

For this experiment, leg and breast muscles were used as sources of lean tissue. Subcutaneous fat was selected to represent fatty tissue and there was a substantial amount of it in the fowl used in this experiment. Since it was not practical to make a clean separation of subcutaneous fat from skin, the latter was included.

In Fig. 3, the total area under the peaks is greater for curve I than for curve II.

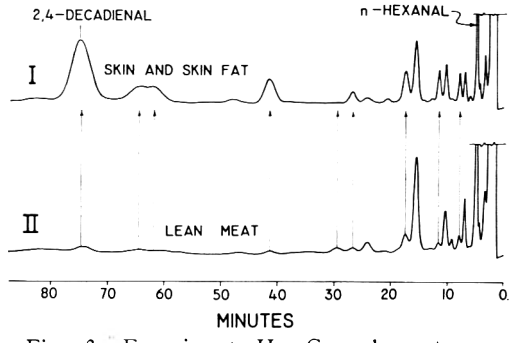

Fig. 3. Experiment II. Gas chromatograms comparing volatiles from fatty and lean tissues. Curve I. Skin and skin fat (chicken). Curve II. Lean chicken meat (leg and breast, mixed).

Since the ratio of the two types of tissue used was the same as that occurring in the oven-ready carcass, it can be concluded that the yield of volatiles per carcass was greater from skin and skin fat than from lean meat. On a weight basis this difference would be greater by a factor of about 4 since, per carcass, there was more than four times as much lean meat as skin and skin fat. The greater yield of individual volatiles from skin and skin fat is reflected in the size of corresponding peaks in the two curves; where there are obvious differences (connected by arrows), the fraction from skin and skin fat is larger 8 times out of 9. The identification of two of the larger peaks as *n*-hexanal and *n*-2,4-decadienal confirms previous findings (Pippen *et al.*, 1958; Pippen and Nonaka, 1960). The much greater

yield of *n*-2,4-decadienal from the fatty tissue of skin than from lean meat also confirms that this aldehyde originates in the fat (Lineweaver and Pippen, 1961).

There is relatively little qualitative difference between the curves. This is not surprising, since lean chicken meat contains an appreciable amount of fatty tissue, which could be expected to give rise to volatile components similar to those from subcutaneous fatty tissue. Fat analyses revealed that about 3 times as much fat was present in the 445 g of skin and skin fat used to obtain curve I as there was fat in the 1859 g of lean meat used to obtain curve II. This strongly suggests that fatty tissue is the major source of volatile components. The most apparent difference between the curves is the presence of a peak at about 29 minutes in lean meat and its absence in skin and skin fat.

Experiment III. Effect of oxygen on the volatile fraction. Previous results (Pippen *et al.*, 1958; Pippen and Nonaka, 1960) have shown that passing air through boiling chicken substantially increases the yield of volatile carbonyls over the yield obtained under more normal cooking conditions. To determine if a similar effect could be detected by gas chromatography, volatiles were isolated from chicken boiled in air and nitrogen atmospheres which were achieved by passing the gases through the mixture during the cooking-distillation period. The results of gas chromatography are shown in Fig. 4. Curves I and II are directly comparable. It is evident that there are more peaks in curve II (air) than in curve I (nitrogen) and that the area under peaks in curve II (air) is much greater than the area under peaks in curve I (nitrogen). It can be concluded that these results show that chicken boiled in air, compared to chicken boiled in nitrogen, yielded a more complex and much larger volatile fraction. It is particularly evident that the peak at about 74 minutes in curve II (air) is much larger than the corresponding peak in curve I (nitrogen). Since, as shown elsewhere, this peak can be attributed to *n*-2,4-decadienal, it is evident that oxygen availability is an important factor governing the production of this compound.

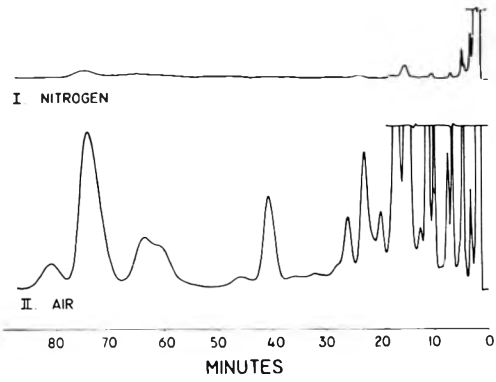

Fig. 4. Experiment III. Chromatograms illustrating the effect of oxidative and inert atmospheres during cooking on the yield and nature of the volatile fraction. Curve I. Volatiles isolated from chicken cooked at 100°C with nitrogen passing through the cooking mixture. Curve II. As in I, except air was passed through the cooking mixture.

While these results leave no doubt that oxygen availability during cooking can have a large effect on chicken volatiles, it will be necessary to have additional information before it can be concluded whether this factor influences the volatile fraction to an appreciable extent at a practical level.

Experiment IV. Comparison of the volatiles of fresh and rancid chicken. Since rancidity in cooked poultry is readily detected by the sense of smell, some of the components responsible are volatile. However, little is known about their chemical nature. Therefore, volatiles of fresh and rancid chicken were isolated and chromatographed.

It was evident during isolation that the volatile fraction from rancid chicken was larger and had greater odor potency than the volatile fraction from fresh chicken, and the greater yield was confirmed by chromatography. In Fig. 5, the area is considerably greater under curve I than under curve II. Corresponding peaks in curves I and II are, with few exceptions, larger in the curve for rancid chicken volatiles. Since 2.4 times more fresh than rancid chicken was used (Table 1, experiment IV), the difference in yield of volatiles on an equal-weight basis would be even greater than that shown in Fig. 5.

n-Hexanal and *n*-2,4-decadienal were identified in two of the larger peaks (Fig. 5,

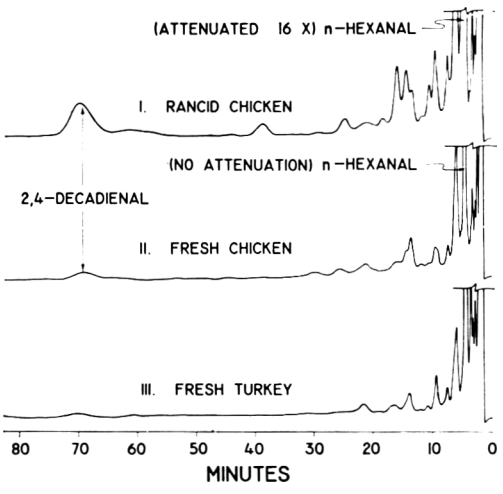

Fig. 5. Experiments IV and V. Chromatograms of the volatile fractions isolated from rancid chicken, fresh chicken, and fresh turkey.

curves I and II), respectively, at retention times of about 4 and 69 min. The much greater amount of *n*-hexanal in rancid than fresh chicken is not as apparent in Fig. 5 as it might be, for space requirements do not permit showing the entire peak. The difference in the amount of *n*-hexanal can be realized better by noting that no attenuation was required in curve II, whereas a 16-fold attenuation was required in curve I to keep the *n*-hexanal peak on the chart. The difference in size in the *n*-2,4-decadienal peaks in rancid and fresh chicken is obvious. These results indicate that *n*-hexanal, *n*-2,4-decadienal, and probably other carbonyl compounds contribute to rancid flavor of chicken. Synthetic *n*-2,4-decadienal has been reported to have a "deep-fat-fried aroma" (Patton *et al.*, 1959). We have noticed also that it can have a desirable odor reminescent of cooked fat or of fried chicken. However, *n*-2,4-decadienal, on exposure to air at room temperature, develops first stale and then rancid odors. Thus, this compound may contribute to desirable aroma but may also be an immediate precursor of stale or rancid odors.

There are many similarities between the curves for rancid chicken (Fig. 5, I) and for chicken cooked in air (Fig. 4, II), suggesting that oxygen availability during cooking rapidly produces many of the same volatile compounds that form slowly as chicken becomes rancid during storage. Most of the peaks in the curve for fresh chicken (Fig. 5, II) are also present in an amplified state in the curves for rancid chicken (Fig. 5, I) and for chicken cooked in air (Fig. 4, II). This suggests that rancid flavor is due primarily to an increase in the amount of the volatile constituents observed in fresh chicken, instead of the appearance of new volatile components. Thus, below a certain level, volatiles may contribute to desirable flavor; above it, they may be recognized as "off" or rancid.

Experiment V. Comparison of chicken and turkey by a sensory method and by gas chromatography. In sensory evaluation (triangle tests) of chicken and turkey broths, 32 of 40 judges were able to detect a difference between the broths, and of those making correct judgments, 21 were able to identify the species from which the broth was prepared. These results are statistically significant. Thus chicken and turkey broth flavors are distinguishable by sensory methods.

Gas chromatography of turkey and chicken volatiles (Fig. 5, curves II and III) reveals that differences do exist even though these curves are generally similar. For example, in chicken peaks at 26, 29, and the shoulder on the peak at 14 minutes are not seen in turkey. Furthermore, the peak at 61 minutes is more distinct in turkey. Further studies are needed to determine more specifically the nature of the differences and to ascertain if they are related to the distinctive flavors of chicken and turkey.

Experiment VI. Chromatography on columns equipped with dual hydrogen flame detectors. The great sensitivity of the hydrogen flame detector to most organic compounds and its lack of sensitivity to water have made it possible to sample vapor directly in flavor studies on potatoes and fruits (Buttery and Teranishi, 1961). The method was therefore tested on poultry.

Volatiles from the chicken and turkey broth used for the sensory evaluations were compared. While these meat and water mixtures were simmering, vapors rising from the cooking vessel were cooled and condensed with an ordinary tap-water-

cooled condenser. The condensates collected amounted to only 2.2 and 3.6% of the weight of turkey and chicken, respectively (Table 1). The condensates were stored in stoppered containers, with a head space amounting to approximately 25% of condensate volume.

Vapor in the head space above the condensate was withdrawn with a syringe and injected directly into the apparatus. Only 10 ml of vapor over the condensed distillates provided enough material to yield the chromatograms shown in Fig. 6. The column

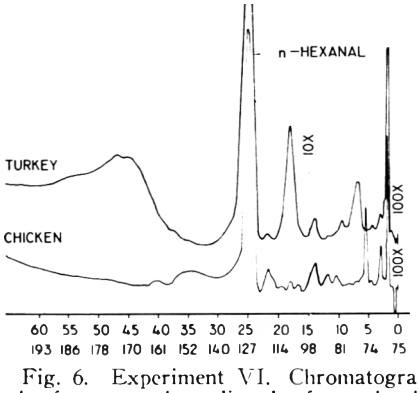

Fig. 6. Experiment VI. Chromatograms of 10 ml of vapor taken directly from the headspace over distillates of chicken and turkey and injected directly into a column equipped with dual hydrogen flame detectors.

temperature was increased to facilitate observation of both low and high boiling fractions within a reasonably short time. No peaks were evident after 60 minutes. Beyond 15 min, peak sizes in both curves must be reduced by a factor of 10 to make them comparable to peaks in the area from zero to 15 min. The prominent peak at 25 min was tentatively identified as *n*-hexanal. Identification was based on the observations that authentic *n*-hexanal had a similar retention time and is known to be a major component of poultry volatiles.

These results illustrate the difference between chicken and turkey volatiles better than the results obtained with thermal conductivity detectors (Fig. 5). In the region from 2 to 25 min (Fig. 6), 8 distinct peaks can be found for turkey, 13 for chicken. Apparently, chicken volatiles are more complicated than turkey volatiles in the lower boiling region. In the region beyond 25 min, more volatile material is indicated in turkey than chicken. Determination of the flavor significance of these differences and the specific chemical components involved will require additional study.

The significance of this experiment extends beyond the limited data obtained. The experiment shows that poultry volatiles, like fruit and vegetable volatiles (Buttery and Teranishi, 1961), can be directly and rapidly sampled and quickly analyzed. This method should make it possible to carry out basic studies on the composition of poultry volatiles that would be impossible or impractical by other methods.

