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CONTENTS

Thermal Destruction of *Streptococcus faecalis* in Prepared Frozen Foods [a]

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Manuscript received January 9, 1960

SUMMARY

The thermal destruction rates of *Streptococcus faecalis*, ATCC 7080, were determined in a selected group of meat and fish precooked frozen products. Data on heat transfer through these products in a hot air household electric oven, were also obtained. Accordingly, the theoretical thermal treatments required to render such products commercially sterile were computed following the procedure of the General method of process calculation for canned foods with some modification. Inoculated product tests were also made to confirm the adequacy of the computed thermal treatments.

It is concluded that the procedure adopted for establishing the thermal treatment was accurate and proved feasible for this type of product. Based on the thermal resistance of our test strain, the heat treatments recommended on the commercial packages seem to be adequate, provided the heating and cooling procedures are similar to those followed in the present experiments. In the case of one product out of the five tested, however, the minimum recommended heating period fell short of the computed thermal requirement for that product.

It has been generally acknowledged that certain prepared frozen foods, meat and fish products in particular, may present a potential hazard from the public health viewpoint. The setting of strict precautionary measures to minimize contamination and to control microbial activities throughout all phases of processing, storage, marketing and consumption has been strongly recommended. Preliminary steps have been taken by certain industrial and regulatory groups in providing such measures: (1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 20, 21, 24).

The present study was initiated in order to: (a) determine the thermal destruction rates of *Streptococcus faecalis*, a relatively thermoresistant non-sporeforming bacterium occasionally associated with food poisoning outbreaks (1, 19, 20), in a variety of precooked frozen foods, and (b) establish the thermal requirements which would render these products practically free of such bacterium, when heated in a hot air oven. This information, based on fundamental quantitative data, is essential to evaluate the potential hazard from either bacterial growth or infection which may be associated with such foods after being heated to be served.

It was shown that the method adopted for determining the theoretical thermal requirements for commercial sterility in these types of products was accurate and feasible. The data also demonstrated, in general, that if the directions on the commercial packages are followed accurately, the problem of infection or intoxication from non-sporeforming bacteria will be adequately controlled. In one case, however, the recommended heating period was short of satisfying the calculated thermal requirement.

EXPERIMENTAL

Test Organism

Streptococcus faecalis, American Type Culture Collection strain No. 7080, was selected as the test organism in view of its comparatively greater tolerance to both cold and heat (*8, 15, 23*).

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Cell Suspension

Various modifications of Trypticase Soy Agar [c] (TSA), through adding different concentrations and combinations of glucose, phosphate and yeast extract, failed to improve significantly the growth of the test organism. Other media such as Eugonagar,[d] Brain Heart Infusion Agar,[d] and Tryptone Glucose Extract Agar,[d] were also tried but showed no improvement over the TSA medium. Thus, all of our cell suspensions were produced on TSA. The stock culture was activated in Trypticase Soy Broth (TSB) for 24 hr at 37° C (98.6° F). The active broth culture was used to spread a number of TSA plates. Plates were incubated for 24 hr at 37° C (98.6° F) and were then harvested in sterile physiological saline. The crude crop was washed once in physiological saline by centrifugation and the final suspension was collected in an 8-oz prescription bottle containing a layer of glass beads.

Initial cell concentrations were determined by both the chamber and the viable plate count, using TSA in the latter case. In making the chamber counts both the total number of individual cells as well as that of cell groupings were determined. The viable plate count agreed with the count of cellular groups and both were about half the individual cell count. It was concluded that the viable plate count can be used safely in establishing death rates without significant interference of cell grouping on either rates or computed survival levels.

The stability of our cell suspensions was also established over a 5-week period. The weekly plate counts indicated a slight increase towards the end of the storage period. Simultaneous chamber counts confirmed this apparent increase in population by showing a steady total individual cell count, but slightly increased counts of cellular groups. Thus, one cell suspension was satisfactorily used over a 4- to 5-week period.

Establishment of the Thermal Requirement

In order to determine the thermal requirement for the sterility of a given food product, two types of data are necessary: (a) bacteriological, which describe the thermal resistance characteristics of the test organism, and (b) physical, which describe the heat transfer pattern through the product under the same heating conditions to which it will be exposed.

The bacteriological data involve the establishment of the thermal destruction rates of the test organism at various heating temperatures. The rate of destruction has been commonly expressed in terms of the "D" value, the decimal reduction time in minutes, or the time in minutes required for 90% destruction.

From the above information the temperature coefficient of the death reaction is determined. This coefficient is usually expressed in terms of the "z" value, which expresses the temperature change in degrees Fahrenheit which causes a 10-fold change in the time required for a given degree of destruction.

A third value "F" has been introduced to describe the heating time in minutes required for practical sterilization at 121° C (250° F). This designation is suitable for bacterial spores which, in general, can still resist exposure to such high temperatures for measurable lengths of time. Dealing with a non-sporeforming organism, we used the same designation "F" qualified with a subscript indicating the heating temperature (° F) and a superscript indicating the level of destruction in terms of numbers of log cycles reduction in the intial population—e.g. "F^{5}_{165}" expresses the time in minutes at 165° F to achieve 99.99999% destruction.

The physical data, on the other hand, involve the establishment of the heating rate of the product in question at the slowest heating zone. In case of products heating mainly by either conduction or convection, the change in delta-T (temperature difference between the product and the surrounding) with respect to "t" (heating time) is found to be exponential, and the slope of such heating curve is expressed in terms of "F_h", namely, the time in minutes required for 10-fold reduction in delta-T (21).

Once the above two types of data are established, various graphical and mathematical methods are available to compute the thermal requirements for the practical sterility of the product in question.

In the present study, the conventional Thermal Death Time (TDT) Tube method was used in determining the death rates (22), and the General or Graphical method (5) was used in computing the theoretical thermal requirements.

Test Products

The following 6 products, representing the precooked frozen foods, were selected.

1. Chicken à la king: a commercial recipe was prepared in volume in the laboratory and frozen in pint jars. This recipe contained cooked diced chicken meat, chicken broth, all-purpose flour, carioca starch, salt, milk (powdered skim), margarine, frozen peas, sliced mushrooms, mushroom broth,

[c] Baltimore Biological Laboratory, Baltimore 18, Maryland.

[d] Difco Laboratories, Inc., Detroit 1, Michigan.

chopped pimiento, chicken à la king seasoning, and water.

2. Beef Pie: a commercial product which consisted of beef broth, cooked beef, potatoes, carrots, peas, wheat flour, starch, tomato puree, salt, flavoring, caramel coloring and water; in a crust consisting of wheat flour, shortening, dextrose, salt and water.

3. Tuna Pie: a commercial product which contained tuna, peas, whole fresh milk, shortening, starch, flour, butter, peppers, onions, liquid turmeric, salt, seasoning, monosodium glutamate and water; in a crust made of flour, shortening, sugar, salt and water.

4. Lobster Pie: a commercial product whose ingredients included lobster, peas, mushrooms, milk, butter, cheese, mustard, cream, shortening, cornstarch, salt, spices and water; in a crust composed of flour, shortening, sugar, salt and water.

5. Fish Sticks: a commercial product made of haddock centers; coated with a special batter which consisted of bread crumbs, wheat and corn flour, salt, leavening, dextrose, spices and water. The final product was French-fried in vegetable oil.

Thermal Destruction Rate Apparatus and Method

The TDT Tube method was used in establishing the thermal death rates of the test strain, heated in various menstrua, made of the 6 test products previously described.

A constant-temperature water bath was used to achieve and maintain the required heating temperatures within $\pm$ 1° F. Death rates were established at 4 different temperatures: 60° C (140° F), 65.5° C (150° F), 71° C (160° F), and 74° C (165° F).

In preparing the various heating menstrua, the ingredients of each product other than crust, were macerated in a Waring blender with equal parts of distilled water for 5 min. Due to the high starch in the frozen codfish cakes, 3 parts of water were used rather than one to facilitate handling with a pipette. One hundred ml each of the blended samples were placed in clean empty dilution bottles. The bottles were capped loosely and sterilized at 121° C (250° F) for 15 min. The pH of all resulting menstrua fell within the low acid range (pH 6.1 to 6.4). All menstrua were prepared on the same day they were used.

The inoculation was performed by aseptically pipetting 1 ml of the cell suspension into the sterilized dilution bottle containing the 100 ml heating menstruum. The resulting cell concentration was between 2 and 3 x 10^7 cells per ml. Two ml of the inoculated menstruum were aseptically transferred into each of the sterile TDT tubes (9 mm OD, 1 mm thickness and 150 mm long—Pyrex glass). The tubes were flame sealed and held in an ice water bath long enough to equilibrate in their initial temperature before heating.

An adequate number of TDT tubes were loaded in pairs into small wire mesh baskets. The baskets were hung on a steel rod and submerged at zero time in the pre-set heating water bath. At the completion of the preselected heating periods, the corresponding basket was removed, immediately immersed in ice water and held until needed for plating. Each tube was then broken open and the contents transferred aseptically into a sterile test tube. Serial decimal dilutions were made and subsequently plated out in triplicate, using TSA containing 0.5% glucose and 0.004% Brom Cresol Purple indicator. Because lower dilutions were difficult to count due to the carry-over of food particles, Brom Cresol Purple was helpful in differentiating the developed colonies. A yellow zone due to acid production by the growing organism was evident around the colonies. No difference in recovery levels was found due to the added glucose and/or indicator.

For the establishment of the thermal destruction rate (TDR) curves, the experimental log counts of survivors were plotted versus time. All heating times were corrected for the heating and cooling lags of TDT tubes at each temperature using similar procedure as described by Sognefest and Benjamin (18). Table 1 presents these computed lag correction factors. The points showing apparent linearity were fitted with a straight line, using the method of Linear Regression (19). The D values describing the slope of the TDR curves were computed from the corresponding regression coefficients.

TABLE 1

Lag Correction Factors Determined for Chicken a la King in TDT Tubes Heated in a Constant-Temperature Water Bath

Heating temperature	Experimental heating time	Computed equivalent holding time	Lag correction factor [1]
(° F)	(minute)	(minute)	(minute)
140	1.50	.71	.79
150	1.50	.74	.76
160	1.50	.79	.71
165	1.50	.76	.74

[1] For intervals less than 1.5 minutes, the corresponding equivalent holding times, as interpreted on the corresponding lethality rate curve, were substituted directly.

For the establishment of the thermal reduction time (TRT) curve, the TDR curves were extrapo-

lated, if necessary, to the F_T^z level and the respective times were then interpreted on the abscissa. The logarithms of these thermal reduction times were plotted against their corresponding heating temperatures. The z value was then interpreted on this curve.

The above procedure was completed in the case of the chicken à la king. In the case of the other market products the various death rates were determined only at 65.5° C (150° F) and the "z" value determined for chicken à la king was used in constructing their own corresponding TRT curves.

Heat Transfer Data

In the conventional method of determining the thermal process for canned foods, the desired sterilization values at a given retort temperature are generally beyond the lethality levels achieved during the come-up and cooling of the food product, thus permitting the point of the slowest heating to achieve maximum temperature at most of the lethality levels tested. However, if lower sterilization values are desired, where maximum temperatures are not reached at the slowest heating point, the immediate water cooling of the hermetically sealed container does not allow an internal rise in temperature upon the termination of the heating period. This makes the over-all composite heating and cooling curve of the canned product sufficient to compute the sterilization values desired.

In the present situation the products concerned, as well as the method of heating and cooling, were different; namely, an exposed frozen food product heated in a hot-dry-air oven and air-cooled at room temperature. In addition, the test organism is of the non-sporeforming type with much lower thermal resistance than the spores conventionally used in the case of canned products. In this situation the internal temperature of the product never reaches that of the oven, and will not exceed several degrees beyond the atmospheric boiling point of water, depending on the kind and percent of the soluble solids. The product come-up and cooling lags are relatively longer and of most importance; high sterilization values are achieved long before the point of the slowest heating (found to be approximately at the geometric center) achieves maximum temperature. Therefore, throughout the heat transfer studies the product had to be removed long before the central point reached maximum temperature. Due to the vast difference in temperature between the outer layer of the product and its central point, and due to the relatively slow cooling, there is always a post-heating temperature rise at the central point upon removal of the product from the oven. This post-heating rise had to be accounted for and integrated as a part of the thermal treatment. This was accomplished by removing the samples from the oven at predetermined times, continuing the temperature readings during the post-heating rise and the subsequent cooling period down to sublethal levels.

Because the temperatures, upon removal from the oven, were considerably different in the replicate samples, a removal time vs sterilization value relationship could not be developed. To overcome this, the removal temperature was used instead and the minimum removal temperature necessary to achieve commercial sterility was determined. The corresponding time for this minimal temperature was interpreted on the slowest heating curve for each particular product.

In this study, the oven of a household electric range was used as the source of heat. The oven thermoregulator was calibrated against our thermocouple measurements. In all but one product the oven temperature was maintained at 218.3° C (425° F), while with the cod fish cakes the temperature was held at 232.3° C (450° F) according to the producer's recommendation. For determining the heat penetration patterns in the individual products, a 24-station Brown indicating potentiometer was used.

The chicken à la king was used as a control product to insure uniformity throughout the entire study. Portions, 175 g each, were filled into 15 Pyrex glass dishes of uniform size (3 inches in diameter and 1½ inches deep). The hot junction of a 24-gauge fiber glass insulated thermocouple wire was placed in the geometric center and held in place by means of a ½-inch wire mesh resting on the circumference of the container. A preliminary study indicated that the geometric center of the products used was the point of the slowest heating. The 15 dishes were placed in a freezer and frozen to a temperature of 0° F. Three frozen samples at a time were placed on an aluminum tray and into the preset oven. Initial temperatures were recorded and readings were taken every 3 min until the lethal temperature range was reached during which time 1-min intervals were used. At the predetermined times, the 3 samples were removed and placed on a table top where they continued to heat at the center and finally cooled. Readings were continued until sublethal temperatures were reached. With the exception of the time intervals for temperature readings and the method of securing the hot junction of the thermocouple, the same procedure was used in determining the heat transfer patterns in the other products. In the pies, the thermocouple lead was forced through the bottom of the crust to the approximate geometric center. The insulated wire was then led between the pie and the aluminum boat which contained the

pie, outside the oven to the potentiometer. In the case of fish sticks and fish cakes the hot junction was inserted through the side of the product at the approximate geometric center.

Thus, 15 sets of heat penetration data were obtained for every product over a wide range of removal temperatures. From these data the corresponding sterilization values were computed by the General method, using the proper F and z values for the respective product; the desired theoretical heating times for the test products were finally interpreted as described previously.

Inoculated Product Test

Fifteen samples of each product were used in groups of threes. One group was given the theoretical heating time, two groups were overheated, and the remaining two underheated. The purpose was to determine how close the calculated time agreed with the actual inoculated pack recovery data. The off-theoretical times were also determined in accordance with the respective sterilization-removal temperature curve.

The entire inoculum was placed in the center (critical zone) of the product and the amount inoculated was based on 10^8 cells per gram. The inoculation was performed by using a sterile syringe and 18-gauge needle. No more than 1 ml was inoculated into each sample. All samples were frozen for a 22-hr period prior to heating in the oven. An exploratory experiment to determine the effect of freezing on the test organism contained in the product indicated 100% survival after 22 hr in a freezer maintained at −23.3° C (−10° F). The temperature of the oven was maintained constant by checking periodically the thermocouple reading.

Each group of samples was heated for the predetermined time interval and placed on a table top for post-heating rise and cooling. Each entire sample was then placed in a sterile Mason jar and an equal amount of sterile distilled water added. Once again, the fish cakes required a 3 to 1 ratio of water to product. A sterile Osterizer blade adaptor was placed on each Mason jar and the sample was macerated at high speed for 2 min. Twenty separate one-milliliter direct transfers from each sample were plated quantitatively, using TSA with the BCP indicator. The plates were incubated at 37° C (98.6° F) for 48 hr and the results were read.

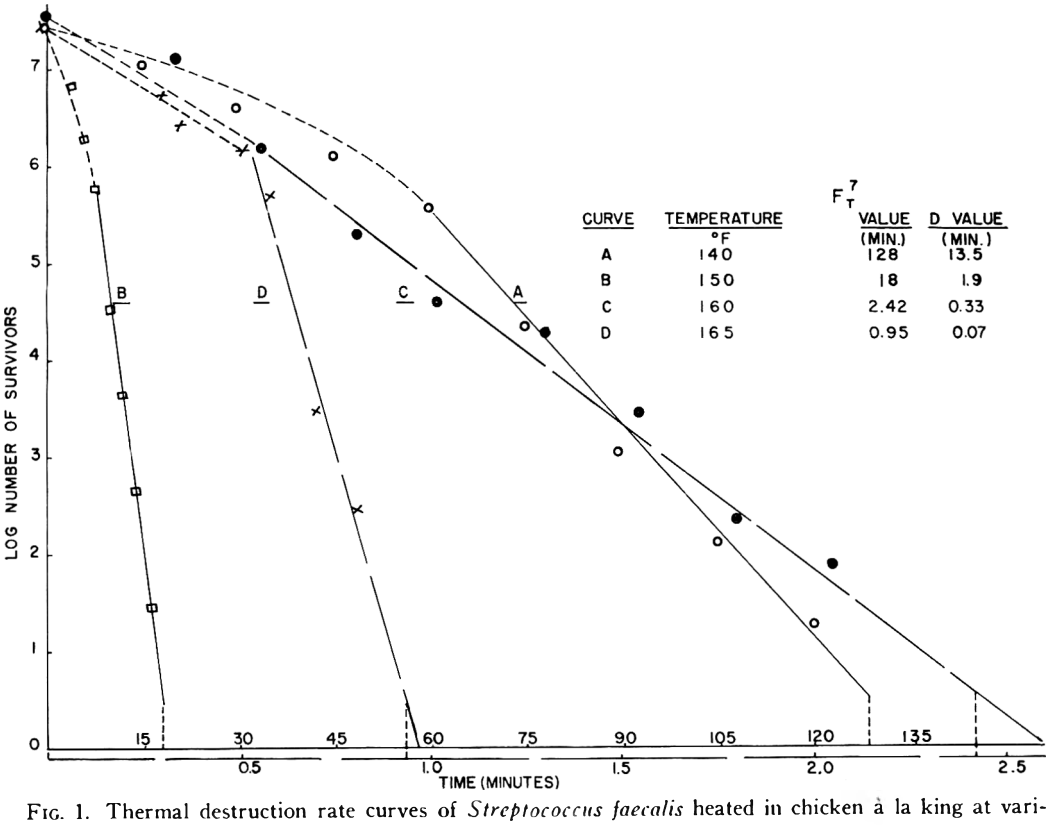

Fig. 1. Thermal destruction rate curves of *Streptococcus faecalis* heated in chicken à la king at various temperatures.

RESULTS AND DISCUSSION

Thermal Destruction in Various Heating Menstrua

Figure 1 shows the TDR curves constructed for the test organism heated in chicken à la king. The solid lines represent the portions of the curves fitted by linear regression, whereas the dotted parts represent the initial lags, which were frequently encountered. It is believed that these initial lags are due, at least in part, to the multicellular grouping and possibly additional clumping of the test organism during the heating. The time intervals on the abscissa were all corrected for the heating and cooling lags, as mentioned earlier.

The "F_T^z" values from Figure 1 were used in constructing the TRT curve for chicken-à la king (Fig. 3, curve A). The "z" value was then computed from the above curve and used to calculate the thermal treatment required for the chicken à la king product. It was interesting that the 4 different "F_T^z" values defined a reasonably straight line without need for the regression fitting.

The same "z" value was used in constructing the TRT curves for the remaining 5 heating menstrua. To obtain an "F_T^z" value needed to anchor each of these curves, destruction rates at 65.5° C (150° F) were determined. The resulting TDR curves are shown in Figure 2, constructed in the same manner, described previously. The interpreted "F_{150}^z" values from Figure 2 show very slight difference among chicken à la king, beef, tuna and lobster pies. Therefore, the TRT curves for these products are all represented by curve A in Figure 3. On the other hand, fish sticks showed a higher "F_{150}^z" value whereas fish cakes showed a lower one, and are thus represented by separate TRT curves (Fig. 3, curves B and C). It should be mentioned, however, that the "D" values as well as the initial lags exhibited by the TDR curves showed greater variation than the final "F_{150}^z" values which are in fact, the

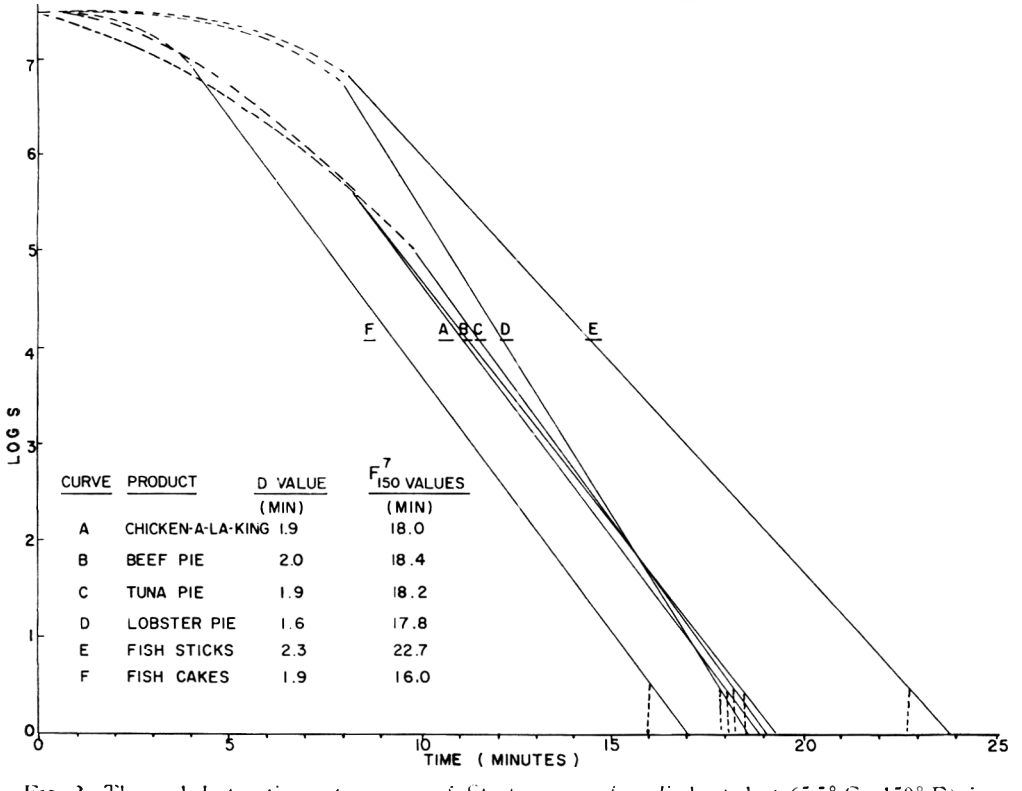

FIG. 2. Thermal destruction rate curves of *Streptococcus faecalis* heated at 65.5° C (150° F) in various heating menstrua.

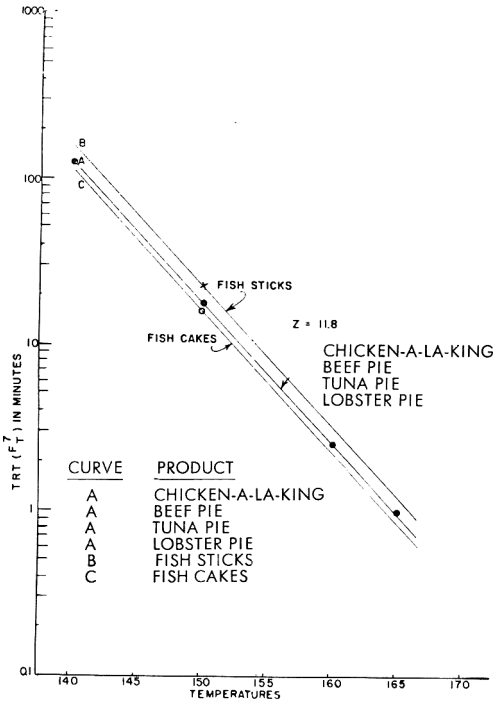

FIG. 3. Thermal reduction time curves for *Streptococcus faecalis* heated in various heating menstrua.

over-all net result of such variations. Table 2 summarizes all the thermal destruction data obtained.

HEAT TRANSFER DATA

Fifteen heat transfer curves were constructed for every test product to furnish the data necessary to establish the subsequent sterility rate curves. These, in turn, were used to construct the final sterilization value vs removal temperature curve. The 5 test products exhibited the following average "F_h" values: chicken à la king, 7.5; beef pie, 3.4; tuna pie, 4.5; lobster pie, 3.8; fish sticks, 1.5; and fish cakes, 3.2 min. Figure 4

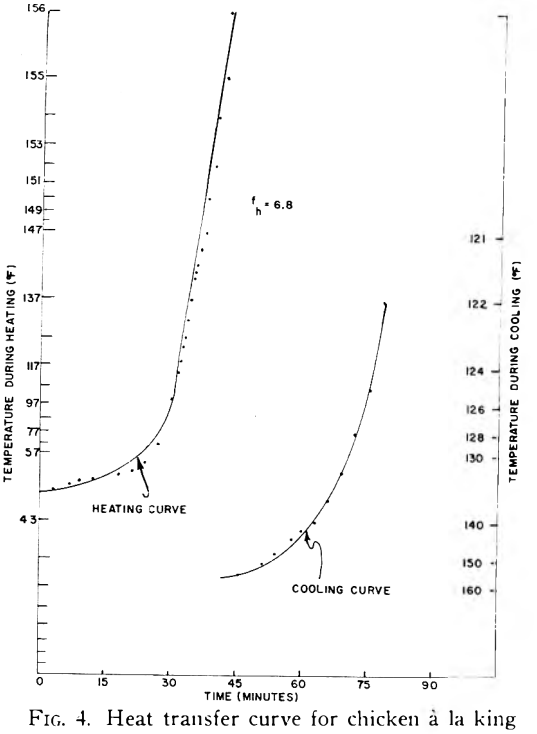

FIG. 4. Heat transfer curve for chicken à la king samples removed from oven at 34 minutes.

TABLE 2

SUMMARY OF THERMAL DESTRUCTION DATA OF STREPTOCOCCUS FAECALIS HEATED IN VARIOUS HEATING MENSTRUA

Product	"D" values [1] at (° F)				"F_T^7" values at (° F)				"z" values computed from	
	140	150	160	165	140	150	160	165	D curve [4]	F_T^7 curve [5]
Chicken-à-la-king	13.50	1.90	.33	.07	128	18.0	2.42	.95	12.3	11.8
Beef Pie	12.20[2]	2.00	.33[2]	.11[2]	128[3]	18.4	2.42[3]	.95[3]	.....	.....
Tuna Pie	11.25[2]	1.90	.28[2]	.07[2]	128[3]	18.2	2.42[3]	.95[3]	.....	.....
Lobster Pie	10.50[2]	1.60	.24[2]	.094[2]	128[3]	17.8	2.42[3]	.95[3]	.....	.....
Fish Sticks	15.70[2]	2.30	.35[2]	.13[2]	157[3]	22.7	3.25[3]	1.20[3]	.....	.....
Fish Cakes	11.25[2]	1.90	.33[2]	.07[2]	112[3]	16.0	2.24[3]	.85[3]	.....	.....

[1] Computed from the regression coefficient for the linear portion of the TDR curve.

[2] Computed from the corresponding "D_{150}" value and the "z" value from the TRT curve for chicken à la king.

[3] Computed from the corresponding F^7_{150} value and the "z" value from the TRT curve for chicken à la king.

[4] TRT curve constructed from log D vs T °F.

[5] TRT curve constructed from long F^7_{150} vs T °F.

presents an example of the heating and cooling curves obtained. This curve was chosen to represent the sample of chicken à la king, removed at the temperature closest to that interpreted later for the computed theoretical thermal treatment for this product.