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Distribution of Pesticides in Fermentation Products Obtained from Artificially Fortified Grape Musts

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SUMMARY

None of 13 pesticides added to grape musts had any measurable effect on fermentation, but their distribution and relative concentrations in the end-products showed wide variation. Five cholinesterase-inhibiting insecticides (demeton, malathion, parathion, Phosdrin, and Sevin) were still present in the finished wine, whereas three cholinesterase-inhibiting insecticides (ethion, Diazinon, and Trithion) and all of the chlorinated insecticides tested (DDT, chlordane, Kelthane, Tedion, and endrin) were not detected in the wines. It was definitely established, however, that all the chlorinated compounds tested were in the sediments or lees removed after fermentation. Three cholinesterase-inhibiting compounds (ethion, parathion, and Trithion) were also more concentrated in the lees than in the original musts. Diazinon was not found in any component after fermentation, probably because it is hydrolyzed in acidic solution. Chlordane, DDT, endrin, Kelthane, Tedion, ethion, malathion, parathion, and Trithion were detected in the distillates from the lees containing these compounds, chlordane being considerably more concentrated than in the original must.

Extended use of chemicals on agricultural crops to be processed in some manner before consumption has created a need for studies on the fate and distribution of these compounds in the finished commodities. Of the large variety of agricultural chemicals used, pesticides currently command considerable attention. After application these compounds, depending on their chemical and physical properties, may be removed, degraded, metabolized, or carried over unchanged into the end-products.

Although numerous residue data have been published for grapes sprayed with various insecticides, the authors are aware of only one report on wine produced from grapes sprayed with a pesticide.[a] Zweig and Archer (1958) found that wine made from grapes sprayed with a formulated mixture of Sevin and sulfur contained a detectable quantity of Sevin.

Research was conducted to determine the effect of pesticides on yeast fermentation through the preparation of wines and to determine the fate and distribution of these chemicals in the fermentation products. Thirteen pesticides recommended for use on grapes were added to grape musts at twice the legal tolerance level (1.0 ppm for endrin where a zero tolerance level has been established). Each chemical was then tested with three cellar treatment variables. For purposes of analysis, the pesticides were classified into two groups: 1) chlorinated compounds, and 2) cholinesterase-inhibiting compounds.

MATERIALS AND METHODS

Preparation and treatment of wines. The wines were prepared in the winery of the Department of Viticulture and Enology, University of California at Davis, with standard procedures and carefully controlled conditions. The musts were inoculated with *Saccharomyces cerevisiae* var. *ellipsoideus*. Cellar treatment variables were: A) fermentation without other treatment; B) fermentation with SO_2 (55–75 mg/L); C) fermentation with SO_2 (55–75 mg/L) followed by bentonite fining. Used in testing chlordane, DDT, parathion, Sevin, and Trithion was Sauvignon blanc grape juice concentrate reconstituted to

[a] See Addendum, at end.

about 20° Brix prior to use. Must analysis: total acids (g of tartaric acid/100 ml) 0.49; pH 3.60; °Brix 20.0. Used in testing the other chemicals was fresh grape must from a mixture of packing-house culls of Thompson seedless and Cardinal grapes. Must analysis: total acids (g of tartaric acid/100 ml) 0.71; pH 3.52; °Brix 16.7. The rate of fermentation in each carboy was followed by daily measurement of the sugar content (°Brix).

Immediately after inoculation of the musts the pesticides, each dissolved in a small quantity of ethyl alcohol, were added to the carboys and the contents mixed by vigorous swirling. Tedion, which is insoluble in ethyl alcohol, was dissolved in acetone. A 250-ml portion of must was then removed from each carboy for residue analysis and stored at −21°C until analyzed. Samples of the lees were removed after the fermentation period and stored at −21°C. The finished wines after cellar treatment were stored at 12°C.

Methods of analysis. The pesticides were extracted from the musts, wines, lees, and distillates of the lees by a single extraction with a mixture of ethanol, ethyl ether, and pentane (b.p. 30–60°C) (Zweig and Painter, 1961); the ratio of sample to extraction solvents was, sample–ethanol–ethyl ether–pentane, 1:1:2:1 by vol. After drying with anhydrous sodium sulfate, the solvent mixture was removed *in vacuo* at or below 45°C. The residue was then made up to volume with redistilled benzene. No further cleanup procedure was necessary.

The cholinesterase-inhibiting insecticides were determined by a modification (Archer *et al.*, 1963) of the method of Giang and Hall (1951). Horse serum was used as the source of cholinesterase for determination of demeton, Diazinon, ethion, parathion, Sevin, and Trithion, and human plasma for malathion and Phosdrin.

The chlorinated compounds were determined by the paper chromatographic method of Mills (1959) as modified by Zweig and Painter (1961) to include dipping of the papers in the chromogenic reagent, made up as follows: To 1.7 g of silver nitrate dissolved in 5 ml of water, add 10 ml 2-phenoxyethanol and 1 drop of 30% H_2O_2. Make up to 200 ml. Add acetone to clear. This reagent can be used repeatedly if stored in the dark in a brown bottle. More acetone should be added if the solution becomes cloudy.

For distillation of the lees, a 100-ml sample was placed in a distilling flask connected to a water-jacketed condenser. The flask and its contents were heated by a Glas-col heating mantle until approximately one-half of the original volume was distilled-over. Because of the quantity of sediment present in the sample fortified with chlordane, only one-fourth the volume of lees was collected.

RESULTS

Effect of pesticides on fermentation. At the levels used, none of the pesticides had any effect on fermentation. Therefore, fermentation data are presented for only the Sauvignon blanc musts (Table 1).

Chlorinated pesticides. Except with chlordane, substantial amounts (85–100%) of each of the chlorinated compounds were recovered in the must samples prior to fermentation (Table 2). None

Table 1. Fermentation record of wines from Sauvignon blanc musts.

Pesticide added	Cellar treatment [a]	°Brix at (day of fermentation):							
		0	1	2	3	4	5	6	7
Parathion	A	20.0	17.4	9.0	3.8	1.0	−0.6	−1.4	−1.4
	B	20.0	18.0	8.2	4.0	1.2	−0.3	−1.5	−1.4
	C	20.0	17.9	8.3	4.1	1.3	−0.3	−1.4	−1.4
Trithion	A	20.0	17.2	7.8	3.8	1.0	−0.1	−1.3	−1.4
	B	20.0	18.0	7.9	3.6	0.7	−0.6	−1.4	−1.4
	C	20.0	18.0	8.1	3.8	1.0	−0.5	−1.6	−1.4
Sevin	A	20.0	17.2	7.8	3.7	1.3	−0.4	−1.5	−1.5
	B	20.0	18.1	8.0	4.0	1.5	−0.1	−1.5	−1.4
	C	20.0	17.9	8.0	4.0	1.1	−0.4	−1.6	−1.5
DDT	A	20.0	17.1	7.8	3.6	1.1	−0.1	−1.5	−1.4
	B	20.0	17.8	7.8	3.7	1.0	−0.5	−1.5	−1.4
	C	20.0	17.8	8.1	4.0	1.4	−0.3	−1.6	−1.4
Chlordane	A	20.0	16.8	7.5	3.8	1.3	−0.3	−1.5	−1.4
	B	20.0	17.7	7.7	3.9	1.1	−0.5	−1.6	−1.4
	C	20.0	17.6	8.1	3.9	1.2	−0.4	−1.5	−1.4
Control	A	20.0	17.1	7.9	3.7	1.2	−0.5	−1.4	−1.4

[a] A, fermented without other treatment; B, fermented after addition of SO_2; C, fermented after addition of SO_2 and fined with bentonite.

Table 2. Distribution of chlorinated pesticides in wine fermentation products.[a]

Cellar treatment [b]	Pesticide added (common or trade name)	Must		Remaining in fermentation product		
		Added (ppm)	Recovered (ppm)	Lees (ppm)	Finished wine (ppm)	Distillate of lees (ppm)
A	Chlordane	0.6	0.3	....	0.0	....
B	Chlordane	0.6	0.3	2+	0.0	5.0
C	Chlordane	0.6	0.3	....	0.0	....
A	DDT	14	12	....	0.0	....
B	DDT	14	12	52+	0.0	8 (decomp. 2 spots)
C	DDT	14	12	....	0.0	....
A	Endrin	1.0	0.9	....	0.0	....
B	Endrin	1.0	0.9	3+	0.0	1.0
C	Endrin	1.0	0.9	....	0.0	....
A	Kelthane	10	9.0	....	0.0	....
B	Kelthane	10	9.0	30+	0.0	1.5 (decomp. 3 spots)
C	Kelthane	10	9.0	....	0.0	....
A	Tedion	10	9.0	....	0.0	....
B	Tedion	10	9.0	30	0.0	1.3
C	Tedion	10	9.0	....	0.0	....

[a] Semiquantitative from paper chromatograms.
[b] A, fermented without other treatment; B, fermented after addition of SO_2; C, fermented after addition of SO_2 and fined with bentonite.

of the chlorinated pesticides was detected in the finished wines.

Further evidence that the chlorinated pesticides were not present in the wines was obtained by analyzing by gas-liquid chromatography the must and wine from must fortified with DDT. Results obtained with a Dohrmann gas-liquid chromatograph Model G-100 indicate that neither DDT nor its degradation product, DDE, is present in the finished wine (Fig. 1).

All members of this group were concentrated in the lees, which indicates that they are removed from the wines with the sediment that settles during the fermentation period. Figs. 2 and 3 show the relative concentrations of Kelthane and Tedion in the musts, wines, and lees from the musts originally fortified with these chemicals.

Because the lees are frequently distilled in industry and because the chlorinated pesticides were concentrated in this fraction, the lees were distilled and the distillates were analyzed by paper chromatography for the chemicals. Detectable quantities of each chlorinated compound were recovered in the various distillates (Table 2). Except with chlordane, the quantities recovered in the distillates did not exceed the levels originally present in the fortified musts. Chlordane was present at about 8 times (5 ppm) the concentration in the original must (must fortified with 0.6 ppm chlordane). However, because of the large amount of sediment present in the sample, only one-fourth the volume was distilled. If one-half the volume had been distilled, as was done with the other samples, the concentration of chlordane in the distillate would have been proportionally less.

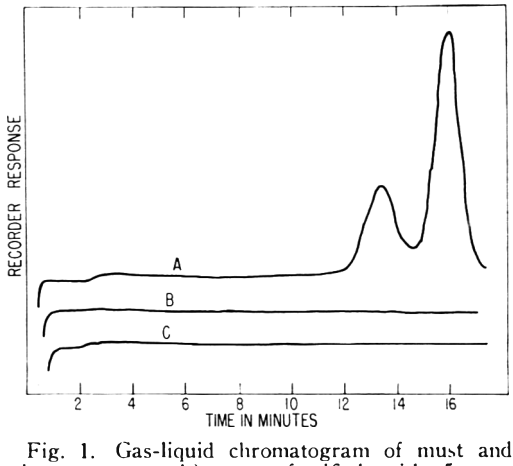

Fig. 1. Gas-liquid chromatogram of must and wine extracts: A) must fortified with 5 ppm DDT; B) must only; C) wine from must fortified with 14 ppm DDT.

Cholinesterase-inhibiting insecticides. Except for Diazinon and Phosdrin, recovery of the cholinesterase-inhibiting insecticides from the musts was good (80–100%) (Table 3). Diazinon, ethion, and Trithion were not detected in the finished wines. However, about 50% of malathion and parathion and 30% of Phosdrin were still present in the finished wines. Demeton and Sevin were present at the original level of fortification.

Also analyzed were lees from musts fortified with ethion, Diazinon, malathion, parathion, Phos-

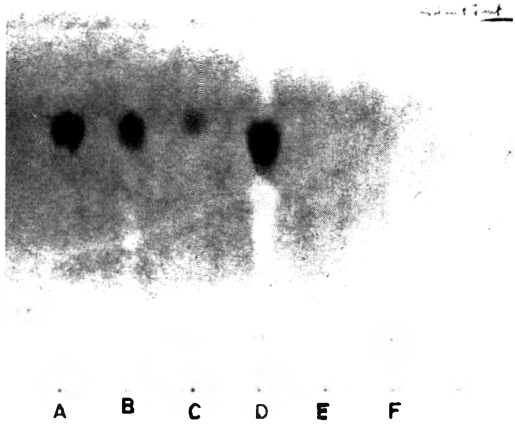

Fig. 2. Chromatogram showing relative concentrations of Kelthane present in must, wine, and lees from must fortified with 10 ppm Kelthane. A) Kelthane standard for reference (5 μg); B) 0.5 ml of must; C) 0.5 ml of wine before racking; D) 0.5 ml of lees; E) 0.5 ml of finished wine; F) 1.0 ml of finished wine.

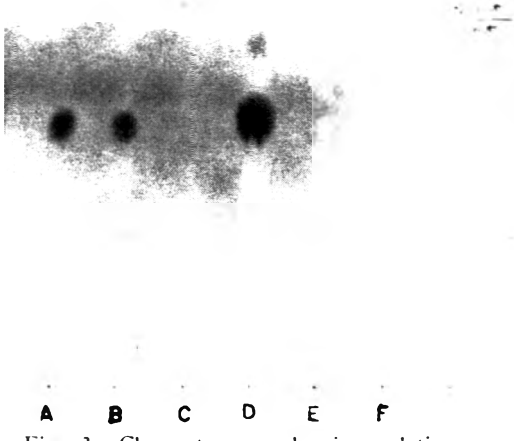

Fig. 3. Chromatogram showing relative concentrations of Tedion present in must, wine, and lees from must fortified with 10 ppm Tedion. A) Tedion standard for reference (5μg); B) 0.5 ml of must; C) 0.5 ml of wine before racking; D) 0.5 ml of lees; E) 0.5 ml of finished wine; F) 1.0 ml of finished wine.

drin, and Trithion. Ethion, parathion, and Trithion were more concentrated in the lees than in the original musts, but Diazinon was not recovered in any component after fermentation (Table 3).

The lees containing ethion, malathion, parathion, and Trithion were distilled as previously described, and the distillates were analyzed for the insecticides. The cholinesterase-inhibiting compounds were detected in the distillates, but the concentrations were not high compared to those in the original must samples, with only ethion and parathion present at levels as high as in the original musts (Table 3).

DISCUSSION

None of the pesticides added to grape musts had any measurable effect on fermentation, but their distribution and relative concentration in the end-products (finished wine, lees, and distillate) showed wide variation. The three cellar treatment variables, however, did not affect the distribution of the compounds in the end-products. The finished wines did not contain Diazinon, ethion, Trithion, or any of the chlorinated compounds, although malathion, parathion, and Phosdrin were present at 30–50% of the original concentration. Demeton and Sevin were carried through into the wines without apparent loss. This is not surprising, since Zweig and Archer (1958) have previously reported the presence of Sevin in wines. They found that grapes sprayed with Sevin produced a wine containing this chemical.

Diazinon was the only compound not found in any fraction after fermentation. Since it is slowly hydrolyzed in weakly acidic solutions (Martin, 1961), failure to find a residue may have been due to the acids present in the must.

Ethion, Trithion, and the chlorinated pesticides were present in higher concentrations in the lees than in the original musts. Many chemicals are readily sorbed by solids, and this may be the reason why these pesticides were concentrated in the lees and not detected in the clear wines. Most of the chlorinated compounds are relatively insoluble or only very slightly soluble in water, and under fermentation conditions the more insoluble chemicals might settle out with the yeast. Distillates from the lees contained detectable quantities of the pesticides present in the lees, with Kelthane and DDT showing evidence of degradation. Chlordane, which has a relatively high vapor pressure, was the only compound present at a higher concentration in the distillate than in the original must. Since many pesticides are not particularly volatile, the presence of low levels of some of these chemicals in the distillates could be due to the method of steam distillation used. Others, however,

Table 3. Distribution of cholinesterase-inhibiting pesticides in wine fermentation products.

Cellar treatment [a]	Pesticide added (common or trade name)	Must		Remaining in fermentation product		
		Added (ppm)	Recovered (ppm)	Lees (ppm)	Finished wine (ppm)	Distillate of lees (ppm)
A	Demeton	2.5	2.8	.....	2.0	.....
B	Demeton	2.5	3.0	.....	1.8	.....
C	Demeton	2.5	2.2	.....	1.9	.....
A	Diazinon	1.5	0.9	.....	0.0	.....
B	Diazinon	1.5	0.8	0.0	0.0	.....
C	Diazinon	1.5	1.0	.....	0.0	.....
A	Ethion	2.0	1.4	.....	0.0	.....
B	Ethion	2.0	1.6	4.0	0.0	2.8
C	Ethion	2.0	1.6	.....	0.0	.....
A	Malathion	16	13	.....	9.3	.....
B	Malathion	16	12	13	7.5	10
C	Malathion	16	13	.....	7.5	.....
A	Parathion	2.0	1.7	.....	1.2	.....
B	Parathion	2.0	1.7	4.0	1.1	2.0
C	Parathion	2.0	1.6	.....	0.9	.....
A	Phosdrin	1.0	0.6	.....	0.3	.....
B	Phosdrin	1.0	0.5	0.5	0.3	.....
C	Phosdrin	1.0	0.5	.....	0.3	.....
A	Sevin	20	20	.....	20	.....
B	Sevin	20	20	.....	19	.....
C	Sevin	20	18	.....	20	.....
A	Trithion	1.6	1.1	.....	0.0	.....
B	Trithion	1.6	1.1	10	0.0	0.6
C	Trithion	1.6	1.0	.....	0.0	.....

[a] A, fermented without other treatment; B fermented after addition of SO_2; C, fermented after addition of SO_2 and fined with bentonite.

are extremely volatile and would be expected to be distilled from the lees.

No attempt was made to identify the pesticide degradation products formed during wine fermentation.

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ADDENDUM

After this paper was submitted for publication a report on Studies of Insecticide Residues on Grapes and in Wines by George W. Still and Jack E. Fahey, United States Department of Agriculture, Agricultural Research Service—33-81 (Jan. 1963), was released, which includes data on lead, arsenic, DDT, parathion, EPN, and methoxychlor.

The Organic Constituents of Food. I. Lettuce

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ABOUT THE SERIES

This is the first of a series of review articles that will endeavor to outline the detailed chemical composition of common foodstuffs so far as it is known. In most instances, this important information has not been compiled previously. It is intended that the present effort should underscore areas where further chemical research is needed, provide a basis for physiological and toxicological study of food components, and suggest the identity of some of the host of reported but unidentified food constituents.

Fulfillment of this last aim, however, must usually await further research. Almost every vegetable and fruit, for instance, can claim its share of "unidentified factors," "growth substances," and "bactericidal fractions." Rather than attempt to catalog this confusing and incomplete mass of data, the present series will discuss primarily those organic constituents whose identity and occurrence have been clearly proved or for which very strong evidence exists. The original literature has been consulted in this regard, and every case that might be open to question has been carefully investigated.

In general, the data presented here are qualitative rather than quantitative. Many factors can influence the level of a particular plant constituent. Among the ones most commonly recognized are genetic variety, degree of maturity, temperature, light, moisture, and soil composition. These factors generally exert less effect on the actual number and character of the chemical components, although the first two may have significance in specific instances. The state of health of the plant has been shown to govern the presence or absence of particular constituents in some cases, but it must generally be assumed that normal sources are employed for chemical examination unless specific information exists to the contrary.

Obviously, there are many ways in which the chemical constituents of food might be classified. The present arbitrary system considers separately the high-molecular-weight polymers such as protein, cellulose, and lignin, and more simple and discrete chemical entities of lower molecular weight. This second group is subdivided to distinguish between substances common to most foods (e.g., fatty acids) and those that are unusual or specific for a limited number of sources.

The unusual compounds are finally classified according to some major structural characteristic:

1) Simple aliphatic compounds (hydrocarbons, alcohols, carbonyl compounds, etc.).
2. Alicyclic compounds (terpenes, carotenoids, steroids).
3) Simple benzenoid compounds (aromatic acids, phenols, etc.).
4) O-Heterocyclic compounds (furans, coumarins, flavonoids, anthocyanins, etc.).
5) Non-protein nitrogen compounds (indoles, pyridines, porphyrins, simple aliphatic and aromatic amines, alkaloids, etc.).
6) Non-protein sulfur compounds (mercaptans, isothiocyanates, etc.).
7) Miscellaneous (compounds not included above).

Subjects are usually placed in only a single category even though they might qualify for others; this category is the one corresponding to greatest complexity (highest number in the preceding list).

No attempt has been made to present a complete bibliography on any of the chemical constituents. Where a large volume of literature exists, the most recent and definitive work has been sought; references to particularly pertinent books or reviews have been included in order to direct the reader

into current research in this field of chemistry.

As stated at the outset, one of the major purposes of this series is to provide a basis for the physiological and toxicological examination of the naturally occurring substances in common foodstuffs that may present a danger to health through continued exposure. A review of this broad subject is in progress and will be published separately.

INTRODUCTION

Lettuce (*Lactuca sativa*, L.) (Compositae) is undoubtedly one of the most important vegetables used in the United States today. The annual per capita consumption of some 16 pounds places it third among all the vegetable foods in popularity. California is the principal production area, and in 1960 the value of the crop to that state alone was $76,054,000.

The species appears to have arisen from the related wild lettuce, *L. scariola*, and has been under cultivation since the days of the great Greek and Roman civilizations, before the time of Christ. Although thousands of varieties have been introduced, they all fall within three general types: head lettuce (variety *capitata*), cutleaf lettuce (variety *crispa*), and Cos (Romaine) lettuce (variety *longifolia*). The so-called "asparagus lettuce" (variety *asparagina*) is encountered only infrequently. Chemical and nutritional investigations of lettuce seldom differentiate between forms, although there seems to be no particular reason to suppose that this lack of distinction is valid.

COMPOSITION

Macromolecular components. The macromolecular components of lettuce—a class of substances that constitute by far the greatest part of the dry weight of the vegetable—have generally been described in terms of a so-called "proximate analysis." In such an analysis of gross composition (Table 1), the term *moisture* actually represents not only water but all other easily volatilized substances, including the lower alcohols, amines, aldehydes, ketones, hydrocarbons, and many of the fatty acids. Until recent years, the chemistry of this volatile fraction has usually been ignored.

Protein is determined by multiplication of the percentage of nitrogen found in the sample by a factor (generally 6.25) that has been shown to vary considerably among plant species. Consequently, all nitrogen-containing constituents, such as nucleic acids, alkaloids, and porphyrins, are included as well as true protein. However, since the protein generally constitutes a large proportion of the nitrogenous solid matter, the error so introduced is probably comparatively small in most cases. Although lettuce proteins represent some 25% of the dry weight of a typical sample, no further characterization of these substances appears to have been made. In fact, not even the component amino acids have been investigated in detail, although it is to be presumed that the normal array would be present. The monumental compilation of the amino acid composition of food by Orr and Watt (1957) indicates analytical data for only three amino acids—methionine, tryptophan, and lysine. When the amino acid composition of even such unusual items as amaranth, Job's tears, and prickly pear has been studied in detail, this lack of information about one of our most common foods is extremely surprising.

Carbohydrate content is usually calculated as the amount necessary to bring the sum of the component protein, ash, and fat to 100%. Obviously, figures derived in this way are open to serious error, and direct measurement of the various forms of true carbohydrate becomes of considerable impor-

Table 1. Proximate composition of lettuce (Howard *et al.*, 1962).