Sterility Rate Curves

The concept 1/t was used to establish these curves, and, therefore, a sterilization value of ONE was the value sought for the theoretical thermal treatment. The 1/t value was computed according to the formula:

$$\frac{1}{t} = \frac{1}{F_{165}^{7}} \log -1\left(\frac{T-165}{z}\right)$$

t = time in minutes for 7 log cycles of destruction at a given temperature T.

Figure 5 represents a sample of the 15 sterility rate curves obtained for each product. This particular curve corresponds to the heat transfer curve shown in Figure 4.

It is very important to note that although the product was removed from the oven when the central temperature was still 137° F (at the end of 34 min) it reached a maximum of 156° F before it started to cool down slowly. Upon integrating lethalities acquired at the measured point, it is apparent that nearly all the sterilization dose was accomplished during the post-heating temperature rise outside the oven. This stresses the significance, in our procedure, of the slow cooling at room temperature. Factors which would accelerate the cooling rate would significantly diminish the sterilization dose of our computed thermal treatment and would result in a still-contaminated product. We cooled the product on the laboratory bench in a still atmosphere at an average indoor summer temperature of 85–90° F.

Sterilization Value-Removal Temperature Curves

Data collected from the sterility rate curves, expressing the various sterilization values corresponding to various heat treatments, were then plotted versus the internal temperatures at which products were removed from the oven. Figure 6 illustrates, as an example, the curve obtained for chicken à la king. The curve appears to be of an exponential nature as contrasted with the linear relationship we get when time is substituted for temperature on the abscissa

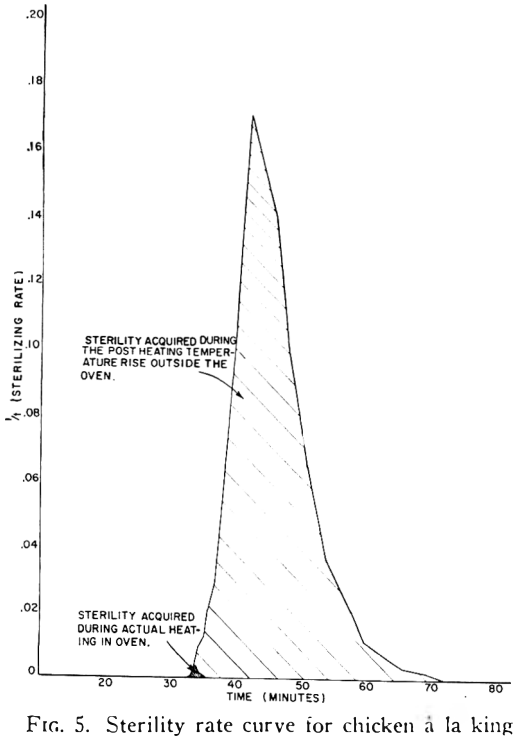

Fig. 5. Sterility rate curve for chicken à la king samples removed from oven at 34 minutes.

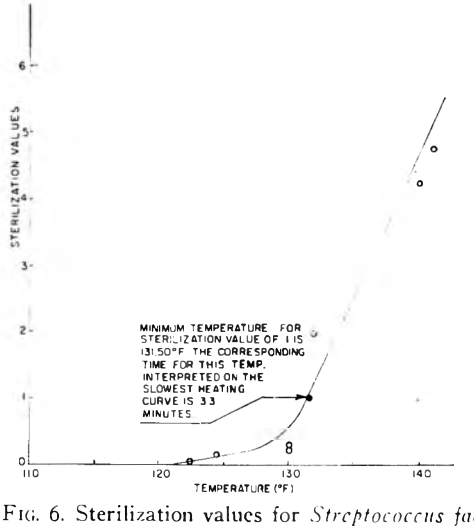

Fig. 6. Sterilization values for *Streptococcus faecalis* corresponding to the temperatures at which chicken à la king samples were removed from the oven.

in a similar plot, the standard practice followed in computing the thermal process for canned foods. This is to be expected, assuming that temperature difference is an exponential function of the heating time.

It should also be mentioned that the wide variation among triplicate samples in the sterilization values acquired, as well as the erratic values we encountered several times, such as lower sterilization values at longer heating times, call for more precise and improved means of locating the thermocouples and maintaining them at the same point throughout the processing as well as more controlled cooling conditions. In constructing these curves, we selected from among the 15 sets of data per product only those we felt are in agreement, and which were adequate in every case to furnish a satisfactory illustration of the relationship in question. On the visually constructed curve, we interpreted the removal temperatures corresponding to a sterilization value of ONE. The process time corresponding to such removal temperature was interpreted on the slowest heating curve of the product in question, and this was the theoretical thermal treatment time sought.

Inoculated Product Test

Based on the times computed for the various theoretical thermal treatments, in the manner outlined so far, 5 different heating levels were selected for each product, two above, one at, and two below the computed sterilization level. The inoculated pack experiment was then run as described previously. Results are presented in Table 3.

As can be seen from Table 3, the recovery data were in fair agreement with the theoretically determined thermal treatments. The chicken à la king, beef pie, and lobster pie showed sterilization by the completion of the theoretical treatment. The 3 remaining products required a few minutes beyond the theoretical heating time. Growth on the positive plates, in all cases, was typical of the test organism macroscopically and microscopically.

In the case of the 5 commercial products used, all but one producer recommended heating times more than sufficient for the destruction of the test organism. The minimum heating period recommended on the package for the lobster pie was 30 min but survival of the test organism was still encountered at 31 min. A one-minute difference can lead to significant reduction in lethality acquired. An added safety factor in these inoculated product experiments was the high inoculation at the center of the product. Although the over-all concentration of bacterial cells was approximately one million cells per gram of product, the entire inoculation was placed in the zone of slowest heating. Assuming some migration of the inoculated cells, a conservative estimate of the concentration at the center of the product would be ten million bacterial cells per gram. Maximum contamination levels reported in the literature were of the latter magnitude.

The choice of the present test strain of *Streptococcus faecalis* was based primarily on its greater thermal resistance (15, 23).

TABLE 3

Recovery Data from the Inoculated Product Test

Chicken à la king		Beef Pie		Tuna Pie		Lobster Pie		Fish Sticks		Fish Cakes	
Time [1]	Recovery [2]	Time	Recovery	Time	Recovery	Time	Recovery	Time	Recovery	Time	Recovery
(min)		*(min)*		*(min)*		*(min)*		*(min)*		*(min)*	
23	+++	22	+++	23	+++	26	+++	3	+++	4.75	+++
28	++−	27	+++	28	+++	31	+++	5	+++	7.75	+++
33	−−−	32	−−−	33	++−	36	−−−	7	+++	10.75	+++
38	−−−	37	−−−	38	−−−	41	−−−	9	+−−	13.75	−−−
43	−−−	42	−−−	43	−−−	46	−−−	11	−−−	16.75	−−−

[1] The theoretical sterilization times are underlined.

[2] Each one of the three signs represents the recovery results in the twenty subcultures made out of each sample.

The + sign indicates growth in one or more subculture.

The − sign indicates no growth in any of the subcultures.

However, further confirmatory studies on the thermal resistance of different strains of the enterococcus group and possibly other related non-sporeforming types are necessary before the final choice of the test strain is made.

LITERATURE CITED

1. Abrahamson, A. E. 1958. Frozen foods; their impact on health. *Association of Food and Drug Officials of the United States*, 22, 7–15.
2. Anonymous. 1959. Prepared frozen foods committee appointed to participate in AFDOUS meeting. *Quick Frozen Foods*, 21, 10, 69.
3. Appleman, M. D. 1957. Symposium on problems in the microbiological analysis of foods. *Bact. Reviews*, 21, 241–244.
4. Appleman, M. D. 1958. Bacteria in food spoilage and food infection. *The Sanitarian*, 20, 177–181.
5. Ball, C. O., and F. C. W. Olson. 1957. *Sterilization in Food Technology*. McGraw-Hill Book Company, Inc., New York, New York.
6. Buchbinder, L., W. Loughlin, M. Walter, and G. Dangler. 1949. A survey of frozen precooked foods with special reference to chicken-a-la-king. *J. Milk and Food Technol.*, 12, 208–213, 231.
7. Canale-Parola, E., and Z. J. Ordal. 1957. A survey of the bacteriological quality of frozen poultry pies. *Food Technol.*, 11, 578–582.
8. Dewberry, E. B. 1950. *Food Poisoning*. Leonard Hill, Ltd., London.
9. Fisher, F. E. *et al.* 1957. Public Health aspects of frozen foods. *J. Milk and Food Technol.*, 20, 145–148.
10. Gunderson, M. F. Dec. 1955. Need for sanitation standards in production and processing. Precooked Frozen Foods — a Symposium. Quartermaster Food and Container Institute for the Armed Forces, Chicago, Ill. pp. 52–55.
11. Huber, D. A., H. Zabrowski, and M. M. Rayman. 1958. Studies on the microbiological quality of precooked frozen meals. *Food Technol.*, 12, 190–194.
12. Husseman, D. L. 1951. Effect of cooking on the bacteriological flora of selected frozen precooked foods. *J. Am. Dietet. Assoc.*, 27, 855–859.
13. Larkin, E. P., W. Litsky, and J. E. Fuller. 1956. Incidence of fecal streptococci and coliform bacteria in frozen fish products. *Am. J. Public Health*, 46, 464–468.
14. Litsky, W., I. S. Fagerson, and C. R. Fellers. 1957. A bacteriological survey of commercially frozen beef, poultry, and tuna pies. *J. Milk and Food Technol.*, 20, 216–219.
15. Proctor, B. E., and A. W. Phillips, Jr. 1948. Frozen precooked foods. *Am. J. Public Health*, 38, 44–49.
16. Rayman, M. M., D. A. Huber, and H. Zabrowski. Dec. 1955. Current microbiological standards of quality for precooked frozen foods and their basis. Precooked Frozen Foods—a Symposium. Quartermaster Food and Container Institute for the Armed Forces, Chicago, Ill. pp. 55–67.
17. Ross, A. D., and F. S. Thatcher. 1958. Bacteriological content of marketed precooked frozen foods in relation to public health. *Food Technol.*, 12, 369–371.
18. Snedecor, G. W. 1950. *Statistical Methods*. The Iowa State College Press, Ames, Iowa.
19. Sognefest, P., and H. A. Benjamin. 1944. Heating lag in thermal death time cans and tubes. *Food Research*, 9, 234–243.
20. Straka, R. P., and J. L. Stokes. 1956. Microbiological hazards of precooked frozen foods. *Quick Frozen Foods*, 18, 181–184.
21. Thatcher, F. S. 1955. Microbiological standards for foods: their function and limitations. *J. Appl. Bacteriol.*, 18, 449–452.
22. Townsend, C. T., I. I. Somers, F. C. Lamb, and N. A. Olson. 1956. A Laboratory Manual for the Canning of Food. Rev. ed. National Canners Association, Research Laboratories, Washington 6, D.C.
23. Webster, R. C., and W. B. Esselen. 1956. Thermal resistance of food poisoning organisms in poultry stuffing. *J. Milk and Food Technol.*, 19, 209–212.
24. Weiser, H. H. 1957. Bacteriology of precooked frozen foods. *J. Milk and Food Technol.*, 20, 33–35.

Loss of Ascorbic Acid During Estimation by the Roe-Kuether Method

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SUMMARY

Application of the 2,4-dinitrophenylhydrazine method of Roe and co-workers to plant extracts, wherein ascorbic acid was present largely or completely in the form of DHA and DKA, resulted in abnormal data, in the sense that the value of AA + DHA + DKA was significantly *lower* than that of DHA + DKA. Such a discrepancy, though not to the same degree, was observed also when the analyses were carried out on solutions of DHA. The loss is believed to take place during H_2S treatment, there being a correlation between the extent of loss and the duration of H_2S treatment, in the majority of the experiments.

The method of Roe and Kuether (7), as modified by Roe and co-workers (8), was adopted by us in studies on vitamin C content and the distribution of AA,[a] DHA and DKA in vegetables (9). The method consists in estimating DHA and DKA together (Step 1) by coupling with DNPH, DKA separately after prior reduction of DHA with H_2S (Step 2) and the combined value of all the three forms (AA, DHA and DKA) after bromine-oxidation of the solution obtained on reduction with H_2S (Step 3). Fresh vegetables and their boiled preparations contained only a small proportion of DHA and DKA, the vitamin being present mostly in the form of AA (9). Freezing over a period of days and subsequent thawing in contact with air led to complete transformation of AA into DHA and DKA in the case of bitter gourd (*Momordica charantia*) and cauliflower (*Brassica oleracea botrytis*). Mills and co-workers (5) reported that the vitamin was present mostly in the form of AA in fresh food material, whereas stored orange juice contained considerable amounts of the oxidized products. In our hands application of the method of Roe and co-workers (8) to vegetables frozen and subsequently thawed, or to homogenates in water of fresh tissue prepared with the aid of a Waring blender, resulted in abnormal data, in the sense that the value of Step 3 (AA + DHA + DKA) was significantly *lower* than the value of Step 1 (DHA + DKA). The treatments were such that AA of the vegetables was transformed almost completely into DHA and DKA. Similar discrepancies were also encountered when the analyses were carried out on solutions of DHA.

[a] The following abbreviations will be used: AA—(reduced) ascorbic acid; DHA—dehydroascorbic acid; DKA—diketogulonic acid; DNPH—2,4-dinitrophenylhydrazine.

MATERIALS AND METHODS

Treatment of Plant Tissue

In studies on storage, bitter gourds were held frozen at −18° C in a deep-freeze and thawed at room temperature after slicing. In other experiments the fresh vegetables were cut and then frozen. The thawed tissue was extracted with 5% HPO_3 containing $SnCl_2$, according to standard practice (1). Homogenates in water were prepared by grinding fresh bitter gourds in a Waring blender; the ground material was filtered through muslin and made to volume.

Preparation of DHA Solution

AA (E. Merck) was dissolved in glass distilled water and the solution was agitated with a drop of liquid bromine. The supernatant was decanted and freed from dissolved bromine, by bubbling water-saturated air. Aliquots of the DHA solution were diluted with HPO_3 containing $SnCl_2$ so as to give a final concentration of 5 μg of DHA per ml in 5% HPO_3 containing 0.5% of $SnCl_2$. Aliquots of this solution were directly utilized for Step 1 analysis. Other aliquots were treated with H_2S, a portion of the resulting solution utilized for Step 2 analysis and the rest oxidized with bromine and used for Step 3 analysis.

In other experiments use was made of a sample of Dehydroascorbic Acid [b] ("93% pure Methanol complex"). The material was received in a sticky condition and was slightly yellowish in color. A small amount of the solid was dissolved in 5% HPO_3 and aliquots of the solution were analyzed according to the 3 Steps outlined above.

The values for DKA, DKA + DHA and AA + DHA + DKA were expressed in terms of mg of AA from which they were derived. A Klett-Summerson colorimeter with filter No 50 was used for comparing the colors.

RESULTS

A. Analysis of Bitter Gourds Stored Frozen and Thawed

Data obtained for the analysis of whole bitter gourds or cut material, stored frozen and subsequently thawed, are given in Table 1. The material used in the analyses was taken from different batches of vegetable; also, the analyses were performed on different days. It is, therefore, not intended to compare the values from the individual experiments. It may also be mentioned that in these experiments special care had not been taken to gas the various samples with

TABLE 1

Analysis of Frozen and Thawed Bitter Gourd

	DKA (Step 2)	DKA + DHA (Step 1)	Total (Step 3)
	mg/100 g raw tissue		
Experiment I (whole vegetable frozen)	20.5	84.0	61.6
Experiment II (whole vegetable frozen)	21.9	95.2	88.0
Experiment III (cut vegetable frozen)	16.9	79.1	70.9

H_2S for identical periods of time. It was observed, in fact, that the time taken for the separation of SnS differed in the various experiments; in some cases the precipitate appeared only after 15 min.

The data in Table 1 show that in every case the value obtained in Step 3 was less than that in Step 1.

[b] Manufactured by Nutritional Biochemicals Corporation, U.S.A.

B. Analysis of Homogenates of Bitter Gourd

The data reported in this section were obtained during the course of experiments in progress in this laboratory on the pathway of transformation of DHA. Homogenates of bitter gourd in water, prepared with the aid of a Waring blender, were incubated and analyzed at regular intervals for AA, DHA and DKA. In a second set of experiments a definite amount of DHA was added to the homogenates before incubation. In these experiments, also, special attention was not paid to keeping constant the period of gassing with H_2S. The data are given in Table 2.

TABLE 2

Analysis of Homogenates of Bitter Gourd

	Period of incubation at 35° C	DKA (Step 2)	DKA + DHA (Step 1)	Total (Step 3)
		mg/100 g raw tissue		
Homogenate	Initial analysis	14.0	95.7	83.4
	30 Min.	23.3	95.7	72.9
	60 Min.	28.0	100.3	72.9
		mg/100 g raw tissue + added DHA		
Homogenate + DHA solution	Initial analysis	38.0	131.0	82.0
	60 Min.	38.0	129.0	78.0

The data show that the differences between the values of Step 3 and Step 1 analyses were even more pronounced than those observed with frozen tissue.

C. Effect of Passing H_2S for Varying Periods of Time

In an effort at elucidating the mechanism of the loss encountered in the above experiments, H_2S was passed for varying periods of time at room temperature through aliquots of solutions of DHA (prepared either by bromine-oxidation of AA or by dissolving commercial DHA). Immediately after H_2S-treatment, the particular solution was degassed and, when the aliquot gassed for the longest period was ready, the final assays were carried out simultaneously on all the samples. In this way any effect of contact with acid was equalized in all the samples,

the only variable being the length of exposure to H_2S. The data are given in Table 3.

TABLE 3

EFFECT OF PASSING H_2S FOR DIFFERENT PERIODS OF TIME

	DKA (Step 2)	DKA + DHA (Step 1)	Total (Step 3)
Readings on Klett-Summerson colorimeter			
Experiment 1[1]: DHA prepared by bromine oxidation of AA			
(a)	15	140	126
(b)	20	140	114
(c)	23	140	108
Experiment 2[2]: DHA prepared by bromine oxidation of AA			
(a)	17	160	140
(b)	18	169	137
(c)	18	169	140
Experiment 3[3]: DHA prepared by bromine oxidation of AA			
(a)	22	138	129
(b)	21	138	116
(c)	20	138	120
Experiment 4[4]: DHA prepared by bromine oxidation of AA			
(a)	14	140	130
(b)	17	140	115
(c)	19	140	120
Experiment 5[5]: commercial DHA; fresh solution			
(a)	54	143	140
(b)	56	143	129
(c)	54	143	119
Experiment 6[6]: commerical DHA; solution stored frozen for 3 weeks			
(a)	222	252	240
(b)	200	252	224
Experiment 7[7]: commercial DHA; solution stored frozen for 3 weeks			
(a)	121	188	157
(b)	127	188	160

[1] (a) H_2S passed for 15 min. (b) H_2S passed for 60 min. Precipitate of SnS appeared after 10–12 min in both (a) and (b). (c) Solution was gassed with H_2S for 3 min during which time no precipitate was formed; delivery and exit tubes were closed with screw clips, and the solution left aside for 42 min. H_2S was then passed for 15 min to precipitate SnS.

[2] H_2S passed for 15 min. (b) H_2S passed for 30 min. (c) H_2S passed for 60 min. Precipitate of SnS appeared after 10–12 min in all three cases. The solution taken for analysis corresponded to 6 μg/ml of DHA instead of 5 μg/ml.

[3] (a) H_2S passed for 15 min. (b) H_2S passed for 60 min. Precipitate of SnS appeared after 10–12 min in both (a) and (b). (c) Solution was gassed with H_2S for 3 min during which time no precipitate was formed; tubes were closed and solution left aside for 57 min during which period the precipitate of SnS appeared after 15 min.

[4] (a) H_2S passed for 15 min. (b) H_2S passed for 60 min. Precipitate of SnS appeared after 10–12 min in both (a) and (b). (c) Solution was gassed with H_2S for 3 min during which time no precipitate was formed. The tubes were then closed and set aside for 57 min; the precipitate of SnS appeared after 20 min.

[5] (a) H_2S passed for 15 min. (b) H_2S passed for 60 min. Precipitate of SnS appeared after 10–12 min in both (a) and (b). (c) Solution was gassed for 3 min during which time no precipitate was formed; tubes were closed and the solution was set aside for 42 min (during which precipitate appeared after 30 min). The solution was gassed again for 15 min.

[6] (a) H_2S passed for 15 min. (b) H_2S passed for 80 min. Precipitate of SnS appeared after 10–12 min in both (a) and (b).

[7] (a) H_2S passed for 80 min; precipitate of SnS was formed after 40 min. (b) Solution was gassed with H_2S for 3 min during which time no precipitate was formed; tubes were closed and the solution left aside for 47 min. H_2S was then passed for 30 min more during which the precipitate of SnS appeared after 10 min.

The data in Table 3 show that the prolongation of the time of passing of H_2S, and, in some cases, delaying of formation of insoluble SnS, led to increased loss of the vitamin. An exception was experiment No 2, in which the increase in the period of gassing did not have any significant effect on the loss of the vitamin.

It will also be noted that solutions of DHA prepared by oxidation of AA with bromine always contained a certain proportion of DKA and that the commercial sample was highly contaminated with DKA. The amount of DKA seemed to increase as a result of storage of the solution in a frozen condition.

DISCUSSION

The experiments reported above show that under conditions where AA of vegetables is transformed largely or completely into DHA and DKA (freezing and thawing; homogenization in water with the aid of Waring blender) the values obtained for DHA +

DKA are always higher than the values for AA + DHA + DKA. This discrepancy, though not to the same extent, was observed also when DHA was analyzed. Since the solutions are gassed with H_2S prior to estimation of total ascorbic acid, the loss may be correlated with the H_2S treatment. The loss can be due to adsorption on the SnS precipitate or to transformation to nonchromogenic material. If H_2S were to transform DHA and/or DKA gradually into products which are nonchromogenic (even after bromine-oxidation), the prolongation of the treatment with the gas may be expected to result in greater loss. Results obtained by the present authors show that in the majority of the experiments there was a correlation between the extent of loss and the duration of H_2S-treatment. However, in one experiment, such a relationship was not clear-cut. Penney and Zilva (6) reported that DKA undergoes decomposition during H_2S treatment. DKA may either be present in solution at the outset or may be formed from DHA during H_2S treatment (4). In experiments No 6 and 7 (Table 3) the greater part of the vitamin was present as DKA; nevertheless, the loss was not more prominent. It would therefore appear that loss in vitamin is not directly related to the amount of DKA.

Since completion of this investigation, the authors came across the papers by Gordon and Noble (2, 3); these workers showed that some vegetables contain non-AA components which react with DNPH. If these compounds were to be altered by H_2S treatment to products which cannot couple with DNPH after bromine oxidation, treatment with H_2S may be expected to lead to diminished chromogenic values for the DNPH derivatives of plant extracts. In this connection, it may be pointed out that the losses encountered by us were more pronounced in plant extracts than in solutions of DHA.

It is emphasized that the loss can be *detected* only when the vitamin is present, either completely or mostly in the form of DHA and DKA. Nevertheless, the loss might be taking place even when solutions consisting largely of AA are assayed, resulting in lower values for the reduced form of the vitamin.

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LITERATURE CITED

1. Association of Vitamin Chemists. 1951. *Methods of Vitamin Assay*. Interscience Publishers, New York, **93**.
2. Gordon, J., and I. Noble. 1959. Suitability of 2,4-dinitrophenylhydrazine method for determining dehydro- and total ascorbic acid in raw and cooked vegetables. I. Dehydroascorbic acid. *Food Research*, **24**, 1.
3. Gordon, J., and I. Noble. 1959. Suitability of 2,4-dinitrophenylhydrazine method for determining dehydro- and total ascorbic acid in raw and cooked vegetables. II. Total ascorbic acid. *Food Research*, **24**, 6.
4. Levenson, S. M., H. Rosen, and G. H. Hitchings. 1951. Kinetics of the reaction of dehydroascorbic acid in the presence of hydrogen sulfide. *Arch. Biochem. Biophys.*, **33**, 50.
5. Mills, M. B., C. M. Damron, and J. H. Roe. 1949. Ascorbic acid, dehydroascorbic acid and diketogulonic acid in fresh and processed foods. *Anal. Chem.*, **21**, 707.
6. Penney, J. R., and S. S. Zilva. 1943. The chemical behaviour of dehydro-l-ascorbic acid *in vitro* and *in vivo*. *Biochem. J.*, **37**, 403.
7. Roe, J. H., and C. A. Kuether. 1943. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. Biol. Chem.*, **147**, 399.
8. Roe, J. H., M. B. Mills, M. J. Oesterling, and C. M. Damron. 1948. The determination of diketo-l-gulonic acid, dehydro-l-ascorbic acid, and l-ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method. *J. Biol. Chem.*, **174**, 201.
9. Tewari, C. P. 1959. Studies on ascorbic acid in bitter gourd and cauliflower. *Ann. Biochem. Exptl. Med.*, **19**, 191.

Changes in Amino Nitrogen, Total Soluble Nitrogen and TCA-Soluble Nitrogen Content of Beef as Influenced by Pre-irradiation Heating, Irradiation Level and Storage at 34° F [a, b]

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SUMMARY

Slices of beef were heated to 130°, 150° and 195° F, irradiated at 0.1 and 5.0 megarads and stored at 34° F. The effect of these variables on free amino nitrogen, total soluble nitrogen and TCA-soluble nitrogen was determined at 15-day intervals during the 60-day storage period. Increases in heating temperature reduced the rate of release of TSN and TCA-soluble fractions. Amino nitrogen was reduced only at highest temperature employed. Irradiation increased the rate of release of these fractions. The release of amino nitrogen began immediately upon storage of raw beef. Major amounts of TSN were not released until after 15 days storage and TCA-soluble nitrogen not until after 45 days storage. This suggests successive fragmentation of the initially bound protein.

A complete study of the proteolysis of muscle has been needed for some time. Recent work by Zender *et al* (11) has begun to shed some light on this little known and heretofore rather precarious subject. By using strict methods to retain naturally sterile conditions these authors allowed lamb and rabbit muscles to undergo proteolysis in an anaerobic environment. A steady increase in the level of the free amino acids and a decrease of glycine-soluble protein was noted during storage at both 25° and 38° C. According to electrophoretic studies it appeared that the protein first split into large fractions and later into amino acids. Quantitatively speaking, however, the protein affected by proteolytic processes appeared to be small. This also tended to be the case with fresh beef muscle when stored for 12–15 days (4, 10).

Previous work by Cain *et al* (2) has shown that gamma-irradiated and bacteriologically sterile fresh beef suffered extensive protein alteration as indicated by increasing amino nitrogen during storage at 72° F for 250 days. An increase in the volume of exudate or "drip" from the meat and the development of tyrosine crystals were noted. On the other hand, identically treated beef which had been pre-cooked to 160° F. internal temperature, suffered no significant proteolytic breakdown.

The present work was initiated to determine proteolytic changes during long-term storage of irradiation-sterilized beef. In succeeding papers the effect of the experimental variables on the release of certain amino acids from beef will be presented.

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EXPERIMENTAL

Preparation of Samples

Three Hereford bulls were selected for approximate uniformity as to age, weight, and conformation. Twenty-four hours after slaughter, the *longissimus dorsi* muscles of each carcass were excised and stripped of all visible fat. The pair of muscles from any one animal was used as one replicate. The muscle was cross sectioned mechanically into slices of one-fourth to three-eighths of an inch in thickness to give samples weighing from 40 to 50 g. Individual samples were placed into polyethylene-coated Saran plastic bags, the entrapped air expelled, by submerging the filled bag in water, and heat sealed. The bags of meat were then randomized within a replication to nullify effects of longitudinal variations in the muscle.

Pre-irradiation Heating

Samples within each replication were subjected to three heat treatments. The final internal meat temperatures selected were 130°, 150°, and 195° F and were measured by thermocouples. The bags of meat were heated in a steam-heated water bath wherein temperatures were automatically controlled from one to two degrees above the final desired internal temperature of the meat. Upon reaching the desired temperature, requiring from 6 to 8 min, the meat was rapidly chilled by immersion of the bags in a cold water bath. Each bag of meat was then sealed into a 307 x 202 "C" enamel can and immediately frozen at −18° F. Unheated control samples were kept in each replication.

Shipment and Irradiation of Samples

All except the non-irradiated samples were packed in dry ice and shipped in insulated containers to the Materials Testing Reactor, Idaho Falls, Idaho. During the 8-day interval between shipment and receipt, the samples were kept frozen at 0° F or under dry ice, except during the irradiation period. The cans were removed from 0° F and immediately irradiated. Some thawing undoubtedly took place during the irradiation period since the water shield was at a temperature of approximately 70° F. The non-irradiated samples were kept at 0° F storage throughout this time.

Irradiation was accomplished by exposing the cans to a gamma grid until a dosage of 0.1 and 5.0 megarad had been acquired. The flux intensity was 1.74×10^6 rads per hour; 3 min and 27 sec were required for the 0.1-megarad dose and 2 hr and 52 min for the 5.0-megarad dose. The samples were then frozen, repacked in dry ice and returned to our laboratory where they were put into storage.

Storage of Samples

The non-irradiated and irradiated meat was placed in storage at 34° F and scheduled for analysis at 0, 15, 30, 45 and 60 days of storage.

Quantitative analyses for moisture, total soluble nitrogen, trichloroacetic acid-soluble nitrogen, and amino nitrogen were carried out for each of the samples.

Moisture Determinations

A representative portion of the meat was weighed into aluminum pans and dried for 24 hr at 70° C and 28 inches vacuum. After drying, the pans were cooled in a desiccator and re-weighed. The calculated weight loss was reported as the moisture content.

Sample Preparation for Amino Nitrogen, Total Soluble Nitrogen and Trichloroacetic Acid-soluble Nitrogen

Ten g of meat were blended with 25 ml of distilled water for one minute in a microblender. The samples and blender were cooled to 40° F before blending. The mixture was filtered through Whatman #12 filter paper and the filtrate was used for the following three determinations:

Amino Nitrogen Determination

Five ml of the filtrate were diluted to 100 ml with distilled water. An aliquot of 5 ml of the diluted solution was used for the amino nitrogen determination of Peters and Van Slyke (9). The final results were calculated in mg per cent amino nitrogen on a dry-weight basis of the fresh meat.

Total Soluble Nitrogen Determination

The micro-Kjeldahl method of the A.O.A.C. (1) with the following modifications was used for the total soluble nitrogen determination. One ml of the filtrate was digested with 2 ml of concentrated sulfuric acid with two selenium-coated Hengar granules as the catalyst. Distillation, using a Kirk-type continuous micro-Kjeldahl unit, was made into 25 ml of 4% boric acid as the receiving solution. The indicator used was that suggested by Ma and Zuazaga (5). The results were calculated in mg per cent on a dry-weight basis of the fresh meat.

Trichloroacetic Acid-soluble Nitrogen

The general protein precipitation method of the A.O.A.C. (1) was used except that 25% trichloroacetic acid was used instead of phosphotungstic acid. The extract used was that indicated under the sample preparation above. One ml of the filtrate

TABLE 1

EFFECT OF TEMPERATURE ATTAINED IN PRE-HEATING, IRRADIATION AND STORAGE TIME ON THE GROSS NITROGENOUS CONSTITUENTS OF BEEF STORED AT 34° F

Pre-heating temperature ° F	(mg per cent dry weight basis)					
	Amino Nitrogen		Total Soluble Nitrogen		TCA [1] Nitrogen	
	Days Storage at 34° F					
a. Non-irradiated beef	0	60	0	60	0	60
Raw	260	340	576	1670	226	983
130	217	310	523	1397	217	640
150	176	253	337	787	150	390
195	90	107	293	393	70	93
b. Beef irradiated to 5.0 megarads						
Raw	253	467	677	1707	280	1020
130	193	407	520	1543	237	783
150	150	333	333	880	207	463
195	100	100	287	467	87	97

[1] Trichloroacetic acid-soluble nittrogen.

containing the TCA-soluble nitrogen was digested using the micro-Kjeldahl method of the A.O.A.C. (1). The results were calculated in mg per cent on a dry-weight basis.

RESULTS AND DISCUSSION

AMINO NITROGEN

Analysis of variance of the data showed that although highly significant interactions occurred among the three main variables—heat treatment, irradiation, and storage—the predominating variable responsible for the changes in the amino nitrogen content of beef was the temperature to which it was heated prior to radiation. The length of storage was shown to be of less importance while the irradiation dosage, although significant, was apparently not as important as the other main variables. The F values were 2381 for temperature, 294 for storage time, and 109 for radiation, with 3, 4, and 2/118 degrees of freedom, respectively. Differences between replications were not significant.

Table 1 shows the mean initial and final values for amino nitrogen of the non-irradiated beef and that irradiated to 5.0 megarads as a function of preheating temperature. These data show that the amino nitrogen decreased with an increase in the heating temperature used irrespective of the radiation variable. This is consistent with previous work (3, 6, 7). Heating the beef to 130° or 150° F did not prevent an increase in amino nitrogen content over the 60-day period at 34° F. In fact, the increase was of the same order. Under the same conditions meat heated to 195° F showed practically no increase in amino nitrogen.

Figure 1 shows the relative increase in amino nitrogen of stored raw beef as affected by the radiation variables. It is to be noted that there appears to be little difference in the rate of increase of amino nitrogen in the radiated samples. The rate of increase was much faster in the irradiated meat than in the control. It may be possible that irradiation, per se, renders some of the meat proteins more accessible to attack by the native enzymes.

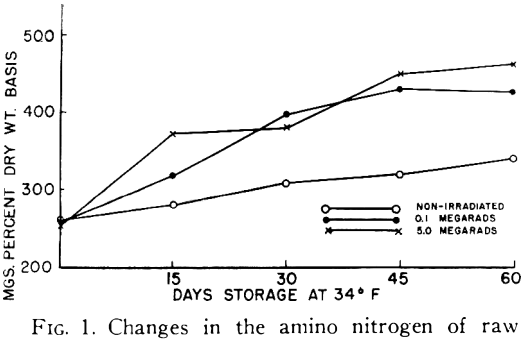

FIG. 1. Changes in the amino nitrogen of raw beef during storage.

TOTAL SOLUBLE NITROGEN

Analysis of variance for total soluble nitrogen revealed that the main effect responsible for the changes was the heating tempera-

ture, despite the fact that significant interactions occurred among the three main variables. Replications were not significant. The storage time was an important secondary source of variation. The irradiation dose, while significant, was not as dominating and influential as the other two main effects. The F values were 3399 for temperature, 1918 for storage time, and 237 for irradiation, with 3, 4, and 2/118 degrees of freedom, respectively.

Table 1 shows the mean initial and final value for total soluble nitrogen of the non-irradiated beef and that irradiated to 5.0 megarads as a function of the heating temperature. As with the amino nitrogen, these data show the importance of the heating temperature in reducing the initial and final level of the total soluble nitrogen.

Figure 2 shows the relative increase in total soluble nitrogen of stored raw beef as affected by the radiation and storage variables. There appear to be only slight differences between the control beef and that radiated at 0.1 megarads. A lag phase was evident which lasted some 30 days and which was followed by an extremely rapid increase in the soluble nitrogen. When irradiated to 5.0 megarads, raw beef exhibited a 15-day lag phase which was followed by a rapid increase in this fraction. The rate of increase was of the same order as in the control sample. Apparently irradiation at the higher level resulted in some alteration of the protein which resulted in degradation of the protein much sooner than in the control samples. Undoubtedly large peptides were formed at a much earlier period in beef irradiated at the 5.0 megarad level.

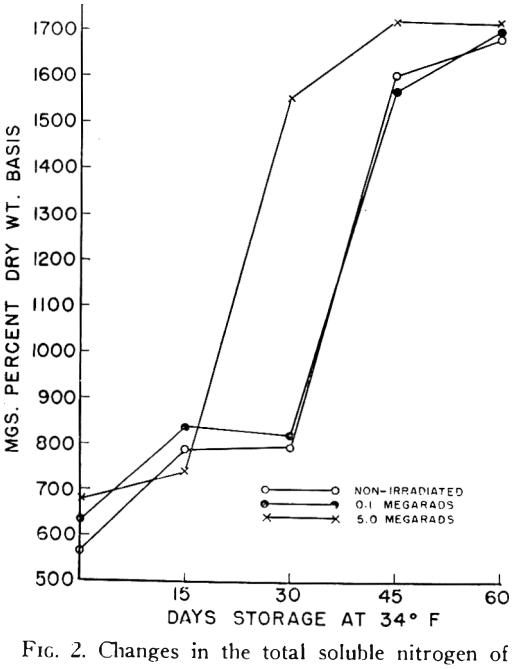

FIG. 2. Changes in the total soluble nitrogen of raw beef during storage.

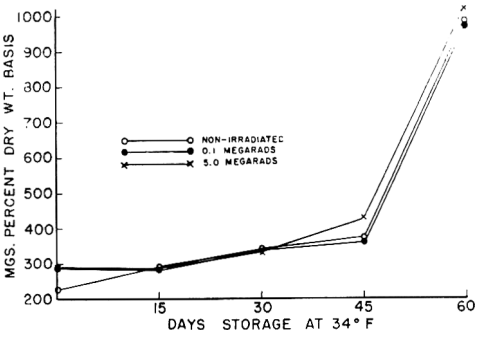

FIG. 3. Changes in the trichloroacetic acid-soluble nitrogen of raw beef during storage.

The increase in total soluble nitrogen of raw, non-irradiated beef stored at 34° F for 30 days was 38%, which is in fairly good agreement with a 41% increase as reported for beef aged 29 days under the usual conditions (8).

TRICHLOROACETIC ACID-SOLUBLE NITROGEN

Analysis of variance for trichloroacetic acid-soluble nitrogen indicated much the same pattern of interactions as was found with the total soluble nitrogen. These data showed that although significant interactions occurred among the three main variables, the predominating factor was the pre-irradiation heating temperature. The storage time was of significant importance though of a secondary nature, while the irradiation dosage, though significant, was not as important a source of variation. Replications were not significant. The F values were 1148 for temperature, 781 for storage, and 11 for radiation, with 3, 4, and 2/118 degrees of freedom, respectively.

Figure 3 presents the relative increase in trichloroacetic acid-soluble nitrogen of raw beef as affected by the radiation and storage-time variables. There appears to be very little difference in the curves, and the slopes

appear to be relatively the same for 45 days of storage. Thereafter one observes an extremely rapid increase in this fraction. This can perhaps be attributed to the breakdown of the large peptides formed before 45 days (Fig. 2) to smaller peptides and amino-acids. Unlike the formation of the larger peptides, however, the release of TCA-soluble nitrogen is not promoted by irradiation.

Husaini *et al* (4) reported a change of 0.442 to 0.445% of TCA-soluble nitrogen of fresh beef 3 to 15 days *post mortem*. The increase in this work was 30%. This may simply be an anomaly due to the lower value for TCA-soluble nitrogen in the fresh non-irradiated beef. It would appear that the value of 226 mg % is somewhat low in comparison to the values obtained for similarly treated irradiated beef.

Upon the application of heat, partial denaturation of the protein apparently occurs. Thus, as the pre-irradiation temperature was increased, the initial value of the constituents analyzed was lowered. Superimposed on this effect was that of irradiation which apparently gave rise to increased sites of attack for the native enzymes. The changes in nitrogenous constituents were not as great in those samples heated to 195° F prior to radiation than when the meat was heated at lower temperatures. The increases which did occur in the beef heated to 195° F may have been due to chemical rather than enzymatic breakdown of the protein.

On the basis of these results it appears that the greatest change in these nitrogenous constituents occurred in the TCA-soluble nitrogen fraction followed by the total soluble nitrogen and amino nitrogen fractions. Storage of fresh non-irradiated beef at 34° F for 60 days resulted in an increase of 335, 190 and 30% for the TCA-soluble nitrogen, total soluble nitrogen and amino nitrogen fractions, respectively. Heating the beef to 130° and 150° F prior to storage reduced the percentage increase of the total soluble and TCA-soluble nitrogen fractions. It did not influence the rate of release of the amino nitrogen fraction.

Sensory changes occurred in the beef. These were due to irradiation and heat treatments both of which were modified by the storage period. In the non-irradiated unheated samples, the microbial growth became apparent in the 30–45-day period. This was offset by increasing the pre-irradiation heating temperature. The effect of microbial contamination in the 0.1-megarad samples was not apparent until between 45 and 60 days and then only in the unheated beef. No evidence of microbial growth could be demonstrated in the beef irradiated at 5.0 megarads.

This study did not attempt to correlate the changes in the nitrogenous constituents with flavor. It is not known if the meat preheated to 150° F would have been acceptable to a panel. It is known that beef roasted to 160° F and irradiated at 3 megarads is acceptable and does not undergo as extensive an alteration in the amino nitrogen pattern as evidenced by these data (2). In this experiment, however, the meat was brought to a specified internal temperature and immediately cooled. If the meat were held at certain of these temperatures for a protracted time considerable differences from these data would undoubtedly have been attained. In any case, the primary result would have been more extensive inactivation of the native enzymes, which, even though the meat was irradiated, could possibly stabilize the meat protein provided the proper temperature/time relationships were met.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Drs. Edward C. Bubl and Peter Nicholls and Messrs. Russell O. Sinnhuber and Samuel R. Cecil for their valuable suggestions. Mr. Orhan G. Artar should be commended for his valuable technical assistance. We would also like to acknowledge the assistance of Mrs. Jean Johnston, Mr. Ricardo Leon and Mrs. Hille Thompson during the preparation of the samples.

LITERATURE CITED

1. Association of Official Agricultural Chemists. *Official Methods of Analysis*. 8th ed. 1955. Washington, D. C.
2. Cain, R. F., A. F. Anglemier, Lois A. Sather, Flordeliza R. Bautista, and R. H. Thompson. 1958. Acceptability of fresh and precooked radiated meats. *Food Research*, 23, 603–610.
3. Ginger, I. D., James P. Wachter, D. M. Doty, and B. S. Schweigert. 1954. Effect of aging and cooking on the distribution of certain

amino acids and nitrogen in beef muscle. *Food Research*, **19**, 410–416.

4. Husaini, S. A., F. E. Deatherage. L. E. Kunkle, and H. N. Draudt. 1950. Studies on meat. I. The biochemistry of beef as related to tenderness. *Food Tech.*, **4**, 313–316.
5. Ma, T. S. and G. Zuazaga. 1942. Microkjeldahl determination of nitrogen. *Ind. Eng. Chem., Anal. Ed.*, **14**, 280–282.
6. Maynard, A. L. and A. V. Tunison. 1932. Influence of drying temperatures upon the digestibility and biological value of fish proteins. *Ind. Eng. Chem.*, **24**, 1168–1171.
7. Newton, R. C., M. M. Piskur, J. M. Ramsbottom, H. E. Robinson, and B. B. McLean. 1937. Review of literature on meat for 1936. *Food Research*, **2**, 581–605.
8. Olsen, H. R. and E. I. Whitehead. 1948. A preliminary study of the water-soluble non protein nitrogen compounds of aged pork and beef muscles. *Proc. S. Dakota Acad. Sci.*, **27**, 180.
9. Peters, John P. and Donald D. Van Slyke. 1932. Quantitative clinical chemistry. Vol. 2. Williams and Wilkins, Baltimore, Maryland.
10. Wierbicki, Eugen, L. E. Kunkle, V. R. Cahill, and F. E. Deatherage. 1954. The relation of tenderness to protein alterations during postmortem aging. *Food Tech.*, **8**, 506–511.
11. Zender, R., C.-Lataste-Dorolle, R. A. Collet, P. Rowinski, and R. F. Mouton. 1958. Aseptic autolysis of muscle: Biochemical and microscopic modifications occurring in rabbit and lamb muscle during aseptic and anaerobic storage. *Food Research*, **23**, 305–326.

Effect of Different Temperatures on Various Bacteria Isolated from Frozen Meat Pies[a]

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SUMMARY

The effect of temperature, particularly storage temperature, is one of the most important factors in controlling microbial populations of frozen foods. Adequate temperature control of frozen foods should limit the growth of microorganisms and insure maintenance of original quality and wholesomeness. This investigation was undertaken to show that correct storage temperatures do inhibit the growth of bacteria which might be considered as the source of potential public health hazard, while certain saprophytic species are able to survive and grow well at the same low temperatures. The cultures were all isolated from chicken pies.

Bacterial counts were determined by plating techniques on Brain Heart Infusion (BHI) agar after various time intervals up to 30 days at −13°, 0°, 5°, 10°, 20° and 37° C, using both BHI broth and chicken gravy as media. Very similar results were obtained in the two media.

Escherichia coli and *Streptococcus faecalis* had a minimum growth temperature between 5° and 10° C for active reproduction. A minimum growth temperature between 5° and 10° C was observed for the culture of *Staphylococcus aureus*. Of two cultures representing variants of *Pseudomonas fluorescens*, one culture followed the growth-temperature pattern shown by the previously mentioned organisms and failed to grow at or below 5° C. The other *P. fluorescens* culture grew rapidly at 5° C. Ability to grow at low temperatures is a constitutive characteristic of the organism and is not common to all types of bacteria. At refrigerator temperatures, the growth of the psychrophilic saprophytic species tested completely outstripped the growth of several bacteria of public health significance. This indicates the great likelihood that a frozen food on defrosting, particularly in a refrigerator, would become completely unacceptable, due to the development of off flavors and odors and deteriorated physical appearance before it became a health hazard.

When foods are cooked, contaminated by handling, and inadequately refrigerated, the food passes from the upper temperature ranges to the lower temperatures slowly; competitive organisms are gone and *Staphylococci* and other organisms increase rapidly. Do *Staphylococci* grow when frozen foods pass from freezing to defrost through a temperature zone where other microbes grow well?

Our present knowledge concerning the growth of organisms from the frozen state to the temperatures of defrost is incomplete and limited. There is little available information regarding the effect of environmental temperatures up to the point of defrost on the growth of bacteria. The present study was made to determine the effect of temperatures of −12° C (8.6° F), 0° C (32° F), and 5° C (41° F) on the growth of bacteria considered to be of public health significance isolated from chicken meat pies, and is part of a continuing study of the microbiology of low temperatures.

EXPERIMENTAL METHODS

For this investigation, the organisms selected were: *Escherichia coli*, *Streptococcus faecalis*, *Staphylococcus aureus*, and two varieties of *Pseudomonas fluorescens*. The last two organisms

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had been associated with defrosted frozen chicken pies. Others were from our culture collection of bacteria isolated from frozen chicken pies.

For our purpose, growth was defined as an increase in the number of viable cells as determined by a plate count. This criterion involved two assumptions: (a) that all the bacterial cells were viable and (b) that all the bacterial cells were single cells and not clumps, and that each single cell gave rise to one colony.

In growth experiments for each of the organisms, brain heart infusion broth and sterile chicken gravy were used as media. The rate of growth of organisms in brain heart infusion broth was used as a control. No attempt was made to maintain the original pH of the medium during the growth of the culture.

The inoculant was prepared by washing a 72-hr brain heart infusion agar slant culture with sterile distilled water. One ml of the cell suspension was transferred to a 99-ml sterile distilled water blank. Portions from the inoculated dilution blank were transferred to other 99-ml dilution blanks and adjusted to give a reading of 85% light transmittancy on a Bausch and Lomb Spectronic 20 set of 400 mμ. Three-tenths-ml aliquots of the final suspension were transferred into each of the growth flasks containing 300 ml of sterile chicken gravy or brain heart infusion broth. The 0.3-ml aliquot gave an original bacterial count of 10^3 to 10^4 organisms per ml of substrate.

Bacterial counts were determined at various time intervals from the substrates incubated at the following temperatures: −13° C (8.6° F), 0° C (32° F), 5° C (41° F), and 10° C (50 F), 20° C (68° F), and 37° C (98.6° F). Plates were incubated at 37° C (98.6° F) for 24 hr for *E. coli*, *S. faecalis* and *S. aureus*, and *P. fluorescens*, strain II. *Pseudomonas fluorescens*, strain I was incubated at 5° C (41° F) for 7 days and 20° C (68° F) for 3 days.

RESULTS AND DISCUSSION

Primary interest in frozen food bacteriology is centered on those factors which affect the length of the lag phase and the rate of bacterial multiplication in the logarithmic growth phase. Stumbo (14) observed that lower temperatures were required to arrest growth in the logarithmic growth phase than to maintain bacteria in the lag phase of the growth cycle. Lawton *et al* (9) noted that the lag phase was not characteristic of the organism, but rather was a physiological condition which could be minimized by increases in temperature. The amount of free water in a food product directly affects the length of the lag period. Increasing amounts of free water shorten the lag period and increase the bacterial growth rate in the logarithmic growth phase.

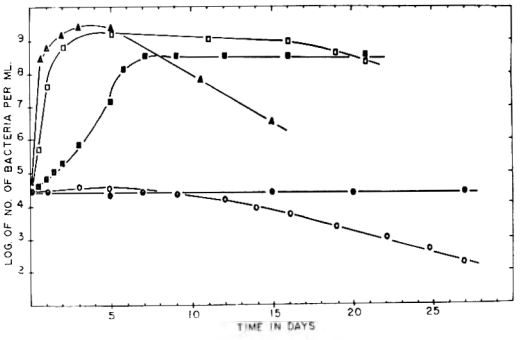

FIG. 1. Growth curves of *Escherichia coli* grown in sterile chicken gravy. ● = −13° C; ○ = 5° C; ■ = 10° C; □ = 20° C; ▲ = 37° C.

Escherichia coli. Figure 1, grown in chicken gravy reached the maximum stationary phase in 3 to 4 days at 37° C (98.6° F) and 20° C (68° F), whereas it required 8 days at 10° C (50° F). A decrease in numbers of this bacterium was noted at 5° C (41° F) throughout the incubation period. At a temperature of −13° C (8.6° F), the counts remained constant. A similar pattern of growth took place in brain heart infusion broth. As would be expected, the culture grew rapidly at 37° C (98.6°F) and at 20° C (68° F). A slower rate of growth of *E. coli* was noted at 10° C (50° F). Barber (2) suggested that *E. coli* did not grow below 10° C (50° F), but his observations were confined to one strain. Dahlberg (6) found that coliform bacteria grew as well as some psychrophiles at low temperatures, as did Burgwald and Josephson (5). A minimum temperature for growth apparently lies between 5° C (41° F) and 10° C (50° F) for the culture employed in this study. Little difference was noted in growth rates between chicken gravy and brain heart infusion broth as culture media in this experiment. Somewhat better buffering action might be expected of the gravy than of the brain heart infusion broth, particularly on extended times of culturing.

Figure 2 illustrates the pattern of growth of *S. faecalis*. This organism exhibited pat-

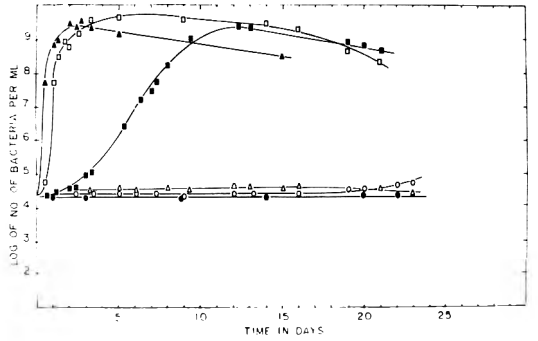

FIG. 2. Growth curves of *Streptococcus faecalis* grown in sterile chicken gravy. ● = −13° C; △ = 0° C; ○ = 5° C; ■ = 10° C; □ = 20° C; ▲ = 37° C.

terns of growth similar to those of *E. coli*. The maximum stationary phase was reached in 2 days at 37° C (98.6° F), in 5 days at 20° C (68° F), and at −13° C counts remained constant. No changes were noted in the growth of *S. faecalis* in brain heart infusion broth except that at −13° C (8.6° F) the counts dropped off sharply. A minimum temperature for growth of this culture lies between 5° C (41° F) and 10° C (50° F). No significant difference was noted between brain heart infusion broth and chicken gravy with the possible exception that chicken gravy provided greater protection to the microorganism at −13° C (8.6° F).

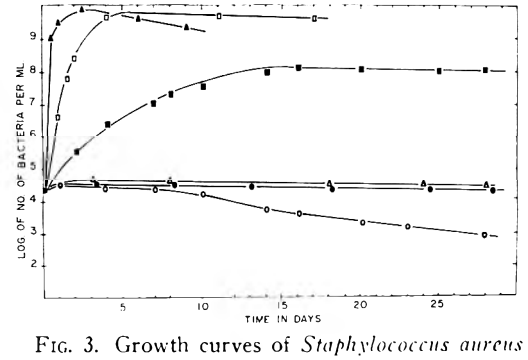

FIG. 3. Growth curves of *Staphylococcus aureus* grown in sterile chicken gravy. ● = −13° C; △ = 0° C; ○ = 5° C; ■ = 10° C; □ = 20° C; ▲ = 37° C.

Figure 3 demonstrates the growth of *S. aureus* in chicken gravy at various temperatures. It is interesting to note that the counts at 5° C (41° F) dropped off in the 30-day period, while the counts at 0° C (32° F) and −13° C (8.6° F) remained somewhat constant. The maximum stationary phase was reached in 48 hr at 37° C (98.6° F), in 6 days at 20° C (68° F) and 15 days at 10° C (50° F). This organism demonstrated similar patterns of growth in brain heart infusion broth and chicken gravy. Again, the minimum temperature for multiplication of this culture was observed to be between 5° C (41° F) and 10° C (50° F).

In a study of staphylococci surviving in frozen vegetables Jones and Lockhead (8) reported that no multiplication occurred at 5° C (41° F), and of 18 enterotoxin-producing strains, none produced toxin at temperatures lower than 20° C (68° F). Nickerson *et al* (11) also stated that *S. aureus* will not grow in food products held below 10° C (50° F).

The presence of *S. aureus* in a mixed culture menstruum, as would be found in foods, results in a competition for growth. When temperatures are brought from the frozen state to the temperatures of defrost (0° C to 5° C), psychrophilic organisms will increase at a faster rate at defrost temperatures than mesophilic bacteria and "competitive inhibition" will cause the psychrophiles to "out strip" the number of *S. aureus*.

Pseudomonas fluorescens, strain I employed in this study was isolated from a defrosted chicken meat pie. *Pseudomonas fluorescens*, strain II was isolated from routine bacteriological examination of a normal chicken meat pie. The organisms were similar morphologically and in some aspects physiologically. Figure 4 illustrates the patterns

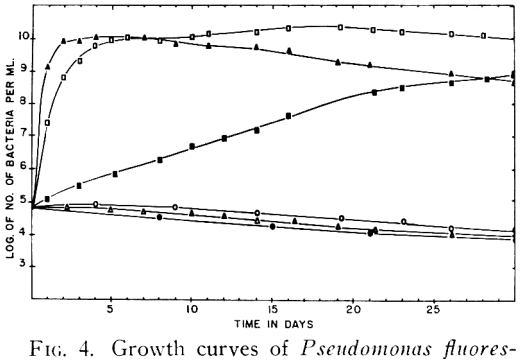

FIG. 4. Growth curves of *Pseudomonas fluorescens* strain II grown in sterile chicken gravy. ● = −13° C; △ = 0° C; ○ = 5° C; ■ = 10° C; □ = 20° C; ▲ = 37° C.

of growth of a *P. fluorescens*, strain II in chicken gravy. This organism demonstrated a pattern of growth similar to the previous organisms studied. At 37° C (98.6° F) the organism reached the maximum stationary stage in 3 days and at 20° C (68° F) it required 5½ days. At 10° C (50° F) the organism was still increasing in total numbers at the end of 30 days. Storage at 5° C (41° F), 0° C (32° F) and −13° C (6.8° F) resulted in a gradual decline in total numbers. However, the growth pattern of *P.*

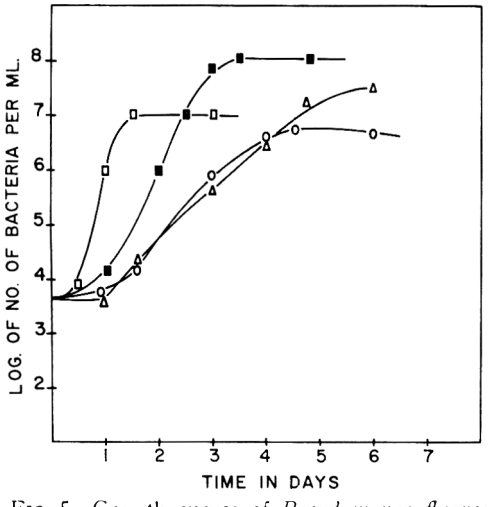

FIG. 5. Growth curves of *Pseudomonas fluorescens* I grown in sterile chicken gravy. No growth was present at 37° C. △ = 0° C; ○ = 5° C; ■ = 10° C; □ = 20° C.

fluorescens, strain I was different (Fig. 5). No growth took place at 37° C (98.6° F). At 0° C (32° F) and 5° C (41° F) the organism reached the maximum stationary stage at the end of 5 days, whereas at 20° C (68° F) it required only 1½ days; and at 10° C (50° F) the organism required 3½ days. *Pseudomonas fluorescens*, strain I, therefore, demonstrated marked "psychrophilic" characteristics.