Variety	Water	Protein	Fat	Minerals (ash)	Total sugar	Other carbohydrates
	Percent of fresh weight					
Crisphead	95	0.8	0.1	0.15	2.2	0.1
Butterhead	96	1.2	0.2	0.37	1.1	0.1
Cos	94	1.6	0.2	0.50	2.0	0.1

tance. The figures in Table 1 were obtained by actual analysis. In the most comprehensive study to date, Williams *et al.* (1940) found lettuce carbohydrates to include cellulose (8.1% of the dry weight), water-soluble hemicellulose (6.9%), water-insoluble hemicellulose (5.8%), and "available carbohydrate" (30.5%). (The factor for conversion to fresh weight was 0.047.) It must be assumed that the term "hemicellulose" includes the pectic substances, gums and mucilages, noncellulosic polysaccharides, polyuronides, and lignin.

The same authors considered "available carbohydrate" to consist of simple reducing sugars such as glucose; fructose; and substances such as sucrose and starch which readily yield reducing sugar on mild hydrolysis. The work of Myers and Croll (1921) helps to define these carbohydrates further by indicating that only "reducing sugar" and "nonreducing sugar" are present; apparently starch is not found in analytically significant amounts. However, beyond this, there seems to be no information about the simple carbohydrates of lettuce.

One other contribution to the subject (Elwell and Dehn, 1939) provides data on lettuce pectins. Principally by alcohol precipitation, water-soluble pectin (4.33% of dry weight), easily hydrolyzed protopectin (9.34%), and difficultly hydrolyzed protopectin (11.32%) were shown to be present. In this case, determination of pectic acids was not carried out.

No information has been published on other macromolecular components such as nucleic acids, lignin, or tannins. Although several other latex-bearing members of the Compositae, notably guayule (*Parthenium argentatum*) and a dandelion (*Taraxacum kok saghyz*), have been found to contain economically important amounts of rubber (a polyterpene), no investigation of this material in lettuce has been reported.

Common components of low molecular weight. Despite the probability that lettuce contains a full complement of common pentoses, hexoses, disaccharides, and other simple sugars, only the cyclic hexitol inositol has actually been shown to occur (Zellner, 1926). Likewise, no specific fats or fatty acids have been isolated or even indicated to be present, although the seeds of wild lettuce (*Lactuca scariola*) yielded both glyceryl trilinoleate (trilinolein) and a mixture of caproic, palmitic, stearic, arachidic, oleic, and linoleic acids (Dhingra and Pershad, 1945). The term *fat* in the proximate analysis (Table 1) generally includes all ether-extractable substances, but significant quantities of true fats and related substances unquestionably constitute a major proportion of this fraction.

Several other common acids have been reported. Malic, oxalic, citric, and levulinic acids were isolated by formation of the insoluble lead salts, esterification, and conversion of the distilled esters to hydrazides (Nelson and Mottern, 1931). The first three of these acids have also been detected in lettuce by other reliable means (Andrews and Viser, 1951; Hartman and Hellig, 1934). The presence of lactic acid has been suggested (Karrer, 1958), but no experimental evidence could be located.

Lettuce appears to contain the usual number of vitamins (Table 2), although very few have actually been isolated. The lack of information on vitamin D is perhaps understandable, but the absence of reference to vitamin K is surprising in view of the amount of research conducted on this substance in other leafy vegetables. The pro-

Table 2. The vitamins of lettuce (Burton, 1959; Mattice, 1960).

Vitamin	μg/100 g	Vitamin	μg/100 g
A	162	Folic acid	4–54
D	....	Pantothenic acid	110
E	500	Riboflavin	80
K	....	Niacin	200
C (Ascorbic acid)	8000	Biotin	3.1
B_1 (Thiamine)	40	Choline	
B_6 (Pyridoxine)	71		
B_{12}			

portions of B vitamins are normal; vitamin B_{12} has not been reported, but the "antipernicious anemia factor" now associated with this vitamin has been suggested to occur (Cary and Hartman, 1948) despite the strong possibility of contamination due to B_{12}-containing microorganisms and the failure of many subsequent investigations to confirm its existence in green leafy vegetables. Choline and *p*-aminobenzoic acid have not been shown to occur in lettuce, although their presence in many other similar sources indicates that undoubtedly they are there.

In 1931, Olcott and Mattill obtained a concentrate of "vitamin E" from the unsaponifiable fraction of lettuce extract. Although they were unable to contribute substantially to investigation of the chemical structure (finally to be determined by Fernholz, in 1938), a crystalline, phenolic antioxidant corresponding to the empirical formula $C_{13}H_{14}O_5$ was isolated. Although the structure of this "inhibitol," as it was called, has never been reported, it is intriguing to speculate that it might indeed be a precursor in the still undefined biosynthetic pathway to the tocopherols.

Although lettuce has been considered to be one of the richest sources of vitamin E, comparison with other vegetables in the light of more recent data shows that it is not particularly outstanding in this regard. Harris *et al.* (1950) showed that while lettuce contains 0.43–0.54 mg/100 g of mixed tocopherols (0.29 mg *α*-tocopherol), turnip greens, for instance, contain 2.30 mg total vitamin E.

The existence of considerable amounts of free (non-protein) amino acids in the leaves of other similar plant species suggests that these compounds also exist in lettuce. However, nothing has been reported on the subject. Indirect evidence does suggest the presence of the tripeptide, glutathione. Otherwise, the literature is silent on the presence of these and the host of other compounds common to most vegetables.

Unusual or specific components. *Aliphatic and Alicyclic Compounds* (Fig. 1). Ceryl alcohol (1-hexacosanol) (I) is the only simple

$$CH_3(CH_2)_{24}CH_2OH$$
I

aliphatic compound to have been isolated from lettuce (Ichiba, 1937). It is notable that the occurrence of this compound was reported at a time prior to the general availability of refined techniques of chromatography, spectroscopy, and other major aids to present-day natural-products chemistry. The occurrence of (+)—camphor (II, Fig. 1) (not the usual (−)—form) and the closely related thujone (III) have been suggested (Karrer, 1958), but no evidence of their characterization was found.

The terpenoid pigments *β*-carotene, neo-*β*-carotene, and xanthophyll were indicated by chromatography to be present in lettuce and several other leafy vegetables (Sadana and Bashir, 1949). Carotene had actually been isolated in 1931 by Olcovich and Mattill (200 mg from 140 kg of fresh lettuce). Those authors also reported the presence of several alcohols of high molecular weight, in particular a phytosterol melting at 146° (acetate, m.p. 126°). Although no further information about this compound was pre-

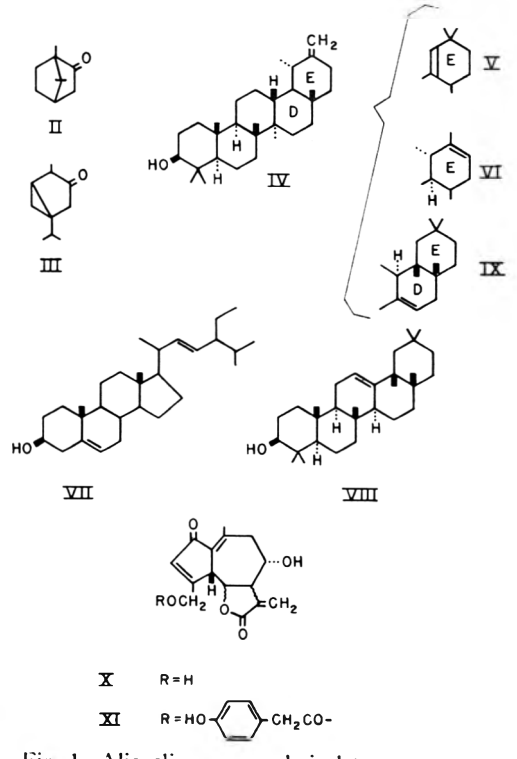


Fig. 1. Alicyclic compounds in lettuce.

sented, it is true that the melting points lie in the usual phytosterol range rather than in either the carotenoid or triterpenoid range. Although steroid mixtures may be difficult to resolve by crystallization such as that carried out by Olcovich and Mattill, the reported constants are similar to those of γ- or δ-spinasterol (Fieser and Fieser, 1949). These substances are isomers of stigmasterol (VII) in which the position of the ring unsaturation is still undefined.

In 1937, Ichiba reported the presence in lettuce of an "amyrin-like" alcohol $C_{30}H_{50}O$. The occurrence of high-molecular-weight alcohols had already been recognized for a long time, particularly in German lettuce (*Lactuca virosa*). For centuries, the dried latex from this plant (known as "Lactucarium Germanicum" or "lettuce opium") had been used throughout Europe as a hypnotic. Since 1846, many investigators have reported research aimed at the isolation and characterization of the active constituents of the drug.

Quite early, the latex was found to contain a mixture of dextrorotatory acetates that came to be known as "lactucerins" or "lactucons." Upon saponification, dextrorotatory alcohols were obtained and were described variously as α- and β-lactucerol or α- and β-lactucol. From 1836 to 1938, the formula for these lactucerols was thought at one time or another to be $C_{18}H_{30}O$, $C_{13}H_{19}O$, $C_{36}H_{60}O$, or $C_{29}H_{48}O$. During this period, there appeared in the chemical literature a large volume of information about the chemistry of α- and β-lactucerol which permitted detailed chemical structures to be assigned to the two compounds. In 1926, Zellner reported that α-lactucerol appeared to be identical with the compound known as taraxasterol (IV), but it was not until 1938 that Burrows and Simpson finally established the formula to be $C_{30}H_{50}O$.

Ten years later, Simpson (1948) reported a re-examination of the unsaponifiable fraction of "Lactucarium Germanicum," a subject which by that time had been popular for over 100 years. His results clearly showed that α- and β-lactucerols actually were extremely complicated mixtures of triterpenoid alcohols that formed constant-melting mixtures. Taraxasterol was shown to be present, though not as a major component. Chromatography on aluminum oxide also showed the presence of β-amyrin (VIII) and a new triterpene alcohol, "germanicol" (V).

With the determination of the stereochemistry of taraxasterol and its close companion pseudotaraxasterol (VI) by Jones' group (Ames *et al.*, 1954), it appears that the fiasco of lactucarium chemistry has finally found a firm footing. The alcohol mixtures from lettuce, German lettuce, chicory, and dandelion all appear to be very similar (Zellner, 1926; Simpson, 1948); only dandelion has been studied in detail. In this case, the latex unsaponifiables were shown to contain (among other things) stigmasterol (VII), sitosterol (stigmasterol with a saturated side-chain), β-amyrin (VIII), taraxerol (IX), taraxasterol (IV), pseudotaraxasterol (VI), and "taraxol" of uncertain structure (Simonsen and Ross, 1957).

However, the lactucerols, backed by the scientific publications of dozens of well-known workers over a period of more than 100 years, appear to be doomed to a slow death. In 1960 a publication appeared on the chromatography of α-lactucerol, and the 1958 compendium by Karrer on the chemical constituents of plants contains full descriptions of these compounds.

Another very interesting terpene alcohol has been found in several species of *Lactuca*, including common lettuce. This lactone, $C_{15}H_{26}O_5$, is known as "lactucin" (X). As was the case with the lactucerols, the investigation of lactucin was first reported in 1847, in connection with a study of the latex of *L. virosa*. After more than 110 years of repeated investigation, the structure of this major bitter principle was reported almost simultaneously in 1958 by British and Czechoslovakian workers (Barton and Narayanan, 1958; Dolejs *et al.*, 1958). Although its stereochemistry is known in considerable detail through investigation of benzal derivatives of the reduction product, hexahydrolactucin, the substance has not yet been synthesized.