Van Der Zant and Moore (16) reported that with a single exception the lag period of psychrophilic pseudomonas did not exceed 24 hr even at 5° C (41° F). We have demonstrated that low temperatures only retard the growth of some bacteria. Marth and Frazier (10) found that psychrophilic bacteria isolated from milk belonged to the following genera in decreasing order of occurrence: *Pseudomonas*, *Achromobacter*, *Aerobacter*, *Alcaligenes*, and *Flavobacterium*. Lawton and Nelson (9) also found psychrophilic bacteria in milk to be principally members of the genus *Pseudomonas*. In a study of psychrophilic bacteria in frozen fruits and vegetables, Hucker (7) found that the most numerous type was a Gram-negative rod of the *Flavobacterium* type. "Paired species of micrococci" which may have been saprophytic species of the genus *Neisseria* were next most numerous, while only a few *Achromobacter* were observed. Ayres *et al* (1) found *Pseudomonas* to be most prevalent in cut-up poultry and that these organisms had lag phases of less than two days at 4.4° C (40° F) and 10° C (50° F). Prescott and Tanner (12) noted the selective action of low temperatures on the ordinary organisms of spoilage and on saprophytes in general. Proteolytic organisms did develop at temperatures between 0° C (32° F) and 5° C (41° F), while carbohydrate fermenters did not develop. In a study of microorganisms growing on frozen meats Sulzbacher (15) found that a majority of bacteria were pseudomonads. Similar results were reported by Berry (3, 4) in a study of frozen fruits and vegetables. He also observed that bacteria of the genus *Pseudomonas* increased in peas stored at −4° C (25° F). In a study of milk Rogick and Burgwald (13) reported that only 4.2% of the psychrophilic bacteria were "true psychrophiles," and all were alkaline-forming Gram-negative bacteria. Of the other facultative psychrophiles, 54% were "inert," 28% were acid-formers, and 18% were alkaline-forming. Thus, according to the literature, the typical psychrophile is a food spoilage organism, but not a food poisoning one.

The frozen-food processor has already recognized the virtues of carrying out all of his processing operations during the lag phase of bacterial growth, and by quick-freezing his product within minutes of manufacture he ensures that bacteria do not begin to multiply actively. Quick freezing also helps to eliminate water of condensation, which as free water, shortens the lag phase.

Nickerson *et al* (11) have stated than "in summarizing the situation of frozen foods

in relation to public health it is evident that these comestibles are no more likely to be involved in causing food infections and food intoxications than are foods processed by other methods of preservation."

LITERATURE CITED

1. Ayres, J. C., W. S. Ogilvy, and G. F. Stewart. 1950. Post mortem changes in stored meats. I. Microorganisms associated with development of slime on eviscerated, cut-up poultry. *Food Technol.*, **4**, 199–205.
2. Barber, J. 1908. The multiplication of *B. coli* at different temperatures. *J. Infect. Dis.*, **5**, 379–400.
3. Berry, J. A. 1933a. Some findings on the microbiology of the cold pack. Canning Age, **14**, 445–446.
4. Berry, J. A. 1933b. Destruction and survival of microorganisms in frozen pack foods. *J. Bacteriol.*, **26**, 459–470.
5. Burgwald, L. H. and D. V. Josephson. 1947. The effect of refrigerator storage on the keeping qualities of pasteurized milk. *J. Dairy Sci.*, **30**, 371–383.
6. Dahlberg, A. C. 1945. The keeping quality of pasteurized milk in the New York Metropolitan area during cool weather as determined by bacterial counts, presence of coliform bacteria and flavor score. *J. Dairy Sci.*, **28**, 779.
7. Hucker, G. J. 1954. Low temperature organisms in frozen vegetables. *Food Technol.*, **8**, 79–82.
8. Jones, A. H. and A. G. Lockhead. 1939. A study of micrococci surviving in frozen-pack vegetables and their enterotoxic properties. *Food Research*, **4**, 203–216.
9. Lawton, W. C. and F. E. Nelson. 1954. The effect of storage temperatures on the growth of psychrophilic organisms in sterile and laboratory pasteurized skim milks. *J. Dairy Sci.*, **37**, 1164–1172.
10. Marth, E. H. and W. C. Frazier. 1957. Bacteriology of milk held at farm bulk cooling tank temperatures, III. *J. Milk and Food Technol.*, **20**, 93–99.
11. Nickerson, J. T. R., B. E. Proctor, and S. A. Goldblith. 1958. Freezing and irradiation. *Am. J. Public Health*, **48**, 1041–1048.
12. Prescott, S. C. and F. W. Tanner. 1938. Microbiology in relation to food preservation. *Food Research*, **3**, 189–197.
13. Rogick, F. A. and L. H. Burgwald. 1952. Some factors which contribute to the psychrophilic bacterial count in market milk. *J. Milk and Food Technol.*, **15**, 181–185.
14. Stumbo, C. R. 1947. Significance of bacterial growth curves in food plant control. *Food Technol.*, **1**, 229–235.
15. Sulzbacher, W. L. 1950. Survival of microorganisms in frozen meats. *Food Technol.*, **4**, 386–390.
16. Van Der Zant, W. C. and A. V. Moore. 1955. The influence of certain factors on the bacterial counts and proteolytic activities of several psychrophilic organisms. *J. Dairy Sci.*, **38**, 743–750.

Reflectance Color Measurements and Judges' Scores for Frozen Cauliflower and Spinach

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SUMMARY

Color data on stored spinach and cauliflower were correlated with judges' scores to determine best sampling procedures for measurements with the Hunter Color and Color-Difference Meter. Spinach stems, rather than leaves or mixtures, changed the most and most uniformly, correlated best with scores, and produced the smallest experimental errors. Cauliflower floret surface, instead of stems or mixture, gave smallest significant differences by judges. Good results on the instrument were obtained for both floret surface and a rapidly prepared slurry from a thin surface layer. Best of six color indices of deterioration were Hunter "a" and hue angle.

Color measurements with the Hunter Color and Color-Difference Meter have been found useful in stability experiments with a number of food products.[b] Data of this type were needed for extensive experiments on frozen cauliflower and spinach. The literature did not report any applicable methods, and our preliminary studies on these products (not published) revealed unsatisfactory reproducibility of measurements. The present experiment was undertaken, therefore, to determine what factors lead to reproducibility.

Measurements with the instrument were made on samples composed of individual parts and blends of each vegetable to increase homogeneity, since the colors of the parts differed substantially. It was required, however, that a sample type should represent the whole vegetable as regards changes observed by the judges. The principal objective was to develop a method for preparing and measuring one or more of the kinds of samples which yield the highest degrees of correlation between the instrument data and judges' color scores.

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

[b] Mention of commercial products does not imply endorsement by the Department of Agriculture over others of a similar nature not mentioned.

MATERIALS AND METHODS

Materials

Retail packages of commercially processed vegetables, Grade A packs of Viroflay spinach and Snowball cauliflower, were used so that results could be applied to commercial material in other experiments. Reference or control samples were held at −20° F. throughout the experiment. Sublots of the spinach were held at 20° F. for lengths of time varying from 1 to 84 days; cauliflower from 1 to 110 days. These holding periods provided a color quality range from excellent to one so poor that consumers would probably object to eating the food.

Judges' Procedures

Judges scored color of upper halves of leaves, stems and mixture of total contents of the spinach package, and of cauliflower floret surface, stems and mixture, each as thawed and as cooked vegetable. Score 1 indicated vivid green spinach and very white cauliflower; these colors were illustrated with a labeled control with each group of samples. Score 5 meant very grayed and/or very yellow green spinach and very dark and/or very yellow cauliflower. Samples of one type (for example, uncooked stems) for all holding periods were scored in one session. Five repetitions by 6 to 8 judges for each group of samples completed the appraisal data, each repetition on a different day.

Hunter Meter Methods

All instrument measurements were made on thawed, uncooked vegetables. The samples were measured in the 9-cm I.D. cells with the rotator

described by Lukens and Creese (3). The spinach samples included upper halves of leaves, the stems, and the mixtures. Cauliflower samples included the floret surface and the slurry prepared from the floret.[c] A photograph of the cauliflower floret surface and spinach mixture is shown in Figure 1.

Special procedures were developed for packing some of the samples into cells. The mixture (complete spinach) sample presented one difficulty. In an attempt to get a representative random distribution of leaves and stems, each leaf was held by its middle portion with the upper leaf and stem freely hanging. It was then simply dropped into the cell and packed with others without rearranging the parts. Steps in preparing the cauliflower florets were: (a) cut off main stem, retaining only enough branch stems to hold florets together, (b) cut florets lengthwise to give pieces with a top diameter of ⅜ to ½ inch, (c) pack pieces closely in cell with floret surface on bottom of cell, (d) fill cell with pieces in order to hold measured area against bottom of cell, remove large air bubbles with the aid of a knife point, and screw lid on cell. Steps in preparing slurry were: (a) cut off thin layer of the top of each floret piece, such that only a few calyces held together, (b) blend 80 g of calyx material and 40 ml of distilled water in pint Waring blender at low speed (50 volts) for 1 min, and (c) put slurry into cell and remove any air bubbles at measured area with rubber spatula. Preliminary studies showed that sufficient deaeration was accomplished by giving each slurry a few sharp jolts.

The reference plate used for standardization of the instrument for spinach measurements was the National Bureau of Standards bath green designated SBC 12 for which Hunter values of Rd = 31.0, "a" = −24.0, "b" = +13.1 have been established. The reference plate for cauliflower was ivory, SBC 31 (Rd = 57.0; "a" = −1.6; "b" = +24.6). Rd was converted to lightness index, "L", by Hunter's formula, "L" = fy Rd, where fy = 0.51 $\left(\frac{21 + 0.20\ \mathrm{Rd}}{1 + 0.20\ \mathrm{Rd}}\right)$. Values were calculated for hue angle ($\tan^{-1} b/a$), saturation index ($\sqrt{a^2 + b^2}$), and total color difference from control, as defined by $\sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$. These procedures of reporting Hunter color data correspond to those described by Hunter (2).

Seven packages of spinach at each holding time were measured; 2 readings were made on each sample (stems, leaves, mixture) from each package. Five packages of cauliflower per holding time and 4 readings per sample provided replicates for this vegetable. The number of repetitions of packages and readings was based on the variability found in preliminary studies. One package of each holding time of a given vegetable was measured on one day.

Statistics

Comparisons of the experimental errors of the several samples and measurements were based on

[c] Preliminary studies showed that the unaltered surface of contents of the spinach package gave unsatisfactory reproducibility of measurements; finely sliced composite was unsatisfactory because color changed during holding in a different way than the original mixture. Also preliminary studies showed that chopped stems or chopped composite of cauliflower did not give satisfactory replicate values.

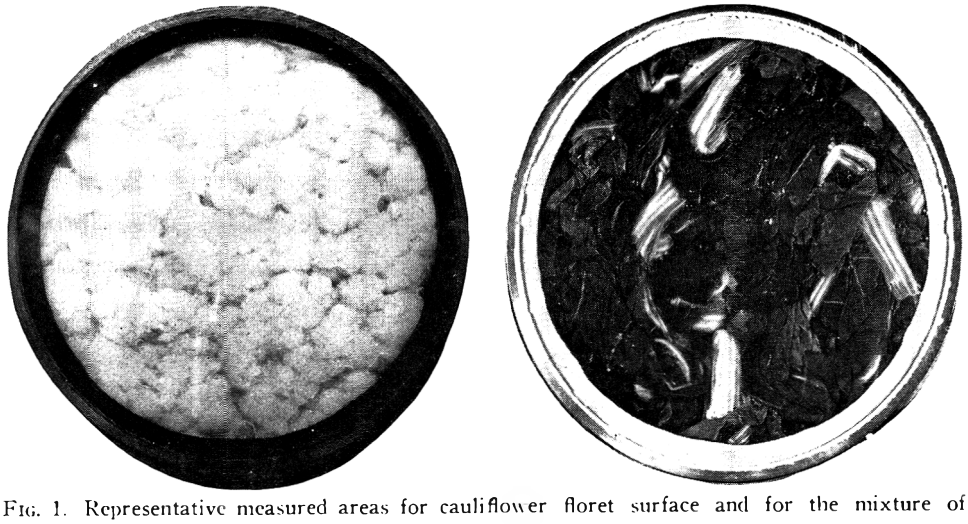

Fig. 1. Representative measured areas for cauliflower floret surface and for the mixture of the spinach package contents.

data obtained from the analysis of variance and the Duncan test (1) at the 5% level of significance. Sources of variability in the Hunter data included treatments (holding times), days of tests, readings, and the interactions with treatments. The same sources of variability applied to scores, except that judges were substituted for the instrument readings. Correlation coefficients for objective and subjective values were calculated.

RESULTS AND DISCUSSION

Figure 2 illustrates the meaning of the terms, hue angle and saturation index, further explained in the figure legend. As previously mentioned, the colors of spinach leaves and stems differed considerably; stems were much more vivid and had a somewhat yellower green hue than leaves. Values for spinach mixture sample (not illustrated) were close to the leaf values, because the leaves constituted 80% of the weight of this pack. Holding the spinach for 84 days at 20° F resulted in yellower and grayer green colors for both samples. Surfaces of the cauliflower florets became yellower during the 110 days holding period, but changed only slightly in saturation index.

Plots of judges' scores (Figure 3) show that throughout the studies judges observed

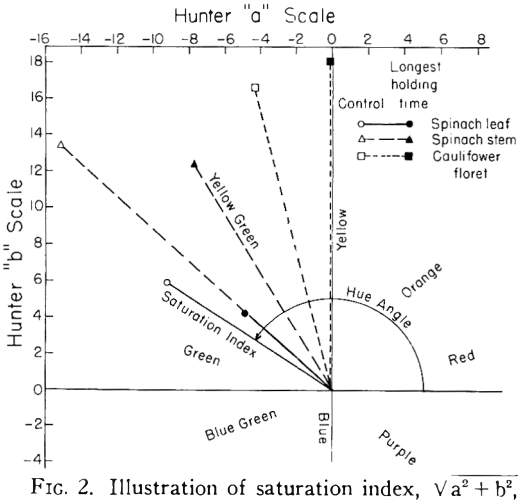

FIG. 2. Illustration of saturation index, $\sqrt{a^2 + b^2}$, and hue angle, $\tan^{-1}$ b/a, for control and most deteriorated sample of stems and leaves of spinach and for cauliflower floret surface. (The radial distance from origin to the point in question is the saturation index, measured in NBS units. The hue angle, measured in degrees, begins at the positive "a" axis and increases counter clockwise to the line extending from origin through the point representing the sample. Lightness index, "L" scale, is not illustrated; it can be visualized as a line perpendicular to both the "a" and "b" scales.)

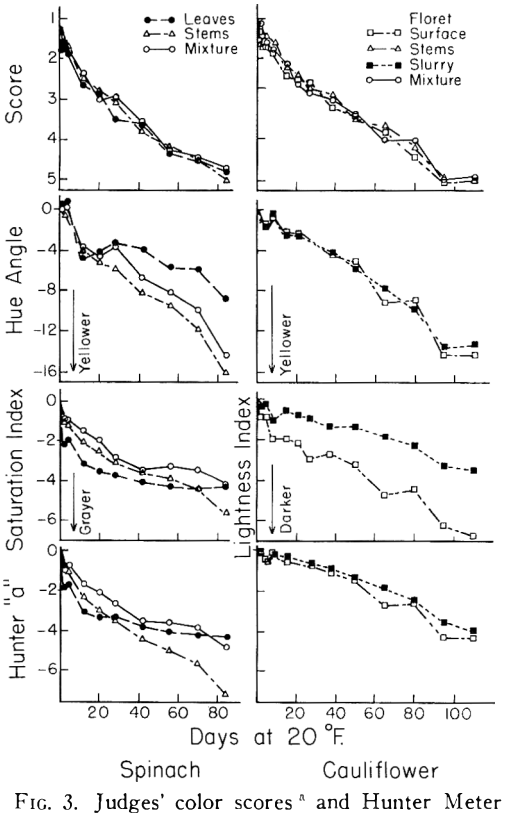

FIG. 3. Judges' color scores [a] and Hunter Meter differences from controls [b] for thawed spinach and cauliflower held varying times at 20° F.

[a] Spinach: Score 1—Vivid green color like labeled control.
" " 5—Very yellow and/or very grayed green.
Cauliflower: Score 1—White like labeled control.
" " 5—Very yellow and/or very dark.

[b] Control Values.

	Hue angle	Satur. index	Lightness index	Hunter "a"
Spinach leaf	147°	10.9	20.9	9.2
Spinach stem	138°	20.2	34.6	15.1
Spinach mixture	143°	12.7	24.2	10.1
Cauliflower floret	104°	17.1	69.2	4.3
Cauliflower slurry	109°	15.1	70.6	4.9

progressive color changes in thawed spinach leaves, stems, and mixture samples and in cauliflower floret surface, stems, and mixtures. Although the curves for a given vegetable are similar, finer differentiation was obtained for the cauliflower floret surface and for the spinach stem samples than for other parts of the vegetables. For the cauliflower florets and spinach stems, significant differences between the samples representing adjoining holding times were obtained in practically all cases. Judges' scores for cooked vegetables agreed closely with those for uncooked vegetables, as indicated by correlation coefficients between 0.97 and 0.99.

The instrument data for each sample will be discussed as regards: (a) changes with holding time, (b) correlation of Hunter values with scores, and (c) experimental errors. The objective is to point out the most satisfactory sample type of each vegetable and the most useful color index for the vegetable.

The spinach data in Figure 3 show that leaf color changed fastest during the initial storage. This tendency was more pronounced for lightness index values (not illustrated). The curves for stems and for mixture of total package contents both show more uniform rates of change than those for leaves. Stems did not darken appreciably at any time, and mixtures behaved more like leaves.

The similarity of the curves for the objective and subjective data is indicated in the graphs. Correlation coefficients above 0.96 were found for stems and above 0.93 for the mixture samples. Leaves gave lower values. The coefficients refer to each of four Hunter functions compared with the scores for mixture. For each function "a," hue angle, saturation index or total color difference, the highest coefficient was associated with stems.

Comparison of sensitivity of the various functions for the three types of spinach samples was obtained by the method of Schumann and Bradley (4). These comparisons showed that for any given function, the stems gave more sensitive results than the leaves or the mixture, and Hunter "a" and hue angle of stems were more sensitive than other functions for stems. The experimental errors for stems were such that when values for two holding times differed by 0.4 in Hunter "a" or 1.5° in hue angle, the difference was significant.[a] These differences were 6% ("a") and 9% (hue angle) of the total changes which occur in the experiment. Therefore, considering the graphs, correlation coefficients and sensitivity, it was concluded that the Hunter "a" and hue angle data on stems provided the best measure of spinach color deterioration.

Cauliflower floret and slurry curves for Hunter "a" and hue angle (Fig. 3) were essentially alike, and these functions changed rather uniformly throughout the experiment. The lightness index for slurry changed less than for florets. The most likely explanation is that slurry contained some internal material with less color change than had occurred on the surface.

Correlation coefficients for any one of the functions Hunter "a," hue angle, lightness index, with judges' scores for whole cauliflower were 0.94 or higher, and the coefficient for total color differences was only slightly lower.

The Schumann and Bradley method (4) applied to the cauliflower data showed that approximately equal sensitivity was obtained with Hunter "a" and hue angle for both florets and slurry. Other functions were less sensitive. Experimental errors for the "a" values and hue angles were at least as small as those discussed above for spinach. Considering all aspects of the cauliflower results, Hunter "a" and hue angle were the best functions, and florets and slurry gave approximately the same results. The experimenter, however, would prefer the slurry because it required much less preparation time.

Reproducibility of the instrument measurements in the present study was considerably better than that obtained previously. Standard deviations of the best data for controls were one-fourth to one-seventh of

[a] S.S.R. by Duncan test (*1*) at 5% level, based on mean square for replicate packages within holding times; this was the largest mean square, other than that for holding times.

those obtained in the preliminary experiments.

LITERATURE CITED

1. Duncan, D. B. 1955. Multiple range and multiple F tests. *Biometrics*, **11**, 1–42.
2. Hunter, Richard S. 1942. Photoelectric tristimulus colorimetry with three filters. Circular of the National Bureau of Standards C 429.
3. Lukens, H. C., and F. G. Creese. 1958. A sample rotator for the Hunter Color and Color-Difference Meter. *Food Technol.* **12**, 570.
4. Schumann, D. E. W., and R. A. Bradley. 1959. The comparison of the sensitivities of similar experiments: Model II of the analysis of variance. *Biometrics*, **15**, 405–416.

The Sodium Content of Hawaii-Grown Fruits and Vegetables in Relation to Environment [a]

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SUMMARY

From four of the major islands in the Hawaiian chain, 19 foods of plant origin, the soil in which they were grown and the water used for irrigation were assayed for sodium. Data on the influence of environment upon the sodium content of fruits and vegetables are given. Most fruits and vegetables were low in sodium. A few, such as papaya, beets, celery, daikon, and sweetpotato, were high and probably should be omitted from very restricted sodium diets. The sodium concentration of some of the foods varied with the soil sodium concentration, which points out the necessity of considering the source of these particular foods before using them in a sodium-restricted diet. The results indicate that variations in sodium should be expected in plant foods, although the differences may not always be nutritionally significant.

The increased use of sodium restriction as a therapeutic practice has produced a great need for accurate determinations of the low quantities of sodium found in plant foods. The literature reveals a wide range of sodium values for a given food of plant origin, but variations in environmental factors have been suggested as the chief cause (7). Few studies have been made on environmental effects on sodium absorption since sodium is not considered essential for growth, although it has been proven beneficial in some plants (6, 7). The Foods and Nutrition Department of the University of Hawaii, as early as 1937, reported that papayas grown in littoral regions not only had a salty taste, but a higher chloride content than those grown some distance from the sea (11). Just what other foods might be similarly affected was unknown. Thus, a project was planned to evaluate the sodium content of fruits and vegetables in relation to soil sodium and proximity to the sea. It was thought that the sodium content of plants, if studied with the object of determining the ranges of variation rather than single values, would be of more value to doctors and dietitians in planning low-sodium meals.

PROCEDURE

SAMPLING

An attempt was made to survey several important plant foods grown in areas of different salinity in the Islands. Collections were from various selected areas on Oahu, the lower slopes on Maui, the seashore region on Molokai, and two areas of high elevation on Hawaii. All plant samples were personally gathered by the senior author applying random sampling among the best-quality produce in a field. For most foods about 12 units of a fruit or vegetable comprised the primary sample. Large items such as cabbages and celery were collected in lots of six to twelve units. For the subsample either opposite eighth or quarter sectors or the entire sample, cut into one-inch pieces, was used, from which 150 g were comminuted and 50 g withdrawn for each assay aliquot. For beans, about 2 lb were gathered and cut into one-inch pieces. For bananas, half of the fruits from 2 to 4 hands taken from 2 to 4 trees was used. Lengthwise halves from each banana were combined and blended to constitute a second subsample; from this portions were removed for analyses. The raw, edible portions of all samples were used for analyses.

[a] Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Paper No. 476.

An attempt to control the horticultural variety through selection of farms was not always possible. In some cases the growers were unable to supply the varietal information, and in other cases it was not possible to find the desired variety being cultivated in the desired environment. Thus, for some foods, the variety could not be kept constant.

Soil samples were obtained from the fields in which the plants were growing, when the fresh fruits and vegetables were harvested. Irrigation water samples were also collected.

Information was gathered on fertilizer and pesticide treatments and distance from the sea and sea breezes.

Chemical Method

The flame photometric method described by AOAC for the Beckman DU (3), but employing hydrogen as a fuel and a photomultiplier attachment, was found to be satisfactory for assaying the low quantities of sodium present in plants. Preliminary work indicated that the effect of interference from ions was negligible, but as a precautionary measure, in each sample calcium and phosphate ions were removed from one of the duplicate aliquots. The recovery of added sodium was tested at intervals and the usual precautions for trace analyses were maintained. A standard curve was determined with each day's operation of the Beckman spectrophotometer and every sample was read twice with checking of the reference standard before and after each reading.

The water samples required only filtering and dilution with distilled water prior to reading (2). The soil was assayed by one of the authors (YK) of the Agronomy and Soil Science Department. The sodium was extracted with normal ammonium acetate, adjusted to pH 7, and analyzed using the Beckman DU spectrophotometer (10).

RESULTS AND DISCUSSION

The results for 19 foods for which 3 or more assays were made and for the corresponding water and soil samples are summarized in the table. Samples for a given food are arranged in order of increasing sodium content in the plant. The moisture content and the source of the samples are also given.

Climatic conditions interacting with soil conditions are known to influence the mineral content of plants (8), but climatic variables are difficult to assess in a study such as this. Thus, the soil was considered the most important environmental factor which would reflect sodium deposition by water, fertilizer and pesticide treatments, and sea breezes. The amount of sodium found in the soil may also vary from day to day, depending principally on such factors as daily variability of sodium in the irrigation water, frequency and volume of irrigation and time of soil sampling, that is, whether the soil is collected immediately before or after irrigation or rain. However, in a general survey such as this, it is expected that the sodium level in the soil will reflect the over-all sodium environment of a given locality and may explain differences obtained in analyses of plant sodium. A direct relationship between the environmental supply of this element and its concentration in plant tissue has been demonstrated for some plants (6, 8).

For the most part, the data given here are in agreement with previous reports. The sweetpotato figures are somewhat higher than published figures and head lettuce and celery values lower (1, 4, 5, 9).

Most fresh fruits and vegetables are normally low in sodium content. Bananas, green beans, green onions, head lettuce, long and round eggplant contained the lowest amounts (less than 5 mg/100 g).

On the other hand, high values (50 mg or more), as well as a range of 25 mg or more, were found in samples of papaya, beets, celery, daikon (*Raphanus sativus*), and sweetpotato. A marked response to differences in soil sodium was apparent in papaya with the highest concentration (76 mg/100 g) being more than twenty times that of the lowest (3.6 mg/100 g). This fruit, an important source of vitamins in Hawaii, could be consumed on a restricted sodium diet of 200 mg per day if obtained from inland areas but not if grown in certain areas near the sea. One serving of papaya from each of the two locations could furnish 4% or 85% of the daily sodium allowance. Beets, celery, and daikon, which are known to be salt-responsive crops (6, 7), were all found by this laboratory to have relatively high-sodium values; sweetpotato had the highest sodium content of any food, although all the samples of these four vegetables were from low-sodium soils.

Head cabbage and Chinese cabbage sam-

TABLE 1

THE SODIUM CONTENT OF HAWAII-GROWN FRUITS AND VEGETABLES IN RELATION TO ENVIRONMENT

Food	Source of Sample [1]				Moisture	Sodium		
	Location	Elevation	Distance from sea	Water supply		Food	Water	Soil
		feet	*miles*		%	*mg/100 g*	*mg/100 g*	*mg/100 g*
Bananas								
1 Chinese	Waiahole	<20	0.2	Stream	76.4	0.4	1.5	25.1
2 Bluefield	Maunawili	150	3.8	Rain, City & County	72.4	0.4	1.4	9.9
3 Brazilian	Maunawili	150	3.8	Rain, City & County	68.2	0.5	1.4	9.9
Beans, Green								
1 Lualualei	Makaha	40	0.5	Well	92.3	0.6	11.2	31.5
2 Lualualei	Kaneohe	50	0.6	Stream	92.0	0.7	3.6	9.4
3 Purple Seeded	Kipapa	500	6.0	Stream	92.7	0.7	2.1	4.8
4 Purple Seeded	Waialua	<20	0.5	Well	91.9	0.9	8.5	30.4
5 Lualualei	Waikane	20	0.1	Stream	91.5	1.0	2.0	4.9
6 Lualualei	Waimanalo	20	0.3	Irrigation ditch	92.5	1.0	2.8	16.7
7 Hawaiian & Maui Wonder	Waimanalo	30	1.0	Irrigation ditch	92.1	1.1	2.8	9.4
8 Lualualei	Kailua	120	1.9	Stream, City & County	93.2	1.7	4.3	9.0
9 Lualualei	Koko Head	<20	1.5	City & County	92.8	2.3	3.7	15.8
Beets								
1 Unknown	Kipapa	500	6.0	Stream	86.6	53.5	1.3	4.9
2 Detroit Dark Red	Moanalua [2]	20	1.0	Well	91.4	64.0	3.9	29.0
3 Detroit Dark Red	Moanalua [2]	20	1.0	Well	88.5	79.5	3.9	8.6
Cabbage, Chinese								
1 Chee Fu	Volcano, Hawaii	3800	12.0	Rain	96.2	2.8	0.5 [3]	1.9
2 Chee Fu	Volcano, Hawaii	3800	12.0	Rain	96.4	5.0	0.5 [3]	2.9
3 Chee Fu	Kamuela, Hawaii	2800	10.0	Reservoir	96.0	6.4	0.6	4.8
4 Chee Fu	Kamuela, Hawaii	2800	10.0	Reservoir	96.4	9.3	0.4	5.9
5 Won Bok	Kipapa	500	6.0	Stream	95.7	10.4	1.3	3.6
6 Won Bok	Waialua	20	0.8	Well	95.8	27.6	13.4	15.3
Cabbage, Head								
1 Copenhagen Market	Kamuela, Hawaii	2800	10.0	Reservoir	94.4	4.2	0.4	7.2
2 Copenhagen Market	Volcano, Hawaii	3800	12.0	Rain	94.0	4.8	0.5 [3]	1.8
3 Copenhagen Market	Kamuela, Hawaii	2800	10.0	Reservoir	93.2	5.1	0.6	6.0
4 Copenhagen Market	Kipapa	500	6.0	Stream	93.1	5.6	1.3	6.2
5 Copenhagen Market	Kailua	120	1.9	Stream, City & County	92.6	6.7	4.3	5.0
6 Copenhagen Market	Kula, Maui	3500	9.2	Irrigation ditch	93.4	10.4	1.9	3.9
7 Copenhagen Market	Ewa	30	1.5	Well	92.0	22.2	42.5	45.1
Celery								
1 Utah Special	Mokuleia [2]	<20	0.5	Well	93.9	27.2	7.4	19.3
2 Utah Special	Kamuela, Hawaii	2800	10.0	Reservoir	95.2	37.0	0.6	12.2

TABLE 1—Continued

	Food	Source of Sample [1] Location	Elevation	Distance from sea	Water supply	Moisture	Sodium Food	Sodium Water	Sodium Soil
			feet	*miles*		%	*mg/100 g*	*mg/100 g*	*mg/100 g*
3	Utah Special	Mokuleia [2]	<20	0.5	Well	95.0	37.2	3.6	5.9
4	Utah Special	Kamuela, Hawaii	2800	10.0	Reservoir	94.2	52.0	0.4	4.0
Cucumber									
1	Unknown	Kailua	120	1.9	Stream, City & County	95.9	2.2	4.3	7.4
2	Burpee's Hybrid	Kipapa	500	6.0	Stream	96.0	2.3	1.3	6.1
3	Unknown	Kaneohe	50	0.6	Stream	95.6	2.5	3.6	8.7
4	Burpee's Hybrid	Mokuleia	<20	0.5	Well	95.4	2.6	7.4	24.2
5	Unknown	Koko Head	<20	1.5	City & County	94.9	4.7	3.7	17.9
6	Unknown	Waimanalo	50	0.8	Irrigation ditch	95.7	6.2	2.8	42.8
7	Unknown	Koko Head	50	1.2	Well	95.4	6.3	68.0	213.0
8	Burpee's Hybrid	Waimea	30	0.1	Well	95.2	9.7	34.0	45.8
Daikon (Japanese Radish)									
1	Mino-Kokonoka	Volcano, Hawaii [2]	3800	12.0	Rain	94.0	5.2	0.5 [3]	2.8
2	Mino-Kokonoka	Volcano, Hawaii [2]	3800	12.0	Rain	94.9	6.6	0.5 [3]	1.8
3	Chinese Radish	Moanalua	20	1.0	Well	94.4	13.4	3.9	13.6
4	Chinese Radish	Koko Head	<20	0.7	City & County	94.6	14.3	3.7	21.6
5	Chinese Radish	Waialua	20	0.8	Well	94.6	14.4	13.4	4.2
6	Chinese Radish	Waialua	35	1.1	City & County	94.9	17.2	8.4	20.2
7	Mino-Kokonoka	Kamuela, Hawaii	2800	10.0	Reservoir	95.0	39.5	0.4	9.7
8	Mino-Kokonoka	Kamuela, Hawaii	2800	10.0	Reservoir	94.4	46.5	0.6	9.2
Eggplant, Long									
1	Unknown	Waianae	100	1.1	Stream	90.7	1.9	2.0	12.1
2	Unknown	Moanalua [2]	20	1.0	Well	92.2	2.2	3.9	15.3
3	Unknown	Kailua	120	1.9	City & County	90.9	2.5	1.4	8.9
4	Unknown	Kaneohe	100	0.7	City & County	91.6	3.1	1.4	5.3
5	Molokai Long	Waialua	<20	0.5	Well	93.0	3.2	8.5	28.3
6	Unknown	Koko Head	<20	1.5	City & County	92.0	3.3	3.7	23.2
7	Long, Giant Honnago	Manoa	90	1.8	City & County	92.6	3.7	3.3	.....
8	Unknown	Moanalua [2]	20	1.0	Well	92.6	3.9	3.9	10.0
9	Molokai Long	Ewa	30	1.5	Well	92.2	4.8	42.5	55.2
10	Unknown	Lualualei	70	2.5	Well	90.5	4.9	1.8	77.5
Eggplant, Round									
1	Black Beauty	Moanalua [2]	20	1.0	Well	92.7	2.1	3.9	69.4
2	Black Beauty	Manoa	90	1.8	City & County	92.1	2.3	3.3	.....
3	Black Beauty	Moanalua [3]	20	1.0	Well	92.9	2.6	3.9	21.6
4	Black Beauty	Ewa	30	1.5	Well	92.0	3.3	42.5	38.0
5	Black Beauty	Waialua	35	1.1	City & County	91.4	4.2	8.4	4.3
6	Black Beauty	Koko Head	<20	1.5	City & County	92.7	4.2	3.7	35.4
Lettuce, Head									
1	Great Lakes	Kipapa	500	6.0	Stream	96.2	2.2	1.3	3.6
2	Great Lakes	Kamuela, Hawaii	2800	10.0	Reservoir	96.8	2.2	0.6	6.4

TABLE 1—Continued

	Food	Source of Sample [1] Location	Elevation	Distance from sea	Water supply	Moisture	Sodium Food	Sodium Water	Sodium Soil
			feet	*miles*		%	*mg/100 g*	*mg/100 g*	*mg/100 g*
3	Great Lakes	Volcano, Hawaii	3800	12.0	Rain	96.0	4.3	0.5 [3]	3.4
4	Great Lakes	Kamuela, Hawaii	2800	10.0	Reservoir	96.2	4.5	0.4	2.7
Lettuce, Semi-head									
1	Manoa	Kailua	120	1.9	Stream, City & County	95.6	4.5	4.3	6.3
2	Manoa	Manoa	200	3.1	City & County	95.7	6.4	3.3	7.2
3	Manoa	Kaneohe	100	0.7	City & County	95.1	6.6	1.4	9.4
4	Manoa	Moanalua [2]	20	1.0	Well	96.5	10.4	3.9	31.0
5	Manoa	Moanalua [2]	20	1.0	Well	95.7	10.8	3.9	12.6
6	Manoa	Koko Head	<20	1.5	City & County	96.3	13.2	3.7	20.1
7	Manoa	Waialua	35	1.1	City & County	95.7	14.8	8.4	10.1
Onions, Dry									
1	Yellow Bermuda	Manoa	90	1.8	City & County	93.0	2.3	3.3	.....
2	Granex Hybrid	Mikilua	50	1.3	Well	92.8	4.8	37.0	34.0
3	Granex Hybrid	Mikilua	50	1.3	Well	94.0	9.6	37.0	49.0
Onions, Green									
1	Green Bunching	Manoa	200	3.1	City & County	91.8	1.2	3.3	6.8
2	Green Bunching	Kailua	120	1.9	City & County	90.8	1.3	1.4	6.3
3	Green Bunching	Waianae	100	1.1	Stream	91.4	1.9	2.0	7.5
4	Green Bunching	Volcano, Hawaii	3800	12.0	Rain	94.6	3.1	0.5 [3]	1.3
5	Green Bunching	Koko Head	<20	1.5	City & County	93.4	3.4	3.7	21.0
6	Green Bunching	Waialua	35	1.1	City & County	90.4	3.7	8.4	9.2
Papaya									
1	Solo	Puna, Hawaii	300	2.5	Rain	86.0	3.6	0.5 [3]	7.6
2	Solo	Kipapa	500	6.0	Stream	85.9	3.9	2.1	7.2
3	Solo	Maunawili	150	3.8	City & County	84.6	4.4	1.4	2.9
4	Solo	Manoa	90	1.8	City & County	88.6	8.3	3.3	.....
5	Solo	Hakipuu	40	0.1	City & County	84.8	9.0	3.4	6.9
6	Solo	Waimanalo	20	0.3	Irrigation ditch	84.6	10.2	2.8	12.6
7	Solo	Waimanalo	80	1.1	Irrigtation ditch	85.4	13.2	2.8	12.7
8	Solo	Waimanalo	20	0.5	Irrigation ditch	85.9	15.0	2.8	14.7
9	Solo	Waianae [2]	35	0.8	Stream	86.4	15.4	5.4	12.6
10	Solo	Moanalua	20	1.0	Well	86.0	17.4	3.9	10.8
11	Solo	Waianae [2]	35	0.8	Stream	86.4	18.6	5.4	11.9
12	Solo	Makaha	<20	0.2	Well	85.0	34.0	80.0	108.1
13	Solo	Kaneohe	<20	0.2	City & County	86.3	45.0	1.4	8.9
14	Solo	Kaunakakai, Molokai [2]	75	0.5	Well	86.7	62.0	40.0	65.2
15	Solo	Kaunakakai, Molokai [2]	75	0.5	Well	85.6	76.0	8.0 [4]	30.5
Pepper, Green									
1	California Wonder	Kipapa	500	6.0	Stream	93.8	1.9	2.1	5.1
2	California Wonder	Waialua	20	0.8	Well	93.7	2.0	13.4	38.9
3	Keystone Resistant Giant	Kipapa	500	6.0	Stream	94.0	2.2	1.3	6.1
4	California Wonder	Makaha	200	1.2	Stream	93.0	2.2	5.5	11.9

TABLE 1—Concluded

Food	Source of Sample [1] Location	Elevation	Distance from sea	Water supply	Moisture	Sodium Food	Sodium Water	Sodium Soil
		feet	*miles*		%	*mg/100 g*	*mg/100 g*	*mg/100 g*
5 Keystone Resistant Giant	Waialua	20	0.8	Well	93.0	3.0	13.4	16.6
6 California Wonder	Kamuela, Hawaii	2800	10.0	Reservoir	93.5	3.6	0.6	8.0
7 Yolo Wonder	Lualualei	80	2.7	Well	93.6	5.6	43.5	106.4
8 Yolo Wonder	Waimea	30	0.1	Well	94.2	8.5	34.0	20.1
Sweetpotato								
1 Unknown	Kailua	120	1.9	Stream, City & County	74.9	15.4	4.3	9.0
2 Onolena	Kipapa	500	6.0	Stream	72.8	18.6	2.1	3.6
3 Kona B	Kipapa	500	6.0	Stream	77.3	28.4	2.1	3.6
4 Unknown	Kaneohe	50	0.6	Stream	64.3	59.0	3.6	4.7
5 Onolena	Waimanalo	100	1.1	Irrigation ditch	74.2	84.0	2.8	7.6
6 Unknown	Kaneohe	50	0.6	Stream	69.6	85.5	3.6	4.7
7 Kona B	Waiahole	<20	0.2	Stream	75.4	108.0	1.5	7.9
Taro Corms								
1 Unknown	Kaneohe	100	1.5	Stream	57.2	16.4	7.1	20.0
2 Unknown	Kaneohe	<20	0.5	Stream	59.1	18.0	1.6	20.8
3 Unknown	Kaneohe	60	1.2	Stream	57.0	26.0	1.7	16.7
Tomatoes								
1 Unknown	Kailua	120	1.9	Stream, City & County	94.8	2.1	4.3	9.5
2 Maui	Makaha	200	1.2	Stream	93.8	2.4	5.5	8.3
3 Unknown	Waimanalo	50	0.8	Irrigation ditch	96.1	5.1	2.8	15.5
4 Anahu, N-5, N-13	Ewa	30	1.5	Well	93.8	5.2	42.5	39.5
5 Anahu, N-5, N-13	Lualualei	80	2.7	Well	94.3	6.4	43.5	80.0
6 Molokai	Lualualei	70	2.5	Well	92.6	6.8	1.8	26.0

[1] Locations are for the island of Oahu unless indicated otherwise. Elevation and distance measured on topographical map.

[2] Same farm, different season for a given crop.

[3] Rain, only source of water. Sodium content estimated to be 0.5 mg/100 g.

[4] Collected from open well after very heavy rain storm.

ples exhibited a slight increase in plant sodium with increase in soil sodium, but the authors were unable to find for comparison, samples which had been grown in high-sodium soils. Cabbages reportedly respond to increases in salt (6, 7). The remaining foods (cucumber, Manoa lettuce, dry onion, green pepper, taro, and tomato) were generally low in sodium and appeared to be non-responsive to a varying sodium environment (see cucumber).

Although many foods were low in sodium, or non-responsive to the sodium environment, the variation between the lowest and highest figures for a given food was a two to tenfold increase. The differences were evident not only between localities but also, to a lesser degree, between seasons on the same farm. (See footnote in table.) The results indicate that variations in sodium should be expected in plant foods, although the differences may not always be nutritionally significant.

Where there was known to be more than one horticultural variety for a given food, the data were examined to ascertain what effect variety might have exerted on the sodium content of plants. There appeared to be little influence.

Waters from streams and the City and

County Board of Water Supply were low in sodium as expected. On the other hand, several wells were producing water containing from 30 to 80 mg/100 g and the soils irrigated by these waters had moderately high concentrations of sodium.

In general, farms near the ocean had higher sodium soils than farms located some distance from the sea.

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LITERATURE CITED

1. Bills, C. E., F. G. McDonald, W. Niedermeier, and M. C. Schwartz. 1949. Sodium and potassium in foods and water. *J. Am. Dietet. Assoc.*, **25**, 304.
2. Board of Water Supply. (Personal Communication).
3. Clifford, P. A., and W. O. Winkler. 1954. Report on the determination of sodium in foods. *J. Assoc. Official Agr. Chem.*, **37**, 586.
4. Dahl, L. K., L. M. Tassinari, and N. Gillespie. 1958. Sodium in foods for a 100-mg. sodium diet. *J. Am. Dietet. Assoc.*, **34**, 717.
5. Davidson, C. S., L. E. Clifcorn, P. A. Clifford, G. J. Gabuzda, and C. Robinson. 1954. Sodium restricted diets. The rationale, complications, and practical aspects of their use. Publication 325. Food & Nutrition Board, National Research Council.
6. Harmer, P. M., and E. J. Benne. 1941. Effects of applying common salt to a muck soil on the yield, composition, and quality of certain vegetable crops and on the composition of the soil producing them. *J. Am. Soc. Agron.*, **33**, 952.
7. Influence of environment on the chemical composition of plants. *Southern Cooperative Series Bull.*, **36**, 198 (1954).
8. Influence of environment on the chemical composition of plants. *Southern Cooperative Series Bull.*, **42**, 123 (1955).
9. Irwin, B. L., and C. Schuck. 1951. Sodium values of a selected group of foods. *J. Am. Dietet. Assoc.*, **27**, 98.
10. Jackson, M. L. 1958. *Soil Chemical Analysis.* Prentice-Hall, Inc., Englewood Cliffs, N. J., 498 pp.
11. Miller, C. D., and R. C. Robbins. 1937. The nutritive value of papaya. *Biochem. J.*, **31**, 1.

Proximate Composition of Nine Species of Rockfish [a]

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SUMMARY

The many species of rockfish found in Pacific Coast waters represent an abundant source of seafood, which is available during all seasons of the year. The edible portion is processed as fillets either fresh or frozen. Composition of these fillets for the more important commercial species have been found to be high in protein and low in both oil and sodium. The values remain quite uniform for the different species regardless of the size of the fish, the season of its capture, or the manner in which it is stored and processed. The nonedible parts provide a valuable source of animal food since they are high in protein, oil, and ash. Thus the entire fish can be utilized in an economical manner.

More than 50 species of rockfish inhabit the waters of the Pacific Coast of the United States. They range in length from 25 to 75 cm, but most of them are less than 60 cm (2). These species of fish represent an increasingly important segment of the Pacific Coast commercial fishing industry. The total catch landed in Oregon, Washington, and California in 1956 was 26 million pounds. Amounts for some of the other species landed in the same area during 1956, also in millions of pounds, were Pacific salmon 54, flounder 52, halibut 22, and Pacific cod 10 (3). The processing of rockfish involves filleting and freezing operations, which have become a major industry in the Pacific Northwest.

The edible flesh of rockfish represents about one fourth of the whole fish. The remainder of the carcass or trimmings is sold to operators of mink and trout farms or is converted to meal for use in poultry and livestock feed. It is important, therefore, to know the composition of the trimmings and of the whole fish, as well as the composition of the edible flesh. In spite of the increasing popularity of rockfish, there are very few data on composition available in the literature for any of the species of the *Sebastodes* group (1, 4).

Chemists at the Seattle laboratory of the Bureau of Commercial Fisheries have obtained composition data for nine species of rockfish. The specimens used represent wide variety in regard to size, season of capture, area of capture, and method of preservation. The purpose of this paper is to compare variations in composition of fillets for the more important species of rockfish with regard to species, size of fish, season of capture, area of capture, and method of preservation, as well as to evaluate the composition data for the nonedible parts and the calculated values for the whole fish.

EXPERIMENTAL

Collection of Specimens

The fish for which data are reported in this paper represent nine of the more common species of *Sebastodes* found in the coastal waters of Washington, Oregon, and northern California (Table 1). They were collected during the years 1950, 1952, and 1957 to 1959. Specimens were obtained during all seasons of the year and were either frozen at once or held in ice in the round on board the fishing vessel. Three series of samples were purchased as commercially packed frozen fillets from local fish houses.

Preparation of Samples

The frozen fish were thawed in lukewarm water, and the iced fish were washed in water. The length and weight of each fish were recorded. Fillets were then removed from each fish, weighed, ground, hermetically sealed in ½-pound cans, and frozen as previously described (5). The commercially frozen fillets were partially thawed at room tem-

[a] Contribution No 576 from the technological laboratories of the Bureau of Commercial Fisheries, Fish and Wildlife Service, U. S. Department of the Interior.

TABLE 1

SOURCE DATA FOR ROCKFISH (SEBASTODES SPECIES)

Series No.	Common name	Scientific name *	Place of capture	Date		Preservation
				Month	Year	
1	Orange rockfish	*S. pinniger*	Puget Sound	Sept.	50	Frozen fillets
2	Orange rockfish	*S. pinniger*	Wash. coast	Jan.	57	Iced round
3	Orange rockfish	*S. pinniger*	Wash. coast	Jan.	57	Iced round
4	Orange rockfish	*S. pinniger*	Wash. coast	June	59	Frozen fillets
5	Orange rockfish	*S. pinniger*	Oregon coast	April	59	Frozen round
6	Orange rockfish	*S. pinniger*	Wash. coast	June	59	Iced round
7	Orange rockfish	*S. pinniger*	Wash. coast	Nov.	59	Iced round
8	Yellowtail rockfish	*S. flavidus*	Wash. coast	Jan.	57	Iced round
9	Yellowtail rockfish	*S. flavidus*	Oregon coast	May	57	Frozen round
10	Yellowtail rockfish	*S. flavidus*	Oregon coast	April	59	Frozen round
11	Yellowtail rockfish	*S. flavidus*	Wash. coast	June	59	Iced round
12	Yellowtail rockfish	*S. flavidus*	Wash. coast	Nov.	59	Iced round
13	Bocaccio rockfish	*S. paucispinis*	Oregon coast	April	59	Frozen round
14	Chili-pepper rockfish	*S. goodei*	Calif. coast	Aug.	50	Frozen fillets
15	Dark blotched rockfish	*S. crommeri*	Wash. coast	Sept.	52	Frozen round
16	Flag rockfish	*S. rubrivinctus*	Oregon coast	Mar.	52	No data
17	Greenstripe rockfish	*S. elongatus*	Wash. coast	Sept.	52	Frozen round
18	Greenstripe rockfish	*S. elongatus*	Oregon coast	April	59	Frozen round
19	Idiot rockfish	*S. alaskanus*	Wash. coast	Oct.	58	Frozen round
20	Rosy rockfish	*S. rosaecus*	Wash. coast	Sept.	52	Frozen round

* S. refers to *Sebastodes* except No. 19 which is *Sebastolobus*.

perature before being ground and canned. To prepare the sample for analysis, the technician thawed the contents of the can by immersing it in lukewarm water. After removal from the can the sample was reduced further in particle size by means of a high-speed cutting blade.

METHODS OF ANALYSIS

The ground samples were analyzed for moisture, oil, protein, ash, sodium, and potassium by standard methods previously described (6).

RESULTS AND DISCUSSION

The data can be treated conveniently in four sections as follows: (1) variations in physical data; (2) variations in the composition of fillets according to: (a) species, (b) size of fish, (c) season of capture, (d) area of capture, and (e) method of preservation; (3) variations in the composition of nonedible parts; and (4) variations in the composition of the whole fish.

TABLE 2

PHYSICAL DATA FOR ROCKFISH SAMPLES

Series No.	Species	No. of fish	Length		Weight		Fillet yield	
			Av	Range	Av	Range	Av	Range
			cm	*cm*	*kg*	*kg*	%	%
1 to 7	Orange rockfish	73	48	40–56	1.60	0.85–3.14	27.6	17.9–40.5
8 to 12	Yellowtail rockfish	49	48	43–62	1.41	0.42–2.82	27.5	17.0–34.9
13	Bocaccio rockfish	15	56	51–58	2.12	1.44–2.56	25.3	20.9–30.8
14	Chili-pepper rockfish	12				Fillets only		
15	Dark blotched rockfish	12	31	27–33	0.59	0.42–0.75		
16	Flag rockfish	6	48	41–56	2.39	1.40–3.69		
17 & 18	Greenstripe rockfish	30	31	27–35	0.44	0.24–0.79	23.7	19.8–29.0
19	Idiot rockfish	13	38	27–47	0.91	0.35–1.55	20.3	16.9–24.4
20	Rosy rockfish	13	24	22–26	0.21	0.15–0.28		
	Average [1]		42	22–62	1.30	0.15–3.69	26.3	16.9–40.5

[1] Averages are for all series of all species.

TABLE 3

COMPOSITION DATA FOR ROCKFISH FILLETS

Series No.	Species	No. of fish	Moisture		Oil		Protein		Ash		Sodium		Potassium	
			Av	Range	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range
			%	%	%	%	%	%	%	%	*Mg %*	*Mg %*	*Mg %*	*Mg %*
1–7	Orange rockfish	73	79.5	75.2–81.8	1.59	0.56–4.10	18.8	17.2–20.6	1.19	1.05–1.27	65	41–92	384	319–459
8–12	Yellowtail rockfish	49	79.5	77.5–81.0	1.53	0.62–3.45	18.9	17.0–20.1	1.20	1.06–1.28	59	40–72	406	342–460
13	Bocaccio rockfish	15	80.0	78.6–81.2	1.04	0.72–1.62	18.6	17.6–19.3	1.20	1.15–1.25	60	53–68	411	371–440
14	Chili-pepper rockfish	12	76.6	75.8–78.7	2.35	1.55–2.90	20.8	19.6–21.6	1.10	1.04–1.20	55	50–58	305	291–320
15	Dark blotched rockfish	12	77.6		2.18		19.7		1.23					
16	Flag rockfish	6	78.8		1.78		18.2		1.10					
17, 18	Greenstripe rockfish	30	79.1	78.3–79.5	0.65	0.56–0.92	20.0	19.6–20.8	1.25	1.21–1.35	68	61–77	414	395–438
19	Idiot rockfish	13	80.7	79.3–81.8	1.66	0.57–3.38	17.3	16.3–18.3	1.07	1.01–1.17	77	65–97	344	336–361
20	Rosy rockfish	13	79.0		0.84		19.3		1.09					
	Average[1]		79.0	75.2–81.8	1.50	0.56–4.10	19.0	16.3–21.6	1.18	1.01–1.35	63	40–97	387	291–460

[1] Averages are for all series of all species.

VARIATIONS IN PHYSICAL DATA

The specimens of rockfish analyzed included a wide variety of sizes, with an average length of 42 cm and a range of 22 to 62 cm (Table 2). Arranged in decreasing order of length, the major species of rockfish were bocaccio, orange, and yellowtail. The same order was found in regard to weight. The average weight for all species was 1.30 kg, and the range was from 0.15 to 3.69 kg. The fillet yield averaged 26.3% and ranged from 16.9 to 40.5% for the skinless, boneless fillets.

VARIATIONS IN COMPOSITION OF FILLETS

Throughout the 20 series of rockfish samples representing 9 different species, the percentages of moisture, oil, protein, and ash were found to be fairly uniform (Table 3). The moisture content averaged 79.0% with variations of ±3%. The oil content averaged 1.50% and usually varied by not more than ±1%. Protein values averaged 19.0% and variations were usually within ±3%. Ash values showed only minor variations from the average of 1.18%. Larger variations were found in the sodium and potassium content, but these values did not deviate by more than 40% from the average of 63 mg per 100 g for sodium or by more than 25% from the average of 387 mg per 100 g for potassium. It is interesting that no specimen in the more than 200 fish analyzed had a sodium content of more than 97 mg per 100 g and that some were as low as 40 mg per 100 g.

When the composition data were arranged according to size of fish (Table 4a), they showed smaller deviations than were found among the specimens of individual species. Even the oil content, which usually varies with size, was practically constant. The composition also did not seem to be affected appreciably by either season (Table 4b) or area of capture (Table 4c). This is an interesting observation, considering that the specimens contained representatives from all 4 seasons and from 5 different years and were collected in an area extending from Puget Sound, Washington, to Eureka, California. It is recognized that to fully evaluate

TABLE 4

COMPOSITION DATA FOR ROCKFISH FLESH

Factor of comparison	Number of fish	Moisture	Oil	Protein	Ash	Sodium	Potassium
		%	%	%	%	*Mg %*	*Mg %*
(a) Size of fish							
Small	75	79.0	1.35	19.2	1.19	70	403
Medium	75	79.8	1.34	18.6	1.18	64	385
Large	34	79.4	1.61	18.6	1.14	62	401
(b) Season of capture							
Winter	18	79.3	1.53	18.9	1.27	68	397
Spring	58	79.5	1.24	18.8	1.22	63	409
Summer	24	80.0	1.15	18.7	1.12	67	370
Fall	83	78.8	1.59	19.1	1.15	61	396
(c) Area of capture							
Oregon coast	71	79.4	1.19	19.0	1.19	63	409
Washington coast	110	79.2	1.56	18.9	1.18	65	394
(d) Method of Preservation							
Frozen round	106	79.4	1.25	19.0	1.19	64	393
Iced round	40	79.4	1.56	19.2	1.21	63	401
Frozen fillets	69	78.2	2.07	19.6	1.11	60	332

the influence of these physical, geographic, and seasonal factors, it would be necessary to include a much broader sampling of each species for size, area, and season. The data do indicate, however, a general trend toward fairly constant composition.