Aromatic compounds (Fig. 2). Another bitter principle of lettuce and other *Lactuca* species (Schmitt, 1940) was described in 1862 and given the name "lactucopicrin" (XI). The occasional bitter taste of lettuce

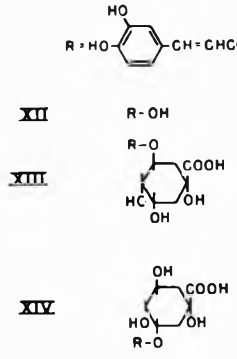


Fig. 2. Aromatic compounds in lettuce.

is probably due primarily to this compound. In 1953, Zinke and Holzer were able to show that lactucopicrin is the *p*-hydroxyphenylacetate of lactucin. In 1960 Michl and Högenauer reported that the primary rather than the secondary hydroxyl group of lactucin is esterified. It appears that the chemical structure of this interesting substance is now complete, two years short of its centenary. However, the pathway of its biosynthesis is unknown, and, in fact, *p*-hydroxyphenylacetic acid has not been observed to occur elsewhere in higher plants except in the related species *Taraxacum officinale* (Karrer, 1958).

Very few other aromatic compounds have been reported to occur in lettuce. Paper chromatography, coupled with fluorescence detection, has suggested the presence of the common aromatic compounds caffeic acid (XII), chlorogenic acid (XIII), and isochlorogenic acid (XIV) (Herrmann, 1957), and a later paper adds ultraviolet spectra to the evidence for these compounds (Butler, 1960). The crystalline phenolic antioxidant ($C_{13}H_{14}O_5$) reported by Olcott and Mattill was mentioned before; no subsequent evidence for its structure has been presented, and, in fact, its isolation was never achieved again (H. S. Olcott, personal communication).

Nitrogenous compounds (Fig. 3). Anyone who has admired a bin of lettuce at the local grocery store would probably be willing to concede that the leaves of this vegetable contain chlorophyll. However, despite the intense interest in the general subject over many years and the popularity of the chromatographic isolation of chlorophyll from leaves as a student introduction to the technique, isolation of the pigment from lettuce apparently has not been described. There can be no doubt of its presence; pheophytin (XV) (magnesium-free chlorophyll) has been isolated from 164 species of dicotyledonous plants (including lettuce) in one investigation alone (Willstätter and Oppé, 1911). In addition, both chlorophyll-a and -b have also been detected spectrophotometrically (Voskresenskaya, 1949).

Spectrophotometric examination has suggested the presence of two other porphyrins in lettuce: protoporphyrin (XVI) and a coproporphyrin (Fischer and Schwerdtel, 1926). Although those authors suggest the latter to be coproporphyrin I, an equally attractive possibility is coproporphyrin III (XVII). These substances have been found to occur widely throughout nature in such varied materials as human blood, egg shells, leaves, and bacterial cells. Considering the important place of this type of compound in the biosynthesis of chlorophyll, it is not surprising to find evidence of their occurrence in green plants. However, the difficulty encountered in distinguishing spectra of coproporphyrin I from those of coproporphyrin III, for instance, makes the importance of other methods of detection apparent (Lemberg, 1954).

Despite the astounding variety of nitrogenous organic compounds that have been found in leafy vegetables, no further information appears to be available in the case of lettuce. Even the widely sought (and widely claimed) 3-indoleacetic acid has not been positively identified in this case (Rugge and Hahn, 1957). There would seem to be no

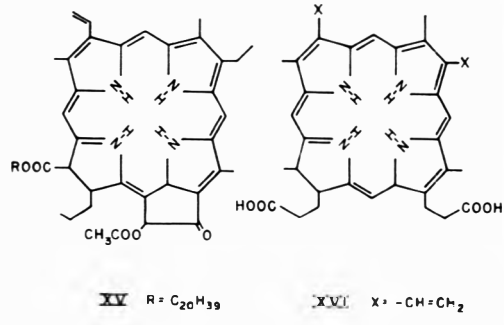


Fig. 3. Porphyrins in lettuce.

reason to doubt that such substances are here, and their presence should be of considerable interest. Their absence would be even more intriguing.

Sulfur compounds. The only unusual sulfur compound to have been isolated from lettuce is the salt of a derivative of methionine. Investigation by Shive's group of a methionine-like growth factor for microorganisms led to the isolation and characterization of *S*-methylmethionine sulfonium bromide (3-amino-3-carhoxypropyl dimethylsulfonium bromide) McRorie *et al.*, 1954). It is unlikely that this compound actually exists as the bromide in the plant cell, and it almost certainly does not exist in the hydroxide form, as suggested on several occasions. Sulfonium derivatives of methionine, *S*-methylcysteine, and other sulfur amino acids have been isolated from such diverse sources as cabbage, asparagus, and parsley (Challenger, 1959). The probable biogenetic relationship of this type of compound to the variety of sulfur-containing flavor components found in cabbage, onion, and garlic raises questions about the obvious absence of these highly odorous substances in lettuce.

DISCUSSION

Without question, the most striking feature revealed during compilation of data on the chemical constituents of lettuce is the lack of information about many types of compounds shown to occur commonly in other plant species. Information that is taken almost for granted on simple sugars, amino acids, fatty acids, lipids, sulfur compounds, simple alcohols, esters, and carbonyl compounds seems to have been ignored in the case of this common vegetable.

Even among the relatively few investigations that have been made, a high proportion are disappointingly incomplete from the standpoint of chemical identification. In view of this paucity of data, it is perhaps not surprising that no distinction has been made among the several types of lettuce in studies of their composition.

The search for uncommon or unusual components also has bypassed lettuce. Even those that have been reported were found in the course of work on other species. Actually, if the frequent observation is true that the occurrence of many types of compounds follows the boundaries of plant families, lettuce should provide a rich source of many interesting natural products.

The family Compositae, perhaps partly because it represents such a large number of species, has provided some of the most interesting problems in structure elucidation. A very small sampling of the substances isolated from composite plants is presented in Table 3 and Fig. 4. Chicory and dandelion, of course, are closely related to lettuce. The other examples shown here, however, are common plants familiar to all—one need not look far to find species that have already revealed their exciting possibilities.

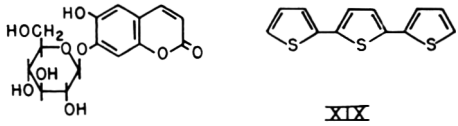
It may be argued that selection of food plants over the centuries generally has removed from human diets those that contain any appreciable amounts of undesirable constituents. Perhaps our garden lettuce has finally come down to us essentially free of the unusual and perhaps deleterious compounds so widely occurring elsewhere among the Compositae. Or has it?

ACKNOWLEDGMENTS

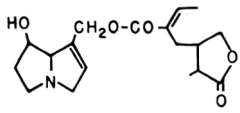
The author is indebted to Miss Phyllis Ferranti for continued assistance in many phases of this review. The project has been supported in part

Table 3. Selected chemical constituents of the Compositae.

Species	Common name	Compound	Structure	Reference
Chicorium intybus	Chicory	Chicoriin	XVIII	Merz, 1932
Taraxacum officinale	Dandelion	Taraxerol	IX	Simonsen and Ross, 1957
Tagetes erecta	Marigold	α-Terthienyl	XIX	Zechmeister and Sease, 1947
Senecio aureus	Golden ragwort	Senecionine	XX	Leonard, 1950
Centaurea cyanus	Bachelor's button	Centaur X_3	XXI	Bohlmann and Mannhardt, 1957
Dahlia variabilis	Dahlia	Aurone	XXII	Nordström and Swain, 1956
Chrysanthemum parthenium	Ox-eye daisy	Parthenolide	XXIII	Sorm, 1961



XVIII XIX



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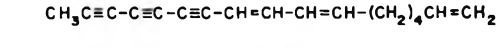

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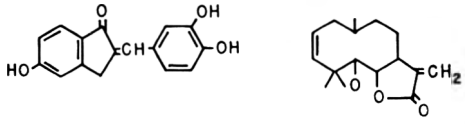

XXII XXIII

Fig. 4. Unusual constituents of Compositae.

by a Research Grant (EF-306) from the U. S. Public Health Service.

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RESEARCH NOTE

Effect of Size Classification and Maturity on the Protein Content of Alaska and Perfection Peas

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Normally, peas are selected for canning or freezing by using quality tests such as the tenderometer or shear press, or starch granule development. The selection is influenced also by the desire for certain sizes of peas. About 35% of commercially canned peas are size classified (The Almanac, 1961). Nutrition tables generally cite protein content of peas without regard to maturity or size (Watt and Merrill, 1950; Lynch *et al.*, 1959; Mellon Inst., 1959), although the protein content varies with maturity. Generally, the content of peas, fresh or canned, increases with increasing maturity while the percent protein in the dry matter decreases (Chitre *et al.*, 1950; Raacke, 1957; Zimmerman and Levy, 1962). The effects of size and variety on protein content have not been reported, although large quantities of peas are marketed on those bases. A study was made to determine the protein content of size-graded Alaska and Perfection varieties of peas separated from garden-run peas freshly harvested at various stages of maturity, as normally selected for canning by tests made with the tenderometer (Food Machinery and Chem. Corp., 1951).

PROCEDURE

Alaska and Perfection varieties of peas were grown in one-third-acre plots. One-fifth-acre areas (excluding border areas) were hand scythed at 1–2-day intervals. The peas were threshed in a commercial pea viner, taken promptly to the university pilot laboratory, washed in cold water, and size graded into USDA sizes 1–5 (sieve sizes 9/32–13/32) in a cylindrical commercial-type pea size grader (U. S. Dept. Agr., 1942; Weckel and Kuesel, 1955). Samples of size-graded and garden-run peas were quickly towel dried and weighed for analyses of protein (Kjeldahl N × 6.25), moisture (dried 5 hr at 98°C at 25 mm pressure), and alcohol-insoluble solids (AOAC, 1955). Tenderometer tests were made on samples of the various lots, although in a few instances the quantity available was insufficient for the tests. All analyses were made in triplicate.

RESULTS AND DISCUSSION

Fig. 1 shows tenderometer values, % moisture, protein, and alcohol-insoluble solids (AIS). Protein content was generally greater in the larger fresh peas than in the smaller and less in the dry matter of the larger fresh peas than in that of the smaller. This was generally true for peas within any one harvest, whether in early or late stages of maturity. The protein content of fresh peas was greater (wet basis) at advanced maturity than in earlier maturity. Protein content was considerably greater in

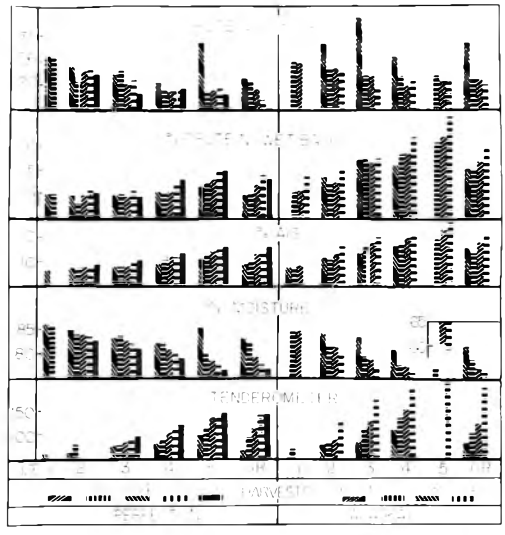

Fig. 1. Analyses of garden-run (GR) and size-graded Alaska and Perfection peas, respectively harvested at four and five stages of maturity, as used for canning or freezing.

Alaska peas than in Perfection peas, whether compared on the basis of size or stage of maturity. The protein content of the fresh peas (wet basis) paralleled the AIS and tenderometer values. In the light of the magnitude of the differences in protein content of peas of different variety, size, or period of harvest, citation of protein content in composition tables might best be qualified.

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Fish Hydrolysates. IV. Microbiological Evaluation

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(Manuscript received November 7, 1962)

SUMMARY

Microbiological evaluation was made of three experimental fish hydrolysates (peptones) prepared in this laboratory from a fresh-water species of fish, *Barbus dubious*. Ten selected microbial cultures grown in an inorganic basal medium with and without yeast or meat extract and containing different concentrations of hydrolysates, showed more or less the same growth in all the hydrolysate media. Media containing the hydrolysates were sensitive to antibiotics. The hydrolysates were quite suitable as ingredients of culture media for biochemical tests like indole and methyl-red reactions. Controls were two commercial peptones, one commercial proteose-peptone, and one commercial tryptone.

INTRODUCTION

The use of hydrolysates of fish proteins as components of bacteriological culture media has been receiving some attention. Tarr (1948) opined that it would be quite simple and perhaps cheaper to prepare peptones from fish muscle and fish waste instead of from meat. Tarr and Deas (1949) showed that a tryptic digest of fish muscle is an excellent medium for certain strict and facultative anaerobes. Akiya *et al.* (1949) used as peptone the preparation from the digestive organs of a whale. Tarr and Deas (1949), preparing fish hydrolysates by enzymes, acid and alkali hydrolyses, observed that only enzyme-hydrolyzed peptones consistently supported the growth of 6 *Streptococcus haemolyticus* cultures and one *Clostridium botulinum* culture. Kheshgi and Saunders (1959) described a method for evaluating the microbiological performance of peptones from diversified high-protein materials. The evaluation was based on growth studies of 8 pure cultures of bacteria as measured by optical densities.

All the investigations referred to above describe only a qualitative appraisal of the value of fish hydrolysates (peptones) for bacterial growth. So far, no serious attempts have been made for a quantitative evaluation. The present study attempts a quantitative appraisal of the possible value of fish flesh in preparing fish hydrolysates (peptones) for microbiological growth.

Commercial peptones are mixtures of meta-proteins, proteoses, peptones, polypeptides, and amino acids, with relatively larger proportions of peptones or polypeptides. Difco (1953) published a fairly detailed analysis of its peptones, but it is by no means clear whether there is any relation between these analyses and the ability of these peptones to support microbial growth. Another object of this study was to examine whether the composition of fish hydrolysates in terms of defined fractions of proteolysis, such as proteoses, peptones, subpeptones, etc., is related to the usefulness of these hydrolysates for microbial growth.

MATERIALS AND METHODS

Experimental fish hydrolysates. Used for evaluation were three papaic digests of fish flesh (A, B, and C) obtained from a fresh-water species of fish, *Barbus dubious*, under conditions of hydrolysis designed to obtain different amounts of proteolytic breakdown products in the hydrolysates. The fractional analysis of the hydrolysates in terms of proteoses, peptones, and sub-peptones used in the studies was the same as reported in a previous study (Sripathy *et al.*, 1963).

Commercial peptones. Two brands of peptone (I and II), and one each of proteose-peptone and tryptone, were four commercial preparations used for control growth studies.

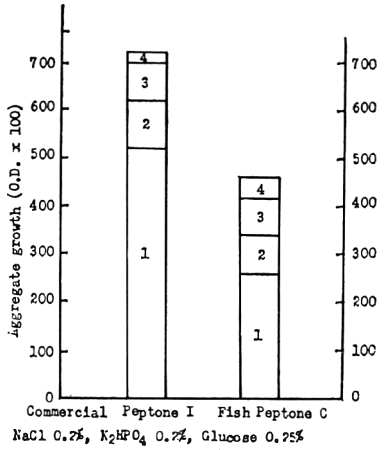
Test organisms. As far as possible, cultures producing a uniform growth or turbidity were selected. The following pure cultures of organisms (aerobic and facultative anaerobic) were used for the evaluation:

1. *Lactobacillus fermenti* (36)
2. *Leuconostoc mesenteroides* (P-60)
3. *Streptococcus lactis* (NTCC 9790)
4. *Lactobacillus arabinosus* (NTCC 17-5)

5. *Streptococcus faecalis* (8043 R)
6. *Escherichia coli* (vitamin B-12/methionine auxotrop)
7. *Lactobacillus helveticus*
8. *Streptococcus lactis* + *Leuconostoc citrovorum* (NDRI)
9. *Lactobacillus acidophilus* (NDRI)
10. *Bacillus subtilis* (NRRL B. 644).

Cultures 1, 2, 4, 5, and 7 were selected because they are used for the microbiological assay of amino acids and vitamins. Culture 3 represented lactic starter organism. Culture 6 was a gas-former, culture 8 was a mixed culture, and 9 was an important fermented milk culture, exacting in its requirements of amino acids and growth factors. Culture 10 was an aerobic spore-former with pellicle formation. Test cultures were maintained in the medium of Barton-Wright (1952), transferred twice weekly with incubation for 24 hr at 37°C.

Media. *Establishment of levels of ingredients of basal medium.* To establish the levels of various ingredients in the basal medium, 10 cultures were grown in media shown in Fig. 1. The variables



Media 1 NaCl 0.2%, K_2HPO_4 0.2%, Glucose 0.25%
Media 2 1 + $MgSO_4$. 7 H_2O(mg/100 ml); $MnSO_4$. 4 H_2O(mg/100 ml); $FeSO_4$. 7 H_2O (mg/100 ml), 1.0
Media 3 2 + additional glucose (0.25%)
Media 4 3 + additional K_2HPO_4 (0.3%)

Fig. 1. Effect of additional salts, glucose, and phosphate on aggregate growth of test cultures.

were: between media 1 and 2, additional salts; between media 2 and 3, additional glucose; and between media 3 and 4, additional phosphate. Each medium was split into 2 media, one containing commercial peptone (I) and one containing fish hydrolysate (C). The histogram in Fig. 1 shows that increased phosphate did not have any appreciable effect on aggregate growth. Additional salts (2) and glucose (3) somewhat increased the growth of cultures. However, 0.25% glucose only was used as source of energy in the experiments. Difco yeast extract (0.3 and 0.5%) was added to commercial peptone (I) and fish hydrolysate (C), and growths of test organisms were determined both in the presence and absence of peptone/fish hydrolysate. The results are shown in Fig. 2. The histogram in Fig. 2 shows that, beyond the level of 0.3% yeast extract, there were not appreciable increases in growth of test cultures, with and without peptone/fish hydrolysate. The increase in aggregate growth at 0.5% level of yeast extract was slightly more with fish hydrolysate than with commercial peptone (I). However, 0.3% yeast extract was used in the studies. In a parallel study, yeast-extract was replaced by meat extract (Lab-Lemco) at the 0.5% level, the concentration used by Difco (1953) and Mathur *et al.* (1962).

Composition of media. As shown in Figs. 1 and 2, traces of certain salts, glucose, yeast extract/meat extract, considerably increased the growth of test cultures. These were therefore included in the basal medium from which different media were derived for evaluation. Each commercial peptone/fish hydrolysate was incorporated at 0.2, 0.5, 1.0, and 2% levels in four basal media. Table 1 shows the composition of the different combinations of media used. All ingredients of media were dissolved in water, pH adjusted to 7.0, tubed in 10-ml quantities and autoclaved at 15 lb pressure for 10 min.

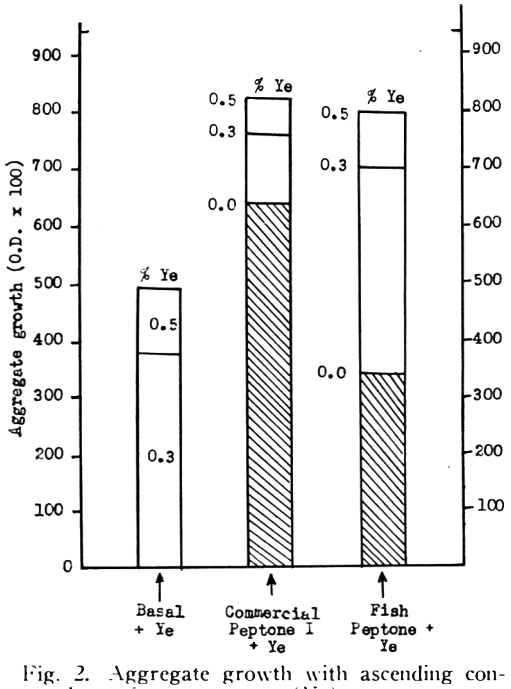


Fig. 2. Aggregate growth with ascending concentrations of yeast extract (Ye).

Table 1. Composition of media used for the evaluation of peptone/fish hydrolysate.

Basal medium	Per liter	I a b c	II a b c	III a b c	IV a b c
NaCl	2.0 g	—Basal—	—Basal—	—Basal—	—Basal—
K_2HPO_4	2.0 g				
$MgSO_4$ 7 H_2O	0.2 g				
$FeSO_4$ 7 H_2O	10.0 mg				
$MnSO_4$ 4 H_2O	10.0 mg				
% glucose		0.25	0.25	0.25	0.25
% peptone/fish hydrolysate		0.20	0.50	1.00	2.00
% yeast extract (Difco)		—0.30—	—0.30—	—0.30—	—0.30—
% meat extract (Lab-Lemco)		— — 0.50	— — 0.50	— — 0.50	— — 0.50

Inoculation and reading of cultures. Test cultures 20 ± 2 hr old were used for seeding tubes (in triplicate) of commercial peptones/fish hydrolysates included in media given in Table 1. The inoculum was transferred into 10-ml culture broths with a calibrated platinum loop (1 mm). Heat-killed inoculated cultures (triplicate) were used as blanks. Cultures were incubated at 37 ± 0.5°C for 20 ± 2 hr. Growths of test organisms were measured as optical densities (O.D.) (Kheshgi and Saunders, 1959; Pradhan and Bhat, 1960) in place of the usual lengthy viable-count technique. The cultures were shaken thoroughly so as to form a uniform turbidity and transferred into special Leitz tubes. Culture 6 was read after all gas was removed from it by stirring with a glass rod. Culture 10 formed pellicle, which somewhat interfered with the measurement of O.D. Optical density was measured in a Leitz Photometer with a 535-mμ optical filter. In the initial stages, growth was measured at 6, 20 ± 2, and 48 hr. There was not any appreciable growth at 6 hr and there was no significant difference in O.S. at 20 ± 2 and 48 hr. So, subsequent O.D. measurements were all made after 20 ± 2 hr of incubation.

Sensitivity to antibiotics was tested by serial dilution of *Micrococcus aureus* (CFTRI 509) against penicillin and aureomycin in assay media constituted of fish hydrolysates and commercial peptone (1).

Suitability of fish hydrolysates as media ingredients for indole and methyl-red tests was determined as follows: 20 ± 2-hour-old pure cultures of *Escherichia coli* (culture No. 6) in 1.0% fish hydrolysate broths were tested for indole production using Kovac's reagent (Am. Public Health Assoc., 1958) and methyl-red reaction with M.R.-V.P. media (Difco, 1953). Production of indole and methyl-red reaction of the culture in 1.0% commercial tryptone water was used as control.

RESULTS

Criteria for evaluation. It may be seen that evaluation of fish hydrolysates was based on:

1) Aggregate growths in media containing 0.2, 0.5, 1.0, and 2.0% of these hydrolysates with and without yeast/meat extract.

2) Sensitivity to antibiotics of assay media constituted of fish hydrolysates.

3) Suitability of fish hydrolysates as media ingredients for indole and methyl-red reactions.

Results of evaluation. Tables 2–5 show both individual and aggregate growths of test cultures in media containing 0.2, 0.5, 1.0, and 2.0% peptone/hydrolysate with and without yeast/meat extract. In each table, *a* represents performance of peptone/hydrolysate alone, *b* performance of peptone/hydrolysate in the presence of yeast extract, and *c* performance of peptone/hydrolysate in the presence of meat extract. Each value represents the mean of triplicate evaluations.