Samples prepared from fish that had been (a) frozen in the round, (b) iced in the round, and (c) commercially prepared as frozen fillets also showed little variation in composition (Table 4d). Even sodium and potassium, which are usually much lower in fish that have been iced than in fish that have been frozen, were essentially the same for the two groups. This might be explained on the basis that the *Sebastodes* group has a

TABLE 5

COMPOSITION DATA FOR NONEDIBLE PARTS AND WHOLE ROCKFISH

Factor of comparison	Number of fish	Moisture	Oil	Protein	Ash	Sodium	Potassium
		%	%	%	%	*Mg %*	*Mg %*
(a) Averages for nonedible parts							
Orange rockfish	26	69.9	7.07	16.5	6.06	136	255
Yellowtail rockfish	43	71.0	6.77	16.5	6.35	166	243
Bocaccio rockfish	15	73.0	6.45	16.3	4.57	139	287
Greenstripe rockfish	14	69.3	5.44	18.4	8.93	181	238
Idiot rockfish	13	75.8	9.66	12.8	2.89	160	201
Average [1]		71.3	7.09	16.2	5.99	157	245
(b) Averages calculated for whole fish							
Orange rockfish	26	72.2	6.19	17.0	4.90	118	292
Yellowtail rockfish	43	73.4	5.29	17.1	4.92	137	284
Bocaccio rockfish	15	74.7	5.08	16.9	3.71	119	318
Greenstripe rockfish	14	71.6	4.30	18.7	7.11	154	380
Idiot rockfish	13	76.7	8.04	13.7	2.52	144	230
Average [1]		73.4	5.66	16.8	4.76	133	294

[1] Averages are for all series of all species.

very tough skin and that the period of time rockfish are stored in ice on board ship is probably considerably shorter than it is with other species, since rockfish usually are captured fairly close to shore. The possibility for leaching of sodium and potassium ions would therefore be greatly reduced. The degree of close agreement in composition between the commercially frozen fillets and the freshly prepared fillets indicates that processing methods do not alter the composition of the fish to an appreciable extent.

Variations in Composition of the Nonedible Parts

Composite samples were prepared from the nonedible parts of all of the fish in nine of the series studied (Table 5a). The average values for the different constituents were moisture 71.3%, oil 7.1%, protein 16.2%, ash 6.0%, sodium 157 mg per 100 g, and potassium 245 mg per 100 g. As in the previous tables, the variations were not large for moisture, oil, protein, and ash and varied a maximum of 25% for sodium and potassium. The nonedible parts of the *Sebastodes* group are sufficiently high in oil, protein, and ash to provide a good source of animal and fish food either in the freshly ground state or as a processed meal.

Variations in the Composition of the Whole Fish

Rockfish are seldom used whole for animal feeding, since the flesh is valuable for human food. The calculated values for the whole fish, however, are interesting by way of comparison with those found for the nonedible parts (Table 5b). The differences are not great; thus the whole fish could be put to the same use if available.

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LITERATURE CITED

1. Iverson, J. L. 1958. Pacific ocean perch—Proximate composition. *Com'l Fish. Rev.*, 20, 22–24.
2. Philips, Julius B. 1957. A review of the rockfishes of California. State Department of Fish and Game, *Fishery Bull.* 104, 20, 21.
3. Power, E. A. 1956. Fishery statistics of the United States. *Statistical Digest*, 43, 16.
4. Sinnhuber, R. O., T. C. Yu, and Neva L. Karrick. 1956. Variation in proximate composition of right and left fillets of rockfish and dover sole. *Com'l. Fish. Rev.*, 18, 24–26.
5. Thurston, Claude E., Maurice E. Stansby, Neva L. Karrick, David T. Miyauchi, and William C. Clegg. 1959. Composition of certain species of fresh-water fish. II. Comparative data for 21 species of lake and river fish. *Food Research*, 24, 493–502.

Determination of the Relative Concentrations of the Major Aldehydes in Lemon, Orange and Grapefruit Oils by Gas Chromatography [a,b]

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SUMMARY

The Girard-T reagent was used to recover the aldehydes from cold-pressed lemon, orange and grapefruit oils for subsequent quantitative gas chromatographic analysis. Optimum conditions for analysis were worked out with a known mixture of citral and the saturated aliphatic aldehydes which occur in major concentration in lemon oil. Isopropanol was used in the preparative step to eliminate acetal formation and isopentane in the final extractions to reduce losses of the lower-molecular-weight aldehydes during vacuum concentration of the sample for gas chromatographic analysis. Typical analyses are given for samples of domestic cold-pressed citrus oils.

Recent work of Weissenberg and Ginsburg (6), and Teitelbaum (5) demonstrated that Girard reagents function satisfactorily for the isolation and regeneration of higher-molecular-weight aldehydes. This is in contrast to observations made in original studies with these reagents by Girard and Sandulesco (2) who recommended them only for use with ketones.

Analysis of the individual carbonyls in lemon and other citrus oils has been hampered by the lack of adequate methods for separating them from other components in the mixture. Conversion to the 2,4-dinitrophenylhydrazones and subsequent column chromatographic separation of the derivatives is time-consuming and may damage the more sensitive components. For example, acids used in preparing the derivatives are known to cause structural rearrangements of the terpenes. Direct column chromatography of the oil mixtures as a primary separation step followed by analysis of fractions with gas chromatography is also time-consuming and does not necessarily guarantee that other non-carbonyl components are absent from the recovered mixtures. Consequently, the Girard reagent procedure, particularly in conjunction with the innovations introduced by Teitelbaum (5), appeared particularly attractive for gas chromatographic analysis of the carbonyls in citrus oils.

To apply the method of Teitelbaum to the recovery of aldehydes from citrus oils for subsequent gas chromatographic analysis it was necessary to investigate several variables. The effects of concentration of Girard reagent in the preparative step, the concentration of formaldehyde, and time and temperature conditions during the regeneration step on the reproducibility of the method for a known mixture of aldehydes were examined. It was also necessary to select the best solvents for the preparative step and the final recovery step (after regeneration of the aldehydes). This analytical procedure was only intended for determining the relative concentrations of the normal saturated aldehydes and citral. Complete recovery of the carbonyls, therefore, was not necessary as long as it could be demonstrated with known mixtures of aldehydes that, within reasonable limits, the relative amounts of the individual aldehydes were unchanged by the recovery procedure.

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Basically the method involves five steps: conversion of aldehydes to water-soluble Girard derivatives, extraction of aqueous reaction mixture with an organic solvent to remove non-carbonyl compounds, regeneration of aldehydes with aqueous formaldehyde, recovery of the regenerated carbonyl compounds by extraction and flash evaporation to remove solvent, and gas chromatographic analysis.

EXPERIMENTAL

General Procedure

Two grams of Girard-T reagent, 200 ml of isopropanol, 0.5 g of IRC-50 ion exchange resin (acid form) and 0.5 g of the known aldehyde mixture (10–50 ml of citrus oil depending on the amount of aldehydes present) were heated under reflux for 1 hr. The reaction mixture was cooled slightly and filtered through coarse filter paper into a separatory funnel. The reaction flask was washed with 2–10-ml portions of isopropanol followed by 250 ml of distilled water and the washings filtered into a separatory funnel. To the material in the separatory funnel was added 75 ml redistilled hexane. The mixture was shaken vigorously and allowed to stand for 2 hr after which the aqueous phase was drawn off and extracted twice with 50-ml portions of hexane allowing 1 hr between extractions for the separation of phases. The aqueous phase was transferred to a 1-liter stoppered bottle, 60 ml of 36% aqueous formaldehyde solution was added and air in the bottle displaced with nitrogen. The contents were thoroughly mixed and the bottle placed in a constant-temperature bath at 37° C for 24 hr. The bottle was then cooled in ice water for 10–15 min, and 40 g sodium chloride and 50 ml isopentane were added. The mixture was swirled until the sodium chloride dissolved and transferred to a separatory funnel. The aqueous phase was then separated and extracted with a second 50-ml portion of isopentane. The isopentane-isopropanol phases were combined and washed with 200 ml of chilled distilled water (to remove the isopropanol) followed by 100 ml of chilled 20% aqueous sodium chloride solution. The isopentane phase was dried over anhydrous sodium sulfate. The isopentane was removed in a rotary vacuum evaporator (bath temperature at 30°, water aspirator vacuum) to a small volume (about 0.5 ml). The residue was analyzed by gas chromatography (column, 10 ft x ¼ in butanediol-succinate coated firebrick, temperature, 205° C, helium flow rate, 25 ml/min; hot wire thermal conductivity detector).

These conditions were selected after considering the results of the following series of experiments.

A mixture of commercial *n*-octanal, *n*-nonanal, *n*-decanal, *n*-undecanal and citral (aldehyde mixture No. 1) was used in developing the optimum conditions for the analytical procedure. These aldehydes are in major concentration in lemon oil (3). Purity of the individual aldehydes was determined by the method of Fritz *et al* (1). Non-carbonyl impurities were assumed to be the corresponding carboxylic acids which would not appear in the gas chromatographic analyses. The aldehydes were analyzed by gas chromatography and found to contain other members of the same homologous series. These aldehydes and the amounts present based on peak areas are shown in Table 1. The per cent composition as total carbonyl, weight of material used in making up the mixture and calculated actual weight of carbonyl used also appear in Table 1. The calculated composition of the mixture appears in column 9 of Table 1. In a final recovery determination, *n*-heptanal (found in lemon oil after the conditions had been worked out) was included in a second mixture (aldehyde mixture No 2). The calculated composition of this mixture, determined in the same manner as for aldehyde mixture No. 1, appears in Table 1, column 13.

Of the four stationary phases, butanediol-succinate, Ucon polar, Apiezon and silicone, which were tested for gas chromatographic analysis with the known aldehyde mixtures, butanediol-succinate was the most satisfactory. The percentage composition of these mixtures as well as all subsequent citrus oil analyses was determined with the butanediol-succinate column using peak areas determined by calculating the areas of triangles circumscribed over the peaks. A relatively fast chart speed (2 inches per min with a Brown strip chart recorder)[d] was used to spread out the peaks to minimize errors in calculating the areas. Because the two forms of citral (neral and geranial) were not completely separated, the citral double peak was cut out and its area determined by weight. In order to account for any variations in the thickness of the paper, a portion of the chart immediately below the citral peak was used to determine the weight-area conversion factor for each run. The gas chromatographic analyses of aldehyde mixture No. 1 appear in Table 1, column 10, as the average of four runs. Also included are the maximum differences between the calculated and found values.

During the early stages of this study, ethanol was used instead of isopropanol as the solvent in the initial preparative step. In the resulting recorder tracings obtained by gas chromatography

[d] The mention of special instruments or materials does not imply endorsement by the Department of Agriculture over others of similar nature.

TABLE 1

ANALYSIS OF ALDEHYDE MIXTURES BY GAS CHROMATOGRAPHY

Aldehyde	% Composition of commercial aldehydes [1]					Composition of aldehyde mixture No. 1						Composition of aldehyde mixture No. 2		
						Wt. comn. aldehyde g	Calculated [3]		Found, % [4]		Found after regeneration % [6]	Calculated %	Found % [4]	Found after regeneration % [6]
	C_8	C_9	C_{10}	C_{11}	Carbonyl [2]		Wt, g	%	Av 4 runs	Max. differences [5]				
n-Heptanal [7]	.....	.....	.....	.....	81.5	.....	.....	.....	.....	.....	.....	5.2	6.0	5.8
n-Octanal	95.5	1.0	3.5	.....	88.9	1.66	1.47	6.0	6.4	0.3	7.7	5.7	6.1	7.1
n-Nonanal	2.4	92.9	2.3	2.3	80.4	3.45	2.61	10.7	11.6	0.3	12.5	10.1	10.6	11.5
n-Decanal	.....	1.0	99.0	.....	72.1	1.69	1.44	5.9	6.6	0.2	6.6	5.6	6.1	6.4
n-Undecanal	.....	0.8	8.2	91.3	88.2	1.66	1.40	5.8	6.3	0.1	6.5	5.5	5.7	6.6
Citral	.....	.....	.....	.....	98.4	17.80	17.51	71.6	69.1	0.5	66.6	67.9	65.5	62.6
Column No 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

[1] Results of gas chromatographic analysis of materials used as components of aldehyde mixtures.
[2] Analysis by method of Fritz *et al* (1) using molecular weight based on gas chromatographic analysis.
[3] Weight of each aldehyde is sum of weights found from compositional data of columns 2, 3, 4, 5 and 6 in conjunction with weights of column 7.
[4] Results by direct gas chromatographic analysis of aldehyde mixtures.
[5] Maximum absolute difference between average value and individual values.
[6] Regeneration from Girard-T derivative according to optimum conditions described in text.
[7] *n*-Heptaldehyde contained no measurable amounts of *n*-hexanal or the higher aldehydes considered in this study.

of the regenerated aldehyde mixtures, peaks appeared which were proportional in height to and following each of the normal saturated aliphatic aldehydes. These peaks were not present in the tracings obtained for the original aldehyde mixtures. The material comprising these small peaks was found to be non-carbonyl. When the material was treated with dilute hydrochloric acid, aldehydes were formed having the same retention times as the original individual aldehydes. In addition, IR spectra indicated the presence of ether linkages. It was, therefore, assumed that the compounds were ethyl acetals of the corresponding normal saturated aliphatic aldehydes. These small peaks were eliminated by substituting isopropanol for ethanol in the initial preparative step.

A series of tests was conducted to determine the quantity of Girard-T reagent necessary to reproduce the aldehyde mixture No 1. Experiments were carried out with the following amounts of Girard-T reagent: 0.5 g, 1.0 g, 2.0 g, 3.0 g, and 4.0 g. All other conditions were held constant. Three grams of reagent exceeded the solubility in the solvent system at reflux temperature. The gas chromatographic analyses are summarized in Table 2. In subsequent experiments 4.0 g of Girard-T reagent was used to assure the presence of an excess of reagent. For general use 2.0 g should be adequate.

The effect of formaldehyde concentration was studied using identical conditions for a series of analyses in which the amount of formalin in the regeneration step was varied from 20 to 100 ml. Results of these analyses appear in Table 3. In all subsequent analyses, and in the recommended general procedure, 60 ml of 36% aqueous formaldehyde solution was used since larger quantities gave only small increases in citral.

Time and temperature were found to be important in obtaining complete regeneration of the aldehydes with formaldehyde. This was demonstrated in a series of controlled experiments in which all conditions were held constant and the time and temperature during the formaldehyde regeneration step were varied. The results appear in Table 4. In order to reduce the time required for an analysis, the conditions chosen for the standardized general procedure were regeneration with 60 ml of aqueous formaldehyde solution at 37° C for 24 hr, although similar results may be obtained with regeneration at room temperature for 4 days.

Isopentane was selected as the extraction solvent for the final recovery step so as to minimize losses of lower-boiling aldehydes during evaporation of the solvent. To test for changes in composition of the aldehyde mixture No 1 during concentration

TABLE 2

EFFECT OF AMOUNT OF GIRARD-T REAGENT ON THE COMPOSITION OF THE REGENERATED ALDEHYDE MIXTURE NO 1 [1,2]

Girard-T reagent, g	Aldehydes, %				
	C_8	C_9	C_{10}	C_{11}	Citral
0.5	3.2	4.9	2.4	2.6	86.9
1.0	8.0	12.4	6.2	6.4	66.9
2.0	7.8	12.4	6.8	6.6	66.3
3.0	7.6	11.9	6.5	6.2	67.7
4.0	7.7	12.5	6.6	6.6	66.6

[1] Regeneration with 60 ml 36% aqueous formaldehyde at 37° C for 24 hr.

[2] Compare with analyses in Table 1, column 10.

TABLE 3

EFFECT OF AMOUNT OF AQUEOUS FORMALDEHYDE (36% SOLUTION) ON COMPOSITION OF REGENERATED ALDEHYDE MIXTURE NO 1 [1,2]

Formalin solution, ml	Aldehydes, %				
	C_8	C_9	C_{10}	C_{11}	Citral
20	8.5	15.8	8.2	7.9	59.6
40	7.6	13.2	7.2	7.2	64.8
60	7.7	12.5	6.6	6.6	66.6
80	7.3	12.4	6.5	6.4	67.4
100	7.3	12.0	6.2	6.0	68.5

[1] Regeneration at 37° C for 24 hr.

[2] Compare with analyses in Table 1, column 10.

TABLE 4

EFFECT OF TEMPERATURE AND TIME DURING FORMALDEHYDE REGENERATION ON COMPOSITION OF ALDEHYDE MIXTURE NO 1 [1]

Temperature & Time	Aldehydes, %				
	C_8	C_9	C_{10}	C_{11}	Citral
Room Temp, 2 hr	14.5	29.6	15.0	16.9	24.0
Room Temp, 6 hr	10.0	19.9	11.4	12.6	46.0
Room Temp, 12 hr	9.4	17.3	9.8	10.1	53.4
Room Temp, 1 day	8.3	13.3	9.0	8.4	58.9
Room Temp, 2 days	7.8	13.7	7.3	6.9	64.3
Room Temp, 4 days	7.6	12.1	6.6	6.5	67.3
Room Temp, 7 days	7.0	11.6	6.8	6.9	67.7
37° C, 1 day	7.7	12.5	6.6	6.6	66.6
37° C, 2 days	7.3	12.1	6.6	6.7	67.3

[1] Compare with analyses in Table 1, column 10.

from isopentane, 0.5 g of the mixture was added to 100 ml of isopentane and the isopentane removed in a rotating vacuum evaporator. The residue (*ca.* 0.5 ml) was analyzed by gas chromatography. The average of 3 runs, and the gas chromatographic analysis of the aldehyde mixture No 1 are given in Table 5 for comparison. The evaporation of isopentane did not change the composition of the mixture, within reasonable experimental error.

To determine the effect of water-washing on the composition of the regenerated aldehyde mixture a 100-ml portion of isopentane containing 0.5 g of aldehyde mixture No 1 was shaken 3× with fresh 100-ml portions of chilled distilled water. The isopentane phase was dried over anhydrous sodium sulfate, the isopentane removed in the rotating vacuum evaporator and the composition of the residue determined by gas chromatography. Similar extraction tests were made with a 50% saturated salt solution and a saturated salt solution. The results appear in Table 5. Extraction with salt solutions and with water had little, if any, effect on the composition of the aldehyde mixture.

TABLE 5

EFFECT OF VACUUM CONCENTRATION ON REMOVAL OF ISOPENTANE AND EFFECT OF WASHING WITH WATER AND SALT SOLUTIONS ON COMPOSITION OF ALDEHYDE MIXTURE NO 1 [1]

	Aldehydes, %				
	C_8	C_9	C_{10}	C_{11}	Citral
Vacuum removal of isopentane	6.6	12.0	6.6	6.2	68.5
Washing 3 x with water [2]	6.3	11.8	6.6	6.2	69.0
Washing 3 x with 50% sat'd salt sol'n	6.3	11.6	6.5	6.1	69.4
Washing 3 x with sat'd salt sol'n	6.4	11.2	6.9	6.2	69.8

[1] Compare with analyses in Table 1, column 10.

[2] Average of 3 analyses.

The aldehyde mixtures No 1 and No 2 were then carried through the recovery procedure with Girard-T reagent, regeneration and gas chromatographic analysis using the selected optimum conditions described above in the general procedure. The resulting analyses appear in Table 1, columns 12 and 15.

The system devised for the aldehydes, however, was tested with a known mixture of ketones and found to be unsatisfactory. A mixture containing methyl heptenone, fenchone, carvone and *n*-octanal (carbonyl mixture No 3) was analyzed by gas chromatography and then carried through the Girard procedure. Regeneration with aqueous formaldehyde was carried out for 24 and 48 hr at 37° C. The analyses are summarized in Table 6. Assuming complete regeneration of *n*-octanal it would appear that only about 50% of the methyl heptenone, 10% of the fenchone and 10% of the carvone were regenerated in 24 hr. There was little improvement when regeneration was extended to 48 hr.

TABLE 6

GIRARD REAGENT RECOVERY AND GAS CHROMATOGRAPHIC ANALYSES OF A MIXTURE CONTAINING *n*-OCTANAL AND KETONES (CARBONYL MIXTURE NO 3)

	Carbonyls, %			
	n-Octanal	Methyl heptenone	Fenchone	Carvone
Original mixture	23.4	24.6	24.9	27.1
Analysis, 24 hr regeneration	54.9	31.3	6.0	7.7
Analysis, 48 hr regeneration	52.0	35.4	5.3	7.3

ANALYSIS OF CITRUS OILS

Representative samples of cold-pressed citrus oils were then analyzed for their major aldehydes using the proposed general procedure. Fifty ml of grapefruit and orange oils, and 25 ml of lemon oil were used. The results of these analyses appear in Table 7. Also included for lemon oil are total carbonyl by the method of Fritz *et al* (1), and the specific citral content by the vanillin-piperidine method (4).

In lemon oil the citral content determined by gas chromatography is consistently about 10% higher than that determined by the vanillin-piperidine method. This is probably because only the major aldehyde components have been considered in the analytical procedure.

One disadvantage of the butanediol-succinate stationary phase is that citronellal is not separated from *n*-decanal, and *n*-dodecanal is covered by the double peak of citral. To analyze for citronellal and *n*-dodecanal another stationary phase must be used. The Ucon-polar stationary phase gives a good separation but is unsatisfactory for quantitative studies because of changes in the base line. The amount of *n*-dodecanal in lemon oil, by rough estimate from the Ucon-polar tracings, was insignificant (judged to be less than 0.1% of the carbonyls). Citronellal, on the other hand, though low in concentration appeared to be present in about one tenth the amount of *n*-decanal. Therefore, the *n*-decanal values for lemon oil determined with the butanediol-succinate stationary phase may be 10% too high because

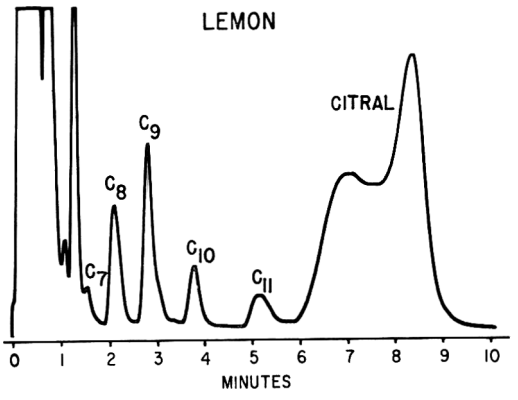

FIG. 1. Gas chromatographic analysis of major aldehydes of lemon oil isolated by Girard method. Column conditions: 10 ft x ¼ in butanediol-succinate stationary phase on crushed fire brick, temperature 203° C, helium flow rate 25 ml/min.

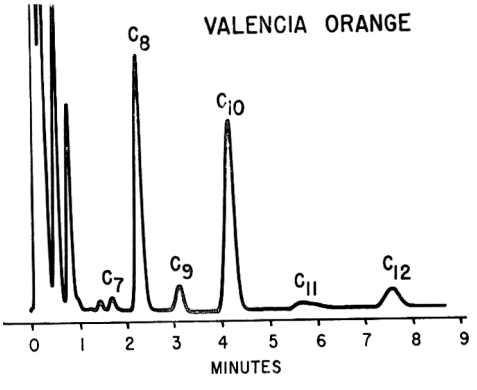

FIG. 2. Gas chromatographic analysis of major aldehydes of Valencia orange oil isolated by Girard method. Column conditions: 10 ft x ¼ in butanediol-succinate stationary phase on crushed fire brick, temperature 200° C, helium flow rate 20 ml/min.

of contamination with the citronellal which accompanies it.

Tracings of gas chromatograms obtained for the aldehyde analyses of lemon, orange, and grapefruit oils appear in Figures 1, 2 and 3. The tracings were made with the Varian Model G-10 recorder [d] for convenience in preparing the illustrations. These tracings were not used for quantitative determinations.

Only the normal saturated aldehydes were considered in reporting the analyses of grapefruit and orange oils. However, there are other carbonyls present which should be

TABLE 7

QUANTITATIVE ANALYSES OF THE MAJOR ALDEHYDES IN COLD-PRESSED ORANGE, GRAPEFRUIT AND LEMON OILS

Citrus Oil	Aldehyde, %							
	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	Carvone[1]	Citral
Valencia orange (Calif.)	2.6	38.6	5.4	41.8	1.8	7.9	2.0	...
Navel orange (Calif.)	3.8	26.9	5.5	40.2	2.9	12.1	8.6	...
Temple orange (Fla.)	1.5	48.6	2.2	34.1	9.3	4.2	...	...
Grapefruit, white (Ariz.)	3.4	34.7	6.4	43.4	2.5	7.7	...	...
Grapefruit, pink (Ariz.)	3.9	16.4	8.8	53.9	6.0	10.9	...	
Lemon[2] (Calif.)	1.1	3.7	5.6	2.8	2.1			84.6

[1] Retention time identical with that of sample of carvone.

[2] Total carbonyl 2.6% by method of Fritz *et al* (1); Citral 1.9% by vanillin-piperidine method (4).

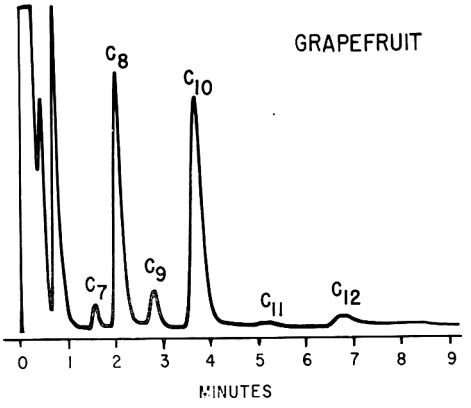

FIG. 3. Gas chromatographic analysis of major aldehydes of grapefruit oil isolated by Girard method. Column conditions same as for Figure 2.

taken into consideration in a more detailed study as, for example, carvone (Table 7), which apparently is not completely regenerated under the conditions established for lemon oil.

As mentioned earlier, complete recovery of constituents was not required in obtaining relative concentrations of the aldehydes by this procedure. Nevertheless, it was of interest to determine whether recoveries were comparable with those reported by Teitelbaum. A total carbonyl analysis (method of Fritz *et al* (1)) was made on a sample of lemon oil and on the carbonyl material recovered after regeneration from Girard-T derivative (total residue after removal of isopentane). It was found that approximately 72% of the starting carbonyl was recovered. This is in good agreement with recoveries obtained by Teitelbaum (5) in large-scale preparative experiments with citral.

The completeness of removal of carbonyls through the formation of the Girard derivative was tested in the following manner. A sample of lemon oil was analyzed for total carbonyl content by the method of Fritz *et al* (1) and found to contain 3.4% (calculated as citral, m.w. 152.23). A 25-ml sample of the oil was then treated with Girard-T reagent as described in the general procedure, the hexane extract separated (first extraction step), dried over anhydrous sodium sulfate, and concentrated in the rotary vacuum evaporator. The residue from the evaporator was then analyzed for total carbonyl content and found to contain 0.1% carbonyl, on the original oil basis. Thus, about 97% of the carbonyls originally in the lemon oil was removed by treatment with Girard reagent.

LITERATURE CITED

1. Fritz, J. S., S. S. Yamamura, and E. C. Bradford. 1959. Determination of carbonyl compounds. *Anal. Chem.*, 31, 260.
2. Girard, A., and G. Sandulesco. 1936. Sur une nouvelle série de reactifs du groupe carbonyle leur utilisation à l'extraction des substances cetoniques et à la caractérisation microchimique des aldéhydes et cétones. *Helv. Chim. Acta*, 19, 1095.
3. Ikeda, R. M., L. A. Rolle, S. H. Vannier, and W. L. Stanley. Aldehydes in lemon oil. Manuscript in preparation.
4. Stanley, W. L., R. C. Lindwall, and S. H. Vannier. 1958. Specific quantitative colorimetric method of analysis for citral in lemon oil. *J. Agr. Food Chem.*, 6, 858.
5. Teitelbaum, C. L. 1958. Isolation of carbonyl compounds under neutral conditions using the Girard reagent. *J. Org. Chem.*, 23, 646.
6. Weissenberg, A., and D. Ginsburg. 1956. Separation of aldehydes and ketones with Girard-P reagent. *Bull. Research Council*, Israel, 5A, 268.

Carotenoid Pigments of Pineapple Fruit. I. Acid-Catalyzed Isomerization of the Pigments [a]

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SUMMARY

The total carotenoid pigments of pineapple fruit contain a high proportion of epoxide groups which are readily isomerized to furanoid forms in an acid but not in an alkaline environment. This isomerization causes a characteristic hypsochromic shift in the absorption maxima of the pigment extract. The absorbance at 425 mμ remains relatively unchanged as isomerization proceeds, and thus can serve as a measure of the total carotenoid pigment regardless of its isomeric form. The sharp maximum at 466 mμ is lost as isomerization progresses. Thus, the ratio of absorbances at 466 and 425 mμ can serve as a measure of the extent of isomerization of the pigments.

The concentration of carotenoid pigments in the flesh of pineapple fruit varies over a wide range among pineapple varieties, distinguishing the golden-fleshed varieties such as Queen or Cayenne from the paler Red Spanish or Cabezona pineapple (2). Within a variety, the intensity of color has served as one measure in quality grading of the processed pineapple.

It has been known for many years that the absorption spectrum of the pigments extracted from canned pineapple differed from that of fresh pineapple. Unpublished work in 1941 by A. J. Haagen-Smit, J. G. Kirchner and A. G. R. Strickland at the California Institute of Technology, in 1944 by G. H. Ellis and his associates at the U. S. Plant, Soil and Nutrition Laboratory in Ithaca, and in 1953 by H. Y. Young in our laboratory showed that the absorption curve of the carotenoids of fresh pineapple is changed and shifted toward the ultraviolet when the tissue is heated. This change obviously has implications in the colorimetric determination of pineapple pigments.

EXPERIMENTAL METHODS

Field-ripe fruit of Hawaiian pineapple, *Ananas comosus* var. Cayenne, were used throughout. Carotenoid pigments were extracted from the edible portions by blending 25 g of tissue with 50 ml of a 1:1 mixture of petroleum ether (BP 60–110° C) and 95% ethanol. The petroleum ether supernatant obtained by centrifugation was used for the absorption spectra measurements in the Beckman DK-2 recording spectrophotometer.

RESULTS AND DISCUSSION

The Spectral Shift

The absorption spectrum of an extract of fresh pigment shows an intense, sharp maximum at 466–467 mμ, a broader and slightly more intense peak at 438–439 mμ, a less intense peak at 415–418 mμ (a shoulder in less than optimal samples) and finally two weak, broad maxima at about 328 and 315 mμ. The isomerized (canned-type) pigment extracts show a weak shoulder at about 470 mμ, a moderately intense absorption maximum at 447–449 mμ, the most intense peak at 425–426 mμ, the next most intense at 401–403 mμ, the third most intense (shoulder) at about 382 mμ, and vague inflections below this wave length (Figure 1).

Since the isomerized pigment has lost the 466 mμ peak and yet retains its absorbance at other lower wave lengths, these changes readily lend themselves to spectrophotometric determination of the relative amounts of the unisomerized and isomerized pigments in an extract. However, the eye is less discriminating and to the human eye these spectral changes are hardly visible. The isomerism

[a] Approved by the Director as Technical Paper No. 271 of the Pineapple Research Institute of Hawaii.

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results in a small shift in the shade of color from a slightly orange-yellow in fresh fruit to a somewhat more lemon-yellow color of nearly the same intensity in the isomerized pigments of canned fruit.

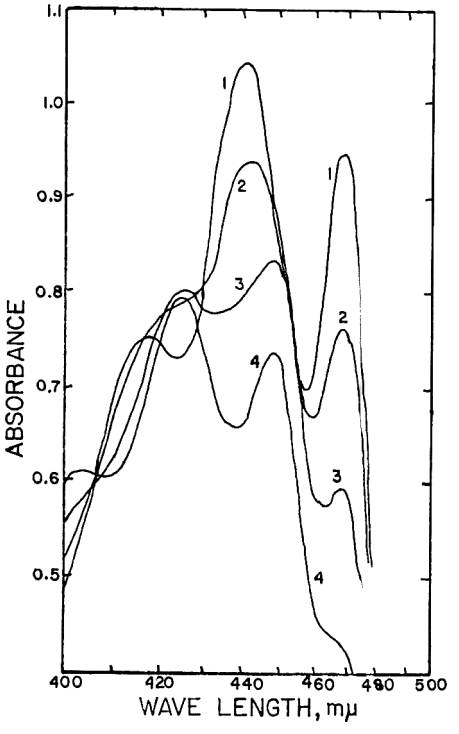


FIG. 1. Change in absorption spectrum of pineapple fruit carotenoids as isomerization progresses, showing especially the loss of the absorbance peak at 466 mμ, the lowered absorbance and shift to higher wave length of the peak at 438 mμ, and the retention of absorbance in the neighborhood of 425 mμ. A tissue homogenate was allowed to stand at 24° C for various periods before extraction of the pigments into petroleum ether. Curve 1 = after 3 min; curve 2 = 1 hr; curve 3 = 2 hr; curve 4 = 4 hr 22 min.

CHEMICAL NATURE OF THE ISOMERIZATION

Carotenoids of undisrupted fresh pineapple fruit tissue isomerize only very slowly, even when cut chunks are allowed to stand for some time. On the other hand, homogenates of the tissue soon exhibit a shifting in the absorption spectra of their extracts. This shift can be prevented by the addition of $CaCO_3$ or NaOH to neutralize the acid of the pineapple. This is illustrated by data in Table 1. Some isomerization occurred before the pH of the blended tissue was adjusted, but thereafter isomerization proceeded only under acid conditions. At the lowest pH normal for the fruit, isomerization was essentially complete after heating.

The type of isomerization that is well known for carotenoids and other polyenes is cis-trans isomerism. This is know to be catalyzed by acids, but is more readily produced by iodine or sunlight. Literature values (1) for the spectral shifts produced by iodine isomerization are of the order of a few millimicrons, whereas the shift caused by canning was considerably higher. Iodine and sunlight treatments or several days' standing at room temperature in the light caused some slight reduction in the absorption intensity of fresh pineapple carotenoid extracts, but did not produce the "canned-type" spectral shift.

There are other indications that the important isomerization is not one of cis-trans change. When the carotenoid extract from fresh pineapple flesh is shaken with strong hydrochloric acid, part of the yellow pigments are converted to a blue solid, soluble in methanol but insoluble in water or petroleum ether. When treated with alkali, the blue material again becomes yellow and soluble in petroleum ether. The absorption spectrum of the residual yellow solution after removal of the blue resembles that of the fresh pigments, whereas the spectrum of the pigments regenerated from the blue solid approximates the "canned-type" spectral shift.

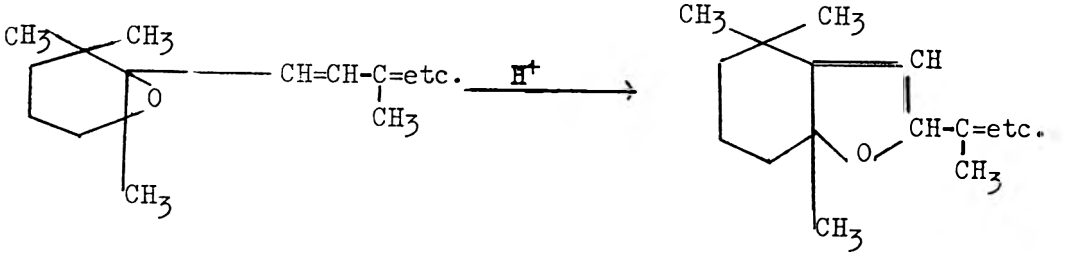
This information coupled with chromatographic and other data characterizing the individual pigments, to be reported later, makes it clear that the spectral shift is caused

TABLE 1

PERCENTAGE ISOMERIZATION OF PINEAPPLE CAROTENOIDS AFTER TISSUE HOMOGENATES ADJUSTED TO VARIOUS pH LEVELS WERE HEATED 30 MINUTES AT 100° C BEFORE EXTRACTION

pH	3.40	4.20	4.60	4.90	5.70	6.05	6.50	7.04
% Isomerized	98	85	84	75	44	38	27	24

by the isomerization of one or more carotenoids containing epoxide groups into their furanoid rearrangement products (2, 3) in a reaction of the following type:



Epoxide pigments are widespread in nature, especially in ripening fruits.

The blue pigment formed with strong acids probably consists of oxonium salts, which would explain its solubility in methanol and not petroleum ether.

Determination of Total Carotenoid Pigment and the Degree of Isomerization

As seen in Figure 1, the unisomerized pigments show a strong peak at 466 mμ which becomes a low shoulder after isomerization. They also have a small absorption minimum at 425 mμ which is changed into the maximum absorbance when isomerization is complete. Absorbance determinations at these two wave lengths can serve as a measure of the degree of isomerization of the carotenoid pigments extracted from pineapple tissue.

A large number of individual determinations on fruit carefully selected and handled to avoid tissue breakdown, and extracted rapidly and in the presence of excess alkali to avoid isomerization, gave an average ratio of absorbances at 466 and 425 mμ of 1.38. This, then, characterizes the "fresh" or unisomerized pigments. Analyses on pigments of heated flesh homogenates or juice gave an average ratio of 0.40 for the absorbances at the two wave lengths.

These two ratios enable one to calculate the degree of isomerization of pigment in a given extract according to the formula:

$$\% \text{ unisomerized} = \frac{\dfrac{(\text{absorbance at } 466\ m\mu)}{(\text{absorbance at } 425\ m\mu)} - 0.40}{1.38 - 0.40} \times 100 = \frac{R - 0.40}{0.0098}$$

The spectra of the pigments before and after the acid-catalyzed isomerization also suggest a means of analyzing for total carotenoid pigment regardless of the isomeric form. This involves selection of an isosbestic-like wave length for the measurement.

A true isosbestic point can exist only when a single compound is being transformed into (or is in equilibrium with) another and they have different absorption spectra. Under these conditions, a sequential series of spectral absorption curves of samples with varying degrees of conversion will all pass through a point of constant absorbance, i.e., the isosbestic point. Although preliminary chromatography had shown that at least three carotenoids were in the natural pineapple mixture, it was hoped that one or more points of nearly constant absorbance could be found as the fresh-type pigment was converted to the canned-type. These pseudo-isosbestic wave lengths could then be used for a colorimetric determination which would give the same reading for fresh *or* canned yellow pigment.

As will be noted from Figure 1, the spectral curves on pineapple carotenoids at various stages of isomerization approach a common absorbance near 425 mμ and at 406 mμ. The 406 mμ point is not on a peak for either the isomerized or the unisomerized pigment spectrum, but rather is in a region of rapid change of absorbance for both. Thus a small wave-length difference could lead to a rather large change in the apparent pigment concentration in the neighborhood of 406 mμ.

Measuring pigment concentration at 425 mμ does not have this disadvantage, since this wave length coincides with a peak on the curve for isomerized pigment and is near a saddle on the spectrum of the unisomerized pigment. Thus, where a mixture of the isomeric forms is being analyzed for

total carotenoid, the absorbance at 425 mμ measured with a narrow band-pass spectrophotometer compared with an appropriate known β-carotene standard measured at the same wave length can give the "ppm carotene" equivalent with satisfactory accuracy.

Effect of Time and Extraction Technique on Isomerization

The marked effect of pH on isomerization of the carotenoid pigments of pineapple has already been referred to (Table 1).

TABLE 2

Percentage Isomerization of Pineapple Carotenoids After Tissue Homogenates Were Allowed to Stand for Varying Time at 24° C Before Extraction

	Time delay before exten.	Per cent isomerized
Blended fruit tissue		
	3 min	8
	11 min	11
	1 hr	38
	2 hr	65
	4 hr 22 min	84
	6 hr	87
Blended with NaOH		
	3 min	5
	1 hr	4
Blended with solvent		
	0 min	9
Blended with NaOH + solvent	0 min	0

At room temperature, isomerization is detectable within a few minutes and proceeds for several hours at the pH of blended pineapple fruit (Table 2 and Fig. 1). An excess of alkali effectively stops isomerization of the carotenoids; no further important spectral change occurs upon standing.

A delay in extraction of the pigments after the fruit tissue is blended can lead to appreciable alteration in the spectrum. If the sampling method requires weighing an aliquot of blended tissue for pigment extraction, the blending should be done in the presence of sufficient alkali to maintain a pH above 7 if the pigments are desired in the isomeric forms present in the original fruit.

No further isomerization of the pigments of the type we are discussing occurs once the carotenoids are extracted into petroleum ether.

ACKNOWLEDGMENT

The technical assistance of Laura Aono and Martha Kent is gratefully acknowledged.

LITERATURE CITED

1. Karrer, P., and E. Jucker. 1950. *Carotenoids.* Elsevier Publishing Co., New York.
2. Magistad, O. C. 1935. Carotene and xanthophyll in pineapples. *Plant Physiol.*, 10, 187.
3. Tsukida, E., and L. Zechmeister. 1938. The stereoisomerization of β-carotene epoxides and the simultaneous formation of furanoid oxides. *Arch. Biochem. Biophys.*, 74, 408.

Carotenoid Pigments of Pineapple Fruit. II. Influence of Fruit Ripeness, Handling and Processing on Pigment Isomerization [a]

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SUMMARY

The acid-catalyzed isomerization of pineapple fruit carotenoid pigments is influenced by any condition leading to loss of integrity of the cells of the fruit tissue. The swollen lower half of fully ripe, yellow, translucent fruit often will contain an appreciable fraction of isomerized pigment. Any post-harvest handling of the fruit that causes bruising of the tissue will lead to pigment isomerization in the damaged areas. Canning completely isomerizes the carotenoid pigments. Frozen fruit contains a high proportion of isomerized carotenoids; after thawing, further change takes place until the spectrum is that of the isomerized or "canned" type pigment.

The first paper in this series (1) demonstrated that pineapple fruit carotenoids undergo isomerization when the tissue is disrupted and the cell-vacuole acid comes in contact with carotenoid-bearing plastids. This isomerization from epoxide to furanoid forms can be measured spectrophotometrically by determining the ratio of the absorbances at 466 and 425 mμ.

It was evident that bruising, freezing and thawing, canning, or even cell wall breakdown due to senescence could lead to this carotenoid isomerization. Accordingly, various of these factors associated with the harvesting and processing of pineapples have been studied for their influence on the carotenoid pigments of the fruit. While data have been obtained only with pineapple, the technique should be applicable to any acidic fruit or tissue with a high proportion of carotenoid epoxides.

EXPERIMENTAL METHODS

Fruit of Hawaiian pineapple, *Ananas comosus* var. Cayenne, were used throughout. Where a tissue homogenate was first prepared, it was generally made by blending large pieces of flesh (trimmed free of adhering shell) with 1.5–3.0 ml of 10 *N* NaOH per 100 g tissue. Some of the experiments were run by blending the tissue with the alkali in the presence of the extracting solvent. The pigments were extracted by blending 25 g fruit tissue with 50 ml of a 1:1 mixture of petroleum ether (B.P. 60–110° C) and 95% ethanol. The centrifuged supernatant was used for absorbance measurements at 466 and 425 mμ in a Beckman DU or DK-2 spectrophotometer.

The proportion of unisomerized pigment in the mixture was calculated (1) from the ratio (R) of these two absorbances:

$$\% \text{ unisomerized} = \frac{R-0.40}{0.0098}$$

RESULTS AND DISCUSSION

Effect of Ripeness

When the entire edible portion of a number of pineapple fruit varying in their ripeness characteristics was used for pigment analysis, the percentage of isomerized pigment found did not exceed 10% regardless of the degree of yellowness of the shell or the translucence of the fruit flesh. However, in an experiment in which various parts of the edible portion of sound fruit were examined, appreciable isomerization was noted in the swollen mid-sections of very ripe fruit. Two fully yellow and fully translucent fruit showed 8–11% pigment isomerization in the top third, 18–24% in the middle third, 7–17% in the bottom third, and 6–9% in the core. By contrast, two fully yellow but

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TABLE 1

MEAN PERCENTAGE ISOMERIZATION OF CAROTENOIDS IN 25-GRAM WEDGES CUT FROM VARIOUS SECTORS OF SOUND PINEAPPLE FRUIT VARYING IN THEIR RIPENESS CHARACTERISTICS

Ripeness		Quarter of fruit represented [1]			
Shell color	Translucence	Top	2nd	3rd	Bottom
Completely yellow	Opaque	6 (1)	0 (1)	0 (1)	
	Semi-opaque	0 (2)	2 (4)	0 (4)	0 (3)
	Intermediate	0 (1)	1 (3)	1 (3)	0 (2)
	Semi-translucent	0 (1)	4 (2)	4 (1)	4 (1)
	Translucent	3 (3)	15 (3)	16 (5)	24 (3)
Mostly yellow;	Opaque				2 (1)
some green near	Semi-opaque		2 (6)	0 (1)	0 (1)
top	Intermediate	6 (1)			
	Semi-translucent	1 (1)	0 (3)		
	Translucent	5 (1)		2 (2)	2 (1)
Three-fourths	Semi-opaque	14 (1)		2 (2)	
yellow	Semi-translucent				2 (1)
	Translucent		0 (2 core samples)		
Half yellow	Semi-opaque	6 (1)			
	Intermediate			0 (1)	

[1] Values in parentheses are the number of determinations entering into the mean value for isomerization.

semi-opaque fruit had only 1–3% isomerization in the top two-thirds of the fruit.

These preliminary indications that isomerization of the carotenoids may occur with certain stages of ripeness led to a more detailed study. These data are summarized in Table 1. They confirm earlier indications that appreciable isomerization occurs only in fully ripe and translucent fruit. The pineapple fruit ripens from the bottom fruitlets upward, with several days' difference in reaching ripeness between the bottom and top. Thus it is not surprising to find that the pigment isomers are found in the lower portions but not in the top part of the ripe fruit.

The data in Table 1 demonstrate that when a fruit is allowed to become fully ripe, the lower portion tends to become overripe. The result is a softening and breakdown of the cell walls, allowing the fruit acids to catalyze isomerization of the carotenoid pigments.

EFFECT OF HANDLING

The change in the spectrum of pineapple carotenoids that occurs with cell disruption suggested that isomerization might serve as a measure of bruising or tissue damage from post-harvest handling of the fruit. If one allows sufficient time for isomerization within the damaged tissue to be complete, the extensiveness of bruising sufficient to cause "leakage" of the cells should be measurable.

Table 2 shows some data obtained in one of the tests of the effectiveness of the per-

TABLE 2

PERCENTAGE ISOMERIZATION OF CAROTENOIDS IN VARIOUS PARTS OF PINEAPPLE FRUIT BRUISED BY DROPPING ON THEIR SIDES ONTO CONCRETE AND ALLOWED TO SIT OVERNIGHT

	Distance dropped, cm	Per cent isomerized pigments			
		Bruised area	Adjacent to bruise	Remainder, bruise side	Opposite to impact
Translucent fruit	33	36	15	10	
	65	45	8	10	8
	98	38	19	14	9
Semi-opaque fruit	33	29	1	3	5
	65	41	5	1	3
	65	37	1	7	2

centage of isomerized pigment as a bruising index. Isomerization was extensive within the area trimmed out as showing bruising, but was not appreciably higher in the adjoining tissue than in the unbruised opposite side of the fruit. The amount of tissue trimmed out as bruised was directly proportional to the distance the fruit was dropped, and greater for the more translucent fruit than for semi-opaque fruit. Thus the total amount of isomerized pigment also increased with severity of bruising. These and other data confirm that the percentage of isomerization of the pigments can serve as a good index of the severity of the conditions causing bruising.

There are conditions in the handling of pineapple fruit that lead to damage without extensive breakdown of the cells. Static pressure sufficient to flatten the fruit causes fruit loss by tearing the flesh apart with minimal cell wall disruption. Under these circumstances, pigment isomerization would not be expected to correlate with damage of fruit in handling. A semi-opaque fruit crushed by a 30-lb weight overnight showed no detectable isomerization; a translucent fruit showed only a normal 8% carotenoid isomers under similar conditions. Both fruit were severely damaged, however.

Effect of Processing

In the first paper in this series (1) data were presented showing that carotenoid isomerization in tissue homogenates required many hours to reach completion at room temperature, hut was complete within 30 min at 100° C. Canning obviously leads to a shift to the isomerized form of the pineapple pigments.

The isomerization observed with the heating of fruit homogenates is also evident in canning of solid-pack items such as slices or chunks.

Freezing and thawing also leads to tissue breakdown. Pigment isomerization is greatly slowed down in the frozen tissue, and thus frozen pineapple chunks contain both forms of the carotenoids. However, as shown in Table 3, the major part of the pigment has been isomerized even before thawing of the frozen chunks. While this does limit the potentialities of using pigment isomerization as a thawing indicator, the pigment data did show a variability in samples at the retail level, indicating that some of the packages had been mishandled either by being allowed to thaw or by being stored too long.

The somewhat erratic data are probably due to sampling, since only 25-g samples were removed at each period. Somewhat different acidity levels in the frozen chunks could account for the observed differences in rates of isomerization after thawing seen in Table 3.

Acknowledgment

The technical assistance of Laura Aono and and Martha Kent is gratefully acknowledged.

Literature Cited

[1] Singleton, V. L., Willis A. Gortner, and H. Y. Young. Carotenoid pigments of pineapple fruit. I. Acid-catalyzed isomerization of the pigments. *Food Research* (this issue).

TABLE 3

Percentage Isomerization of Carotenoids in Commercial Frozen Pineapple Chunks and in the Chunks Following Thawing

		Thawed and held at 24° C			
	Frozen	2.5 hr	3.5 hr	5 hr	23 hr
Samples from packer's warehouse					
A	79	78	96	86	....
B	84	96	98	100	100
C	78	78	78	92	93
D	88	81	83	86	100
Samples from grocer's shelf					
E	89	95	95	98	100
F	75	78	84	78	....
G	100	100	100	100	100
H	100	100	100	100	100

Pineapple Stem Acid Phosphatases.[a] I. Effect of pH, Anions, Cations and Sulfhydryl Reagents on Crude Preparations

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Manuscript received March 28, 1960

SUMMARY

The pineapple stem is an excellent source of two acid phosphatases. Although the enzymes are stable between pH 4.0 and 8.5 and are not readily surface-denatured, yet the assay values are not too reproducible. Micelle formation may account for the erratic results. The enzyme shows optimum activity at pH 6.0 and requires no metal cofactor. Certain metals, such as magnesium, stabilize the enzyme. Molybdate and hydrogen peroxide are the most potent inhibitors. Dibasic phosphate is about three times as potent an inhibitor as dibasic arsenate.

Although acid phosphatases probably occur in all higher plants and fungi, only a few plant tissues contain enough readily isolable phosphatases to make them convenient sources of these enzymes. In addition to the sources mentioned by Roche in his review article (10), an excellent new source is the juice obtained from the stems of the pineapple plant (6). This juice is available in a dried form as an acetone-precipitated powder. It contains a mixture of proteases, two acid phosphatases, a diesterphosphatase and a peroxidase.

We wish in this and succeeding papers to describe some of the properties of the acid phosphatases in the protease preparation stem bromelain, certain techniques for the partial purification of these phosphatases, and possible applications of these enzymes in the food industry.

MATERIALS AND METHODS

Enzyme Preparations

The plant tissue used as the source of enzyme was the 4- to 6-year-old stem of the pineapple plant. After stripping the leaves and pressing the stems in a roller mill press, the juice was clarified and the acidity adjusted to pH 4.0–pH 4.3. In some experiments this juice was used as the enzyme. In other experiments the colloids were partially purified by precipitation.

In the fractionation work with either ammonium sulfate or acetone, the least soluble fraction was discarded. This contained iron and calcium as well as a carbohydrate polymer and some protein. The enzymatic activity of this fraction was low. The next fraction collected, that soluble in 40% saturated ammonium sulfate or 45% v/v acetone but insoluble in 60% saturated ammonium sulfate or 60% v/v acetone, contained most of the protease activity and between one-half and two-thirds of the total acid phosphatase activity. The last fraction collected, the most soluble fraction, contained negligible protease activity. However, the specific acid phosphatase and peroxidase activities were very high, even though the weight yield of the acid phosphatases was low.

The assays of some typical fractions are given in Table 1.

Assays

Three substrates were used, glycerophosphate (52% alpha, 48% beta), phenylphosphate, and p-nitrophenylphosphate. In routine assays with glycerophosphate, neither buffer nor activating ions were used. To 25 ml of 0.04 M substrate adjusted to pH 6.0, 1 ml of enzyme solution was added. The enzyme was incubated 1 hr at 25° C and the action then stopped by adding sulfuric acid. The liberated phosphate was estimated by Sumner's method (11).

Phenylphosphate, containing no added buffer, was adjusted to have a pH of 6.0 and a substrate concentration of 0.01 M. Either the liberated phenol was estimated from the blue color formed with the Folin Ciocalteau reagent (5) or the liberated phosphate was estimated by Sumner's method (11).

[a] Presented at the Twentieth Annual Meeting of the Institute of Food Technologists, May 16, 1960.

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TABLE 1

ACID PHOSPHATASE AND PROTEASE ACTIVITIES OF PROTEINS OBTAINED FROM PINEAPPLE STEMS

Code	Method of Preparation	% Ash	Acid Phosphatase [1]	Protease M.C.U.[2]
S.J.C.	Stem juice, crude	2.3	1.94 ml^{-1}	35 ml^{-1}
S.J.D.	Stem juice, dialyzed	0.3	1.23 ml^{-1}	28 ml^{-1}
Ac-2D	Soluble in 45% v/v acetone; insoluble in 60%; dialyzed two days	3.58	.054 mg^{-1}	3.8 mg^{-1}
Ac-3D	Soluble in 60% v/v acetone; insoluble in 80%; dialyzed two days	0.96	.026 mg^{-1}	neg
Am. Sul. 2D	Soluble in 40% saturated ammonium sulfate; insol. in 60%. Dialyzed	.....	.046 mg^{-1}	4.0 mg^{-1}
Am. Sul. 3D	Soluble in 60% saturated ammonium sulfate; insol. in 80%. Dialyzed	.....	2.35 mg^{-1}	0.07 mg^{-1}
Commercial	Commercial bromelain	23.0	0.082 mg^{-1}	3.0 mg^{-1}

[1] Micromoles p-nitrophenol liberated per minute from a 0.015 M substrate at 37.5° C.

[2] Milk clotting units = 1/min to clot 5 ml of a 5% skimmilk solution of pH 5.3 at 37.5° C.

p-Nitrophenylphosphate, made up in a 0.001 M citrate buffer and adjusted to pH 6.0, was used at a concentration of 0.015 M. In some experiments ethylenediaminetetraacetate was also incorporated in the substrate to give a final concentration of .001 M. The incubation temperature was 37.5° C and the time of digestion generally 15 min or less. This method is similar to that described in Fishman and Davidson's review article (5). The amount of nitrophenol liberated was estimated from the molar extinction coefficient of 17,000 (2).

RESULTS AND DISCUSSION

SOME FACTORS AFFECTING THE RELIABILITY OF ASSAYS

Although acid phosphatases as a class are quite sensitive to relative minor changes in assay procedures, the pineapple acid phosphatases appeared to be particularly sensitive. Increasing the length of the incubation period, changing the concentration of enzyme, over-shooting the desired pH value, holding the enzyme at different temperatures before assaying the enzyme frequently affected the specific activity. In an attempt to identify and eliminate these sources of variation, we tested many of the causes of variation reported by other investigators.

Tsuboi and Hudson, in one of their series of papers on the acid phosphatase from erythrocytes (12), found that adding a non-ionic surface-active material eliminated the variation caused by surface denaturation. In our tests we found no beneficial effect from surface-active materials. At low enzyme concentrations surface-active agents actually decreased the specific activity.

Although some enzymes require either monovalent cations or anions for maximum activity—for example, lysozyme requires sodium ions (7) and salivary amylase requires chloride ions—pineapple acid phosphatase was not appreciably affected by rather large variations in salt concentration. At moderate salt concentrations (10^{-3}—10^{-4} M) the specific activity was slightly higher than at very low salt concentrations. Only when salt was added to the substrate in large amounts (to give an 0.5 M solution) did it inhibit some samples.

The composition of the solution used to dilute the enzyme had an appreciable effect on the specific activity of the enzyme. At high dilutions of the enzyme certain diluting solutions gave a preparation which had a high specific activity—for example, water, ethylenediaminetetraacetate, or ethylenediaminetetraacetate plus Triton X-100. Other diluting solutions, citrate and citrate plus Triton X-100, did not give this increase (Fig. 1).

The results shown in Figure 1 can be explained on the assumption that dilution and ions in the diluting solution affected the size of micelles and the orientation of mole-

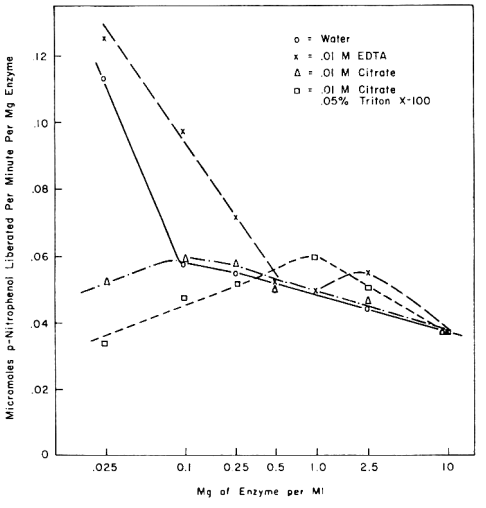

FIG. 1. Effect of different diluting solutions on the specific activity of pineapple acid phosphatase. (One other curve, .01 M EDTA + .05% Triton X-100, was similar to the .01 M EDTA curve and was not included.)

cules on the surface of the micelles. If this assumption is correct, then very subtle variations in handling technique or in the changes in the ratio of ions would be expected to have a large effect on the assayed activity.

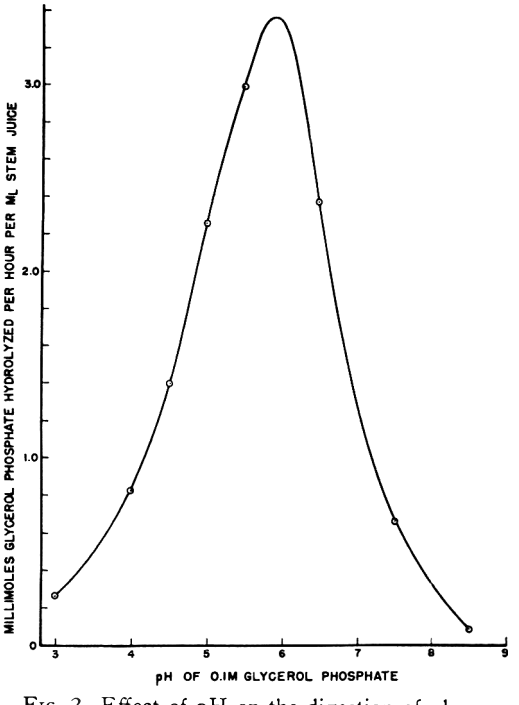

FIG. 2. Effect of pH on the digestion of glycerophosphate by diluted stem juice. Curves of other preparations were similar.

OPTIMUM pH FOR DIGESTION

Although stem colloids contain two acid phosphatases of very dissimilar isoelectric points (6), the pH-activity curves determined on fourteen different preparations were remarkably similar and were all fairly symmetrical. Figure 2 illustrates one such curve. With glycerophosphate as the substrate and an incubation period of one hour at 25° C the average optimum pH was 6.02. With p-nitrophenylphosphate as the substrate and a shorter incubation period at 37.5° C the average optimum pH was 5.93. At this temperature if the incubation period was increased from 15 to 30 min, the optimum pH dropped to 5.8. This decrease in the optimum pH is in agreement with the greater stability of many of the enzyme preparations held at pH 5.0 than at pH 6.0 (Fig. 4).

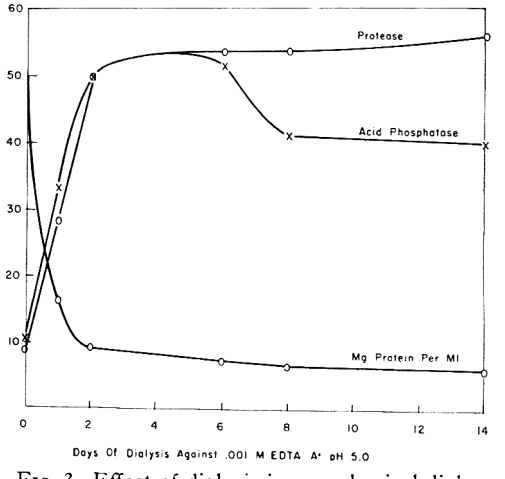

FIG. 3. Effect of dialysis in a mechanical dialyzer at 4° C against twice-daily changes of buffer. Protease values are expressed as milk clotting units per mg protein x 20; protein as mg per ml x 1; and acid phosphatase as micromoles p-nitrophenol liberated per minutes per mg protein x 500.

In addition to the pH 5.9 peak, some preparations showed a small shoulder at pH 6.2 and a suggestion of activity at pH 9.5. (Compare a similar alkaline peak in curve A, Fig. 5.)

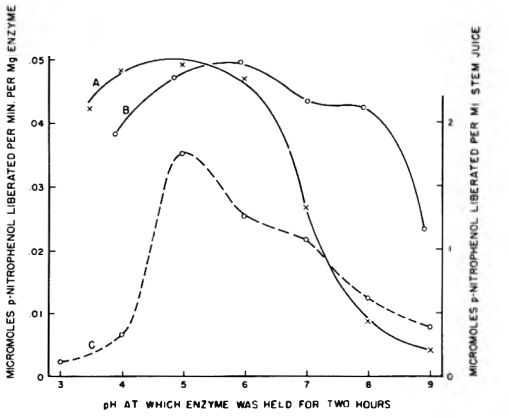

FIG. 4. Effect of holding phosphatase samples for two hours at different pH values before readjusting to pH 6 and assaying with glycerophosphate as the substrate. (A x—x dialyzed stem colloids; B o—o commercial grade bromelain; C o--o stem juice).

EFFECT OF DIALYSIS ON ACTIVITY

Normally a phosphatase requiring a metal as an essential cofactor can be at least partially inactivated by dialysis or by adding a metal-chelating agent. However, since pineapple colloids retain ions very tenaciously (see, for example, Chittenden's work [3]), such simple tests cannot be used to furnish an unequivocal answer regarding the role of divalent cations.

To increase the likelihood of removing all bound ions, we used the technique of Boroughs (1) and dialyzed our preparations against ethylenediaminetetraacetate solutions adjusted to low pH values or against acid salt solution. In the experiment recorded in Figure 3 dialysis of stem juice for 13 days in a mechanical dialyzer against twice-daily changes of pH 5.0, .001 M ethylenediaminetetraacetate solutions did not reduce the specific activity appreciably. With the volumes of solute and dialyzing water used, each period of dialysis should have removed 99.7% of all the dialyzable ions.

In another experiment samples of stem juice were dialyzed against acidified water or acidified 0.1% sodium chloride solution. The latter, which would be expected to have the greater replacement of ions, showed the most activity.

These and other dialysis experiments indicate that the acid phosphatases which we were measuring probably did not require a metal ion as a cofactor. The experiments reported in the next sections further support this conclusion.

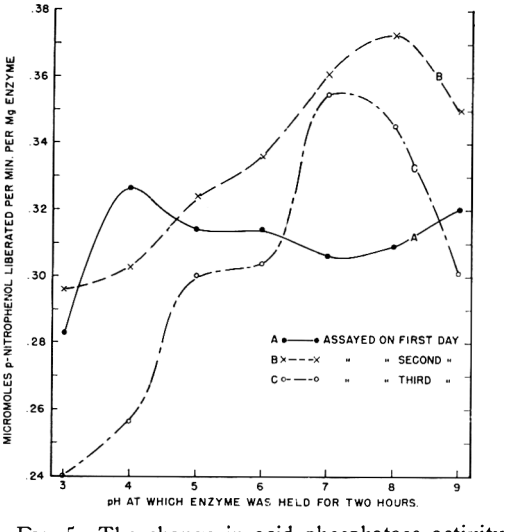

FIG. 5. The change in acid phosphatase activity with time of samples adjusted to pH 6 from the pH values shown on the abscissa. (Two hours of adjusting were required to get the pH values shown. They were held at these pH values two additional hours.)

EFFECT OF CATIONS ON ACTIVITY

In general alkaline and diesterphosphatases require divalent cations as essential cofactors whereas most acid phosphatases do not (10). However, even with acid phosphatases certain ions may stabilize the enzyme and thus cause what appears to be activation. For example, Cohen, Bier and Nord (4) found that cobalt and zinc stabilized both wheat germ acid phosphatase and chicken liver alkaline phosphatase. These metals were not cofactors for the acid phosphatase.

Plant phosphatases differ greatly in their response to different ions. Potato acid phosphatase, according to Naganna *et al* (8), was not inhibited by 2×10^{-3} M cobalt but was completely inhibited by the same concentrations of cooper or zinc. Wheat leaf phosphatase, according to Roberts (9), was not inhibited by 2×10^{-2} M zinc, copper, cobalt or nickel. Pineapple stem phosphatases appear to be similar to potato phosphatase in their reactions to inhibiting metals.

Magnesium, the most general activator of the alkaline phosphatases, "activated" certain of our phosphatases under certain conditions. For example magnesium chloride at .001 M caused the largest activation (142%) of sample Ac-3D. Manganese chloride at .01 M was the next best activator (22.5%). By contrast neither these cations nor nickel, cobalt or zinc activated sample Ac-2D (Table 2).

TABLE 2

EFFECT OF CATIONS ADDED TO GLYCEROPHOSPHATE SUBSTRATE AT pH 6.0 ON THE PHOSPHATASE ACTIVITY OF TWO FRACTIONATED SAMPLES INCUBATED AT 25° C FOR ONE HOUR

	Cation Concentration for 50% Activity	
Salt	Enzyme Ac-2D	Enzyme Ac-3D
$MgCl_2$	0.13 M	(activated)
$MnCl_2$	0.007	Slight Activation
$NiCl_2$	0.002	.004 M
$CoCl_2$	0.002	.....
$ZnCl_2$	0.0003	.002

On enzyme Ac-3D $MgCl_2$ at .001 M activated 142%. $MnCl_2$ at .01 M activated 22.5% and $CoCl_2$ caused 30% inhibition at all concentrations tested.

Another comparison was made of the effect of different cations at 55° C and 38° C on the acid phosphatase activity of acid-dialyzed stem juice. At 10^{-3} M magnesium chloride "activated" 90% at 55° C but had no effect at 38° C. At 10^{-4} M copper chloride "activated" 33% at 55° C but had no activating effect at 38° C (Table 3).

In still another experiment an attempt was made to "flush out" all adsorbed cations by dialyzing stem juice for a few days with twice-daily changes against weak acid (pH 3.5 and pH 4.5) or against 0.1% sodium chloride solutions at pH 3.5 and pH 4.5. The most active sample was that dialyzed against 0.1% sodium chloride adjusted to pH 4.5. Magnesium chloride caused only 10% activation of this sample at 5×10^{-3} M.

In spite of our finding that some samples were strongly "activated" by magnesium we do not believe that magnesium is an essential cofactor. The rather extensive and drastic dialysis experiments showed that no dialyzable factor could be removed. Instead of being an activator we believe that magnesium, and to a lesser extent some of the other ions, functioned as stabilizers.

Those samples which were strongly "activated" by cations were those which were unstable. For example, preliminary experiments showed that the activity of sample Ac-3D decreased more rapidly during the time required to set up the experiment, than that of Ac-2D. Therefore the sample was made up in ice water and left in ice water until it was used. Similarly the other experiment in which dramatic activation was shown was the temperature comparison experiment. The activity at 55° C was conservatively estimated to be less than 60% of what it should have been if the enzyme had been stable.

The exact mechanism of stabilization is still unknown. If the cations affected the size and shape of the micelles, then the differences between divalent cations should have been small. A direct or indirect effect on an enzyme which can destroy acid phosphatase is another possibility.

The effect of other cations will be mentioned only briefly. Cobalt was a strong inhibitor of dialyzed juice at 55° C but not at 38° C. On the most soluble fractionated phosphatase, sample 3AcD, cobalt inhibited only 30% at all concentrations tested, whereas on the less soluble phosphatase, sample 2AcD, it gave a normal inhibition curve. Nickel gave a normal inhibition curve with an I_{50} of 2.4×10^{-3} M. Manganese inhibited only one sample, sample Ac-2D. Otherwise, it had little effect on the acid phosphatase activity. Iron and tin caused no inhibition. Copper was a fairly strong inhibitor. However, of all the cations tested zinc was the most powerful inhibitor.

EFFECT OF ANIONS ON ACTIVITY

In his review article (10) Roche mentions that molybdate, fluoride, and oxalate were all strong inhibitors of Class II phosphomonoesterases. Naganna *et al* (8) found that 1×10^{-3} M fluorine and 2×10^{-6} M molybdate gave over 75% inhibition of potato acid phosphatase.

Not many of the anions which we tested were marked inhibitors of pineapple acid phosphate. However, molybdate was a strong, and fluoride a moderate, inhibitor.

The amount of inhibition was similar to that reported by Naganna. Bisulfite, cysteine, cyanide and oxalate inhibited only at relatively high concentrations (Table 4).

TABLE 3

EFFECT OF CATIONS ON THE ACID PHOSPHATASE ACTIVITY OF STEM JUICE DIALYZED AGAINST .01 M EDTA AT pH 4.8 AND THEN AGAINST DISTILLED WATER

	Cation Concentration for 50% Activity	
Salt	38° C	55° C
$CoCl_2$	.....	.00063
$CuCl_2$	.00068	.0013
$ZnCl_2$	.0025	.00020

$MnCl_2$, $MgCl_2$, $CaCl_2$, $ZnCl_2$ and $FeSo_4$ had no inhibiting effect at either 38° C or 55° C. $MgCl_2$ "activated" 90% at 55° C but not at 38° C.

TABLE 4

EFFECT OF ANIONS ON THE ACID PHOSPHATASE ACTIVITY OF STEM JUICE

Salt	I_{50}M
EDTA	Activated
Sodium Sulfhydride	0.17
Aurin tricarboxylate	0.18
Sodium oxalate	0.056
Sodium cyanide	0.040
Sodium bisulfite	0.025
Sodium fluoride	0.004
Ammonium molybdate	0.00005

I_{50} = Anion concentration for 50% activity.

The effect of phosphorous and arsenic compounds on pineapple phosphatase was interesting (Table 5). Orthophosphate, a material which should act as a competitive inhibitor, inhibited only at high concentrations ($I_{50} = 0.03$ M). Certainly in the normal assay procedures, at pH 6.0 the effect of the liberated phosphate on the rate can be disregarded. Arsenate, on the other hand, was a moderately active inhibitor. However, since at pH 6.0 orthophosphate is about 93% in the monobasic form and 7% in the dibasic form, whereas by contrast, arsenate at this pH exists principally in the dibasic form, the two materials should be compared at similar concentrations of dibasic ions. When this is done, phosphate is a more potent inhibitor than arsenate (for $AsO_4^=$ I_{50} 8.0×10^{-4} M; for $HPO_4^=$ I_{50} 2.5×10^{-4} M).

TABLE 5

EFFECT OF PHOSPHORUS AND ARSENIC COMPOUNDS ADDED TO P-NITROPHENYLPHOSPHATE SUBSTRATE ON THE ACID PHOSPHATASE ACTIVITY OF PRECIPITATED ACID PHOSPHATASE

	I_{50}
Sodium Phosphate	0.031 M
Sodium Pyrophosphate	0.78
Sodium Hypophosphate	No inhibition
Sodium Metaphosphate	No inhibition
Sodium Arsenite	>1 M
Sodium Arsenate	0.0014

EFFECT OF SULFHYDRYL AND DISULFIDE REAGENTS

Although some types of phosphatases have been reported to require free sulfhydryl groups (10), none of the pineapple acid phosphatases belong to this class. None of the typical sulfhydryl agents tested, such as menadione, N-methylmaleimide or p-chloromercuribenzoate caused inhibition. Instead almost all caused a small but consistent increase in activity, especially at low concentrations. Arsenite, a specific reagent for dithiols, caused no marked inhibition. Diisopropylfluorophosphate caused no inhibition.

Reagents which can reduce disulfide bonds were inhibitory. Thus even cysteine was inhibitory ($I_{50} - .05$ M). Bisulfite, a better disulfide-reducing agent, had an I_{50} of .025 M. On the other hand, sulfide ion was not a potent inhibitor.

Hydrogen peroxide, which can of course affect many other groups in addition to sulfhydryl groups or disulfide groups, was strongly inhibitory ($I_{50} - .0005$ M). It is interesting that low concentrations of peroxide inhibited the acid phosphatase but not the protease activity.

EFFECT OF pH ON ACTIVITY AND STABILITY

Attempts to run standard pH stability curves gave a variety of differently shaped curves. All curves showed one peak of maximum stability between pH 4.5 and 5.5 (Figs. 4, 5). In addition many also showed a second peak of maximum stability on the alkaline side. Since repeated experiments failed to give a consistent set of curves, it was apparent that the adjustment of the pH was affecting some unknown factor or factors

more strongly than it was affecting the stability of the acid phosphatase.

We have evidence that two other factors were affecting the results of our pH stability experiments. One of those was an oxidase or a peroxidase which permanently inactivated the acid phosphatase. The other was micelle formation. In most of the experiments we believe that micelle formation was the principal factor affecting the assay values.

Since the micelles, when once formed, are extraordinarily well buffered and are slow to reach equilibrium with ions in solution, adjustment of the pH was difficult and tedious. For example, in one experiment 2 hr after a 1% enzyme solution had been adjusted to pH 9, 8 and 7 the pH drifted to pH 7.2, 7.1 and 6.9. Part of the lack of consistent curves could certainly be attributable to the difficulty of getting the pH adjusted.

By spending 2 hr adjusting the pH values until no further drifting occurred and then holding for an additional 2 hr at these pH values the very interesting sequence of curves shown in Figure 5 was obtained. These changes can be explained on the basis of micelle formation. Since all solutions were readjusted to pH 6.0 before assaying (an operation which was easy to perform in contrast to the trouble in making the original adjustments) all solutions would contain micelles whose composition would reflect the pH at which they had been held before adjustment. The initial micelle formed would represent a micelle which had not reached its most stable form. In fact at the pH extremes there is a strong possibility that free enzyme molecules still existed in solution. On longer holding the molecules in the micelle would rearrange to give a more stable structure. Some of these rearrangements might have exposed more enzyme sites than had been originally present.

By illustrating the extreme importance of previous treatment on activity, a phenomenon which we believe is attributable to micelle formation, these pH-stability experiments may be of more value than if they had given unequivocal answers to the effect of pH on stability. This phenomenon may be restricted to certain plant enzymes or it may be of more general occurrence. It is certainly a factor which must be considered in any isolation work, inhibition study or kinetic study.

LITERATURE CITED

1. Boroughs, Howard. 1954. Studies on the acid phosphatases of green leaves. *Arch. Biochem. Biophys.*, **49**, 30–42.
2. Burch, Helen B. 1957. Fluorimetric assay of cocarboxylase and derivatives. *In Methods of Enzymology*, Vol. III (Colowich, S. P., and Kaplan, N. O., ed.) 946, Academic Press Inc., New York.
3. Chittenden, R. H., E. P. Joslin, and F. S. Meara. 1891. On the ferments contained in the juice of the pineapple. *Trans. Conn. Acad. Sci.*, **8**, 281–308.
4. Cohen, W., M. Bier, and F. F. Nord. 1957. Mechanism of enzyme action. LXII. Acetylation of alkaline phosphatase. *Arch. Biochem. and Biophys.*, **67**, 479–489.
5. Fishman, W. H., and H. M. Davidson. 1957. Determination of serum acid phosphatases. *Methods of Biochem. Anal.* Vol. 4 (Glick, D. ed.) 257–284. Interscience.
6. Heinicke, R. M., and W. A. Gortner. 1957. Stem Bromelain, a new protease preparation from pineapple plants. *Econ. Botany*, **11**, 225–234.
7. Mounter, L. A., and B. A. Shipley. 1958. Inhibition of plasmin by toxic phosphorus compounds. *J. Biol. Chem.*, **321**, 855–861.
8. Naganna, B., A. Raman, B. Venugopal, and C. E. Sripathi. 1955. Potato pyrophosphatases. *Biochem. J.*, **60**, 215.
9. Roberts, D. W. A. 1956. Wheat leaf phosphatases. I. A survey of the inhibitors at pH 5.7. *J. Biochem.*, **219**, 711–718.
10. Roche, J. Phosphatases, Chapter 11. *In The Enzymes*, Vol. 1, Pt. 1 (Sumner and Myrbäck, eds.)
11. Sumner, J. B. 1944. Method for the colorimetric determination of phosphorus. *Science*, **100**, 413.
12. Tsuboi, K. K., and P. B. Hudson. 1955. Acid Phosphatase. II. Purification of human red-cell phosphomonoesterase. *Arch. Biochem. and Biophys.*, **55**, 206.

Composition of Honey. VI. The Effect of Storage on Carbohydrates, Acidity and Diastase Content

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SUMMARY

Quantitative examinations have been made of the changes that occur in honey while stored at room temperature. Though honey is a relatively stable commodity, it has been found to change in composition and biochemical activity even when stored at 26 ± 3° C. During two years of such storage about 9% of the monosaccharides are converted per year into more complex disaccharides and higher sugars. The ratio of fructose to glucose increases markedly as the free glucose content declines more rapidly than the free fructose content. All samples examined showed such changes. Significant increases were noted in acidity during storage, but some samples showed no change. Evidence for the enzymic nature of the change is given. Diastase values of unheated honey decline in storage at room temperature (23–28° C), with diastase showing a half-life of 17 months under these conditions of storage. Cool or cold storage and expeditious handling are recommended for preservation of diastase in honey for export to Europe.

Honey is considered to be a relatively stable foodstuff, with only minor changes in flavor and color taking place during several years storage. It is well-known that properly ripened honey is not susceptible to spoilage by microorganisms, with the exception of osmophyllic yeasts, and then only above moisture contents of 17% (9). Granulation of honey does increase the possibility of such spoilage since it results in an increase in the moisture content of the liquid portion. A comprehensive study of the effect of storage at elevated temperature and of heat processing on the color of honey has been described by Milum (12).

Both physical and chemical actions are involved in transformation of nectar into honey, with the activity of enzymes being most prominent. Since these enzymes remain in the honey, their action may continue at a declining rate. The long-noted (3) decrease in the sucrose content of honey after extraction has been ascribed to a continuing action of the invertase added by the bee. Sucrose content does not reach zero after several years of storage, however, even though a honey may still contain an active invertase.

It was recently shown (22) that honey contains a transglucosylase which produces several oligosaccharides, including maltose and isomaltose, from sucrose. Austin pointed out (2) that because of this enzymic activity the "maltose" (actually reducing disaccharide) content of a honey will depend to some degree on methods of apiary management, storage temperature, and density of honey. He did not predict the effect of storage in general on the maltose content of honey.

De Boer (5) examined a number of yearly honey samples stored for up to 22 years; nearly all were white clover and all were stored in the unheated state. He pointed out that the same changes in composition that take place on heating of honey also occur in storage. He concluded that polarization is unchanged and the change in sucrose content negligible, implying no changes in the sugars. The amounts of glucose and fructose and their ratio remained unchanged; no relative increase was noted in fructose content, contrary to previous reports (1).

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Diastase was found to decrease with age, 3 Gothe "steps" in 10 years. The acidity was unchanged, but the Fiehe test for hydroxymethylfurfural (HMF) became positive and after 10 years HMF could be determined gravimetrically.

Armbruster, quoted by deBoer (5), reported that aging of as little as 2½ months sometimes causes a noticeable decrease of diastatic activity, while other types of honey show no loss after 2–5 months. After 2½ years a considerable decrease was found in one type of honey.

The recent introduction of a more comprehensive method of carbohydrate analysis of honey (20, 23) has made it possible to learn more about the carbohydrate make-up of honey. Whereas older procedures reported only glucose, fructose, sucrose and dextrin, the newer procedure allows determination of reducing disaccharides, which in itself provides more accurate values for glucose and fructose. Better values for higher sugars ("dextrin") also result because reducing sugar contamination of dextrins, common to older precipitation procedures, is eliminated.

During an extensive analytical survey of the composition of American honey by the newer methods, we have re-examined the effects of storage on the composition of honey. We have studied the effect of room-temperature storage of up to 3 years on unheated and mildly heated honey, determining changes in glucose, fructose, reducing disaccharides, sucrose, higher sugars, diastase, free acidity, lactone and total acidity. Significant changes were found for nearly all of these constituents, contrary to previous beliefs.

MATERIALS AND METHODS

Honey samples used in this work were some of those collected from producers for the analytical survey noted above; they will be described in detail when the results of the survey are published. In general, unheated samples were received, and divided into 3 portions. One was stored at about −20° C within one day of receipt, a second heated in a closed jar in a water bath at 55° C for 30 min and cooled (essential pasteurization without enzyme inactivation), and the remainder left unheated. The latter two portions were stored in the dark at room temperature (23–28° C). Samples from frozen storage were allowed to reach room temperature overnight before analysis.

Analyses of corresponding samples of a set were carried out on the same day; sets were selected at random.

Carbohydrate Analysis

Where necessary, samples were liquefied by the new AOAC procedure (20). Carbohydrates were determined by the selective adsorption method (20). Moisture was determined by refractive index using the Chataway table (11). Results for each set were calculated to the moisture content of the sample kept in cold storage.

Acids

Free acidity, lactone, and total acidity were determined by a recently developed method (24). All samples were unheated; aliquots stored at room temperature and −20° C were analyzed; the cold-storage samples in duplicate and others in triplicate.

Diastase

The method of Schade, Marsh and Eckert (14) slightly modified as adopted by the AOAC (20) was used. Samples which were granulated were made as homogeneous as possible by stirring before sampling; no heat was used. Samples upon which carbohydrates and diastase were determined were all liquid and did not require heating before carbohydrate analysis.

RESULTS AND DISCUSSION

Carbohydrates

Table 1 shows the values obtained for each type of storage for 5 honey samples, each set calculated to the moisture content shown for the cold-storage sample. The moisture values in parentheses are those actually found for the samples. The column under "age" gives the number of months of storage for the samples after receipt at the laboratory.

The data in Table 1 were analyzed statistically by the analysis of variance. Each set of 15 values for each sugar was examined and the variability due to sample and storage was calculated and tested statistically. The variance and F values are shown in Table 3. The differences due to storage are shown in all cases to be significant at the 1% probability level, except for the unanalyzed portion, where the change is significant at the 5% probability level.

TABLE 1

EFFECT OF STORAGE ON HONEY SUGARS[1]

No.[2]	H_2O	Fructose	Glucose	Maltose	Sucrose	Higher Sugars	Unanalyzed