It will be seen from Tables 2–5 that in general, the growth of cultures in all media increased with increased concentrations of peptone/hydrolysate. However, the rates of growth were lower in fish hydrolysates than in other peptone media tested. Growth of culture no. 10 was somewhat limited in all media, probably because of its pellicle formation, which interfered with the measurement of O.D. Addition of yeast/meat extract to media resulted in increased growth of cultures, the increase being more in media with lower concentrations of peptone/hydrolysate. This was more significant in fish hydrolysate media than in others. There were no significant differences in increase of aggregate growths with yeast and meat-extract additions, although there were slight differences in growths of individual cultures.

Based on criteria of growth in different concentrations of peptone/hydrolysate, the final evaluations of commercial peptones and fish hydrol-

Table 2. Growth (O.D. × 100) of test cultures in media containing 0.2% test peptone/hydrolysate.

Test-peptone/ hydrolysate		Test cultures										Aggregate growth
		1	2	3	4	5	6	7	8	9	10	
Commercial peptone (I)	a	49	55	50	51	52	50	28	27	37	20	419
	b	77	76	80	77	69	59	71	76	76	33	694
	c	77	79	79	77	73	61	57	63	67	28	661
Commercial peptone (II)	a	21	31	21	24	25	51	15	16	18	30	252
	b	66	66	76	69	61	57	75	75	72	33	650
	c	71	73	72	66	67	59	63	67	61	21	620
Commercial proteose peptone	a	31	33	27	31	30	43	26	23	23	14	281
	b	74	72	80	72	70	59	74	79	72	23	675
	c	73	71	84	75	76	70	79	81	75	20	704
Commercial tryptone	a	47	46	28	49	47	59	21	21	20	32	370
	b	78	78	81	75	77	64	89	88	84	33	747
	c	76	76	88	80	65	61	73	69	68	28	684
Fish hydrolysate	a	15	14	16	14	13	49	17	23	12	18	191
A	b	67	65	92	69	65	67	63	71	74	25	658
	c	63	61	85	73	60	71	58	65	72	27	635
B	a	14	12	13	12	12	52	13	14	13	11	166
	b	77	75	83	75	76	73	75	71	69	34	708
	c	70	68	89	77	69	74	71	68	63	25	674
C	a	12	12	14	11	13	46	15	14	14	16	167
	b	71	70	84	71	67	67	73	71	69	29	672
	c	65	63	79	65	71	70	65	67	65	23	633

Table 3. Growth (O.D. × 100) of test-cultures in media containing 0.5% peptone/hydrolysate.

Test-peptone/ hydrolysate		Test cultures										Aggregate growth
		1	2	3	4	5	6	7	8	9	10	
Commercial peptone (I)	a	54	61	62	60	54	54	46	49	48	21	509
	b	75	85	80	82	85	77	81	73	67	34	719
	c	88	92	99	92	86	73	80	79	81	23	793
Commercial peptone (II)	a	44	44	43	45	58	59	43	41	51	20	448
	b	83	74	87	85	75	76	74	73	68	28	723
	c	73	72	81	72	78	66	87	69	64	23	685
Commercial proteose peptone	a	47	55	63	55	53	52	52	41	55	17	490
	b	71	73	89	83	86	75	75	68	63	29	712
	c	85	87	99	85	84	90	82	77	83	20	792
Commercial tryptone	a	52	53	65	57	61	62	60	40	63	24	537
	b	80	80	94	82	84	84	88	83	70	30	775
	c	77	76	98	80	72	77	83	82	70	20	735
Fish hydrolysate	a	25	24	51	29	58	54	34	33	38	16	362
A	b	76	78	86	83	73	78	85	76	68	21	724
	c	64	63	95	64	61	73	80	78	67	23	668
B	a	12	13	51	13	54	62	25	23	17	18	288
	b	75	74	84	56	59	91	72	70	64	24	669
	c	69	75	98	69	72	78	82	76	67	21	707
C	a	13	13	40	18	45	74	43	36	37	17	336
	b	76	74	82	79	76	88	84	84	78	22	743
	c	67	67	95	63	67	74	80	81	72	20	686

Table 4. Growth (O.D. × 100) of test cultures in media containing 1.0% test peptone/hydrolysate.

Test-peptone/ hydrolysate		Test cultures										Aggregate growth
		1	2	3	4	5	6	7	8	9	10	
Commercial peptone (I)	a	81	84	73	81	75	76	59	56	65	28	678
	b	81	94	86	94	85	77	78	73	67	34	769
	c	90	90	93	90	93	79	74	71	66	30	776
Commercial peptone (II)	a	78	71	84	76	77	80	57	57	48	22	650
	b	82	78	81	78	75	81	74	71	73	29	722
	c	82	84	84	95	82	81	74	73	64	30	729
Commercial proteose peptone	a	80	77	87	77	84	70	58	46	46	20	645
	b	81	79	83	80	81	73	72	68	63	29	709
	c	98	88	98	96	98	83	75	70	65	27	798
Commercial tryptone	a	79	73	81	73	72	73	46	50	47	21	615
	b	87	82	86	89	86	84	83	75	72	30	774
	c	84	84	95	88	92	84	85	79	76	28	795
Fish hydrolysate	a	27	31	58	31	21	65	41	33	32	21	360
A	b	79	79	83	79	83	73	80	65	62	28	711
	c	74	71	88	83	74	78	72	69	68	27	704
B	a	23	62	61	28	23	61	32	26	21	20	357
	b	81	81	61	78	78	81	81	69	55	27	692
	c	55	58	81	55	59	85	71	63	64	23	614
C	a	26	33	52	36	26	65	36	31	26	21	352
	b	84	80	85	82	80	80	78	70	68	28	735
	c	79	73	83	78	73	82	79	69	65	30	711

Table 5. Growth (O.D. × 100) of test cultures in media containing 2.0% test peptone/hydrolysate.

Test-peptone/ hydrolysate		Test cultures										Aggregate growth
		1	2	3	4	5	6	7	8	9	10	
Commercial peptone (I)	a	85	84	95	84	84	84	68	70	64	28	746
	b	91	90	99	93	92	90	81	81	93	32	842
	c	88	87	98	90	86	93	78	76	69	31	796
Commercial peptone (II)	a	80	80	72	84	84	84	75	74	69	24	726
	b	91	91	97	96	98	96	84	82	71	29	835
	c	83	80	89	87	89	91	79	73	67	30	768
Commercial proteose peptone	a	89	88	94	86	88	80	61	69	65	35	755
	b	93	98	98	91	98	97	83	83	77	33	851
	c	87	81	93	88	91	89	76	72	70	32	779
Commercial tryptone	a	78	74	91	73	76	83	73	68	66	21	703
	b	89	85	97	87	90	92	82	80	74	30	806
	c	81	83	91	89	88	87	77	70	64	29	759
Fish hydrolysate	a	27	27	91	25	21	70	53	61	46	19	440
A	b	57	53	99	54	41	90	81	79	61	23	638
	c	54	49	87	56	45	80	79	77	61	38	626
B	a	26	22	90	32	22	64	55	53	53	23	440
	b	84	64	98	82	84	87	80	82	71	31	763
	c	63	52	88	78	82	84	75	80	71	28	701
C	a	47	43	94	36	44	74	65	65	51	27	546
	b	53	68	98	37	43	75	75	80	61	41	631
	c	56	61	92	53	47	86	73	78	60	34	640

ysates are given in Table 6 in descending order of over-all growth performance. It is seen that there was no significant difference in the performance of commercial peptones. Fish hydrolysates were only next to commercial peptones in extent of microbial growth promotion. Fish hydrolysates did not differ in themselves in performance, thereby showing that the composition of these hydrolysates in terms of proteoses, peptones and subpeptones did not affect their abilities to support microbial growth.

The sensitivity to penicillin and aureomycin as indicated by zones of inhibition are shown in Table 7. From the data, it is observed that the extents of inhibition against the antibiotics were more or less the same for fish hydrolysates and commercial peptone (I).

Escherichia coli (culture no. 6) gave good indole and methyl-red reactions in fish hydrolysate broths.

DISCUSSION

Peptones/protein hydrolysates are such complex materials, that it will be impossible to develop a foolproof method for the evaluation of all peptones. Quite often, a peptone/hydrolysate that is suitable for the growth of an organism may not be suitable for the production of a desired end-product of the same organism, and vice versa.

The methods and materials used in the manufacture of different peptones are not usually available. It is quite possible that some peptones are fortified with substances that are foreign to them (Kheshgi and Saunders, 1959). This is probably the reason why commercial peptones have given better growth than fish hydrolysates. In the light of newer knowledge of nutrition, it would be reasonable to assume that the ability of a given peptone or protein hydrolysate to promote microbial growth would depend more upon its content of essential amino acids, B-vitamins, and other growth factors than merely on the degree of hydrolysis of protein used in its preparation. That it is so, has been shown by the results of the experiment. It is quite possible that fish hydrolysates are either deficient in some growth-factors or contain certain growth inhibitors (Traxler and Lankford, 1957).

It may be seen that although fish hydrolysates were only second to commercial peptones in microbial growth promotion, they were on a par with commercial pep-

Table 6. Comparative performance of test peptone/hydrolysate based on growth of test cultures in different concentrations.

Test peptone/ hydrolysate	Aggregate growth (O.D. × 100)												Sum of aggregate growth					
	0.2%			0.5%			1.0%			2.0%			0.2%	0.5%	1.0%	2.0%		
	a	b	c	a	b	c	a	b	c	a	b	c	a+b+c	a+b+c	a+b+c	a+b+c		
Commercial peptone (I)	419	694	661	509	719	793	678	769	776	746	842	796	1774	2021	2223	2384		
Commercial proteose peptone	281	675	704	490	712	792	645	709	798	755	851	779	1660	1994	2152	2385		
Commercial tryptone	370	747	684	537	775	735	615	774	795	703	806	759	1801	2047	2184	2268		
Commercial peptone (II)	252	650	620	448	723	685	650	722	729	726	835	768	1552	1856	2101	2329		
Fish hydrolysate A	191	658	635	362	724	668	360	711	704	440	638	626	1484	1754	1775	1704		
B	166	708	674	288	669	707	357	692	614	440	763	701	1548	1664	1663	1904		
C	162	672	633	336	743	686	352	735	711	546	631	640	1467	1765	1798	1817		

Table 7. Sensitivity limits (zone of inhibition in mm at 24 hr) to antibiotics in assay media constituted of fish hydrolysates vs. commercial peptone (I). Test organism: *Micrococcus aureus* (CFTRI 509).

Assay medium		Penicillin (μg/ml)				Aureomycin (CTC) (μg/ml)			
		10.0	1.0	0.1	0.01	10.0	1.0	0.1	0.01
Commercial peptone		27	22	Slight	....	15	....	....	....
Fish hydrolysates	A	25	20	Slight	....	15	....	....	....
	B	24	19	Slight	....	14	....	....	....
	C	29	25	Slight	....	15	....	....	....

tones in sensitivities to antibiotics, and in suitability as media ingredients for indole and methyl-red reactions.

The test cultures used were rather exacting in their amino acid requirements and growth factors, needing a variety of nutrients. Fish hydrolysates, which have supported the growth of all these cultures, can be expected to be suitable for general microbiological media preparations.

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Fish Hydrolysates. III. Influence of degree of hydrolysis on nutritive value

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SUMMARY

Three vacuum-dried papaic hydrolysates (A and B, short-term hydrolysis; and C, long-term hydrolysis) of fish muscle were prepared from a fresh-water fish, *Barbus dubious*. They had, in order, α-amino N contents of 22.5, 28.2, and 42.5%, and sub-peptone N content of 29.3, 32.6, and 60.9% of total N. The lysine and methionine contents of the three hydrolysates were nearly the same, ranging from 8.88 to 10.53 and 3.11 to 3.50 g/16 g N, respectively, while the tryptophan contents of hydrolysates A and B (0.66 and 0.69 g/16 g N) were much less than that of hydrolysate C (1.16 g/16 g N), indicating slower release of tryptophan than of lysine and methionine during hydrolysis. The protein efficiency ratios of hydrolysates A, B, and C were 2.01, 1.87, and 2.88 at 5% level, and 2.31, 2.39, and 2.95 at 10% level of protein intake, compared with 2.92 and 3.19 for the proteins of skim-milk powder. Weight gain response by the rat repletion method, using adult rats, showed a similar trend for the nutritive value of the hydrolysates. The superior nutritive value of hydrolysate C may be due to its higher tryptophan content.

INTRODUCTION

Protein hydrolysates are being used extensively in therapeutics as a source of readily assimilable form of protein in gastro-intestinal and liver disorders and in the treatment of severe cases of protein malnutrition (Elman, 1947; Trowell *et al.*, 1954; Bose and Guha, 1961). These have been prepared by the hydrolysis of liver, meat, fish, casein, and vegetable proteins (Ramasarma *et al.*, 1955; Esh and Som, 1959; Rastogi *et al.*, 1961; Mathur *et al.*, 1962). Fish is an important source of proteins of high nutritive value in several developing countries where there is a shortage of liver, meat, and milk (Nutrition Division, FAO, 1958). It therefore can serve as a cheap raw material for the preparation of protein hydrolysates. In view of this, it was considered of importance to study the nutritive value of protein hydrolysates prepared from fish. Earlier publications from this Institute reported the results of studies on the factors affecting the degree of hydrolysis and the fractional analysis of certain papaic hydrolysates of fish flesh (Sen *et al.*, 1962; Sripathy *et al.*, 1962). Esh and Som (1959) reported that the degree of hydrolysis had a significant effect on the nutritive value of beef hydrolysates. They observed considerable differences in the nutritive value of meat digests produced by treatment with different proteolytic enzymes. It was therefore considered of interest to assess the nutritive value of fish hydrolysates containing varying amounts of the different protein breakdown products.

MATERIALS AND METHODS

Preparation of hydrolysates. A fresh-water species of fish, *Barbus dubious*, was handled in 40-kg batches. It was treated with papain for different periods at suitable temperature and pH designed to obtain hydrolysates containing varying amounts of protein degradation products. The fish were dressed, filleted, minced, mixed thoroughly with an equal quantity of water, adjusted to required pH, and brought to the temperature of hydrolysis. A preparation of papain used in earlier studies (Sen *et al.*, 1962) was dispersed in water and added at the rate of 0.1% papain by weight of the total substrate. The slurry was maintained at the required temperature for the duration of the hydrolysis. At the end of hydrolysis, the digest was brought quickly to boiling, boiled for 15 min, and decanted to remove bones and larger

pieces of undigested residue. The decanted liquid was clarified in a Westfalia Clarifier to remove a major portion of the suspended material, pressure-filtered with Hyflo-supercel used as a filter-aid to get a sparkling clear hydrolysate, vacuum-concentrated at 25 in. Hg and 3 lb steam pressure from a solids content of about 8% to one of about 30% (at this stage, Dow Corning Silicone Antifoam C was used to control excessive frothing), vacuum-dried at 27.5 in. Hg and 45–60°C. The dried material was powdered and stored in air-tight bottles until used.

The hydrolysates were analyzed for total N, coagulable protein N, proteose N, sub-peptone N, and α-amino N by the methods used in the previous study (Sripathy *et al.*, 1962).

Amino acid assay. Three essential amino acids (lysine, methionine, and tryptophan) were estimated. Standard microbiological procedures (Barton Wright, 1952) were followed, using *Lactobacillus mesenteroides* P-60 for the determination of lysine, *L. fermentii* 36 for methionine, and *L. arabinosus* 17/5 for tryptophan. Colorimetric determinations were also made of methionine (Horn *et al.*, 1946) and tryptophan (Spies, 1950).

Animal experiments. The nutritive value of the hydrolysates was assessed by two methods: rat growth method and rat repletion method.

Rat growth method. The protein efficiency ratio of the hydrolysates at 5 and 10% levels of protein equivalent intake was determined by the rat growth method of Osborne *et al.* (1919). Weanling albino rats, about 28 days old, were allotted to the required number of groups according to randomized block design. The hydrolysates were incorporated as the sole source of dietary nitrogen in otherwise adequate experimental diets and were fed *ad libitum* to the rats for 4 weeks. The composition of the diets is given in Table 1. Records of food intake of individual rats were maintained for all the groups, and the animals were weighed weekly.

Rat repletion method. Wizzler *et al.* (1947) and Frost and Sandy (1948) described methods for the assay of protein hydrolysates based on weight gain response to protein hydrolysates of adult rats maintained on protein-free diets. A similar procedure was adopted in this investigation. Female adult albino rats weighing 160–180 g (about 13 weeks old) were maintained on a diet with no added source of nitrogen for 4 weeks. Then they were allotted into comparable groups according to randomized block design on the basis of their initial weight and loss in weight during depletion. They were fed for 9 days on diets (Table 1) containing 10% protein equivalent from the three fish hydrolysates; records were kept of increase in body weight and food intake of the different animals during this period.

In the above experiments, the rats were housed in individual raised wire-screen-bottom cages. For each set of experiments, a comparable group of control rats were fed a diet containing skim-milk powder as the sole source of protein.

RESULTS AND DISCUSSION

Table 2 shows that the three hydrolysates (hydrolysates A, B, and C) have increasing amounts of α-amino N, expressed as percentage of total N, 22.46, 28.20, and 42.48, respectively; and of sub-peptones, 29.25, 32.55, and 60.89%, respectively.

Table 3 gives the results of growth experiments with weanling rats at 5% and 10% levels of protein intake. The protein efficiency ratios of the two short-term digestion hydrolysates A and B were not signifi-

Table 1. Percentage composition of diets.

Ingredients	5% protein equivalent				10% protein equivalent				
	Fish hydrolysates			Skim-milk powder	Fish hydrolysates			Skim-milk powder	Depletion diet
	A	B	C		A	B	C		
Source of dietary nitrogen	5.6	5.7	5.7	15.6	11.2	11.4	11.4	31.2	
Corn starch	79.4	79.3	79.3	69.4	48.8	48.6	48.6	28.8	60
Sucrose	----	----	----	----	25	25	25	25	25
Estimated level of protein (N × 6.25) on moisture-free basis	5.72	5.97	5.63	5.77	10.23	10.80	10.58	10.54	0.74

Ingredients common to all diets: vitaminized starch, 1%;[a] salt mixture, 2%;[b] peanut oil, 10%; cellulose powder, 2%.

[a] Chapman, D. G., R. Castillo, and J. A. Campbell. 1959. *Canad. J. Biochem. Physiol.* **37**, 679.

[b] Hubbel, R. B., L. B. Mendel, and A. J. Wakeman. 1937. *J. Nutr.* **14**, 273.

Table 2. Conditions of hydrolysis and composition of three fish hydrolysates.

	A	B	C
I. Conditions of hydrolysis			
pH	7	5	5
Temperature (°C)	40	55	55
Concentration of papain in the medium (%)	0.1	0.1	0.1 + 0.05 [a]
Duration of hydrolysis (hr)	2	2	17 hr [b]
II. Composition of the hydrolysates			
Total N	14.5	14.2	14.2
Coagulable N, as % of total N	0.8	0.5	0.7
Proteose N, as % of total N	32.8	9.1	2.6
Peptone N, as % of total N	37.2	57.8	35.8
Sub-peptone N, as % of total N	29.2	32.5	60.9
α-NH_2N, as % of total N	22.5	28.2	42.5

[a] A second quantum of papain, half of the first, was added at the end of 6 hr of digestion.
[b] The hydrolysis was carried out until there was no further increase in α-amino nitrogen. The hydrolyzing medium was left in cold-storage (35–38°F) during overnight periods.

Table 3. Comparison of nutritive value of fish hydrolysates with skim-milk powder.

Source of dietary nitrogen	Protein equivalent level	No. of rats	Av. initial weight (g)	Av. gain in weight (g)	Av. protein intake (g)	Av. PER		Least significant difference at P = 0.05	P = 0.01	P = 0.001
Series I										
Fish hydrolysate A	5	5 F [a]	44.4	14.4	7.24	2.01				
B	5	5 F	44.4	14.6	7.40	1.87	±0.25			
C	5	5 F	44.4	24.4	8.53	2.88	(12 d.f.) [b]	0.77	1.08	1.53
Skim-milk powder	5	5 F	45.0	39.0	9.96	2.92				
Series II										
Fish hydrolysate A	10	6 M	50.2	45.0	19.52	2.31				
B	10	6 M	50.2	51.5	21.50	2.39	±0.07			
C	10	6 M	50.0	83.3	28.27	2.95	(15 d.f.) [b]	0.21	0.29	0.40
Skim-milk powder	10	6 M	50.0	87.7	27.05	3.19				

[a] F = female; M = male.
[b] d.f. = degrees of freedom from the analysis of variance.

cantly different in nutritive value from each other at either level of protein intake. The long-term digestion hydrolysate C, however, was significantly superior in nutritive value to the other two hydrolysates ($P < 0.05$) for 5% level of protein intake and ($P < 0.001$) for 10% level of protein intake. The protein efficiency ratio of hydrolysate C was slightly inferior to the proteins of skim-milk powder at either levels of protein intake, the difference being significant ($P < 0.05$).

The weight gain response (Table 4) of protein-depleted rats was less with hydrolysates A and B than with hydrolysate C, though not significantly so. This may be due to the fact that the amino acid requirements of adult rats are less than those of young rats (Mitchell, 1959). As compared with the proteins of skim-milk powder, hydrolysates A and B were significantly inferior ($P < 0.01$) whereas hydrolysate C was not significantly different in nutritive value for the protein-depleted adult rats.

The differences among the three hydrolysates in nutritive value may, to some extent, be due to the fact that, during the hydrolysis of fish proteins, there may possibly be a differential release of essential amino acids into the hydrolysates, with the result that all the essential amino acids present in fish flesh are not proportionately released into the hydrolysates. The tendency will be toward larger amounts of different amino acids being released into the hydrolysate

Table 4. Repletion experiment with fish hydrolysates at 10% protein-equivalent level (8 female adult rats per group).

Source of dietary nitrogen		Av. initial weight (g) before depletion	Av. weight (g) after depletion for 4 weeks	Av. gain in weight (g) after repletion for 9 days	Av. protein intake (g)	Av. weight (g) gain per gram protein intake		Least significant difference at		
								P = 0.05	P = 0.01	P = 0.001
Fish hydrolysate	A	168.9	133.8	18.0	6.70	2.60	±0.21			
	B	168.3	131.2	19.0	6.96	2.67				
	C	168.0	131.5	27.7	8.50	3.22		0.63	0.88	1.21
Skim milk powder		167.3	130.3	28.2	7.89	3.55	(15 d.f.)[a]			

[a] d.f. = degrees of freedom from the analysis of variance.

when the period of digestion is longer. Such a tendency was observed by Ambe and Sohonie (1957) when shark and skate muscle was hydrolyzed with trypsin. The results of amino acid assay (Table 5) indicate that, although the lysine and methionine contents were nearly equal in the three hydrolysates, the tryptophan contents were very much less in hydrolysates A and B than in hydrolysate C. This clearly indicates that degree of enzymic hydrolysis influences the amount of different essential amino acids in hydrolysates.

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Table 5. Lysine, methionine, and tryptophan contents of fish hydrolysates (g/16 g N).

Fish hydrolysate	Lysine M [a]	Tryptophan M [a]	Tryptophan C [a]	Methionine M	Methionine C
A	8.88	0.66	0.64	3.11	3.31
B	10.53	0.69	0.67	3.50	3.30
C	10.53	1.16	0.91	3.50	3.39

[a] M = microbiological; C = colorimetric.

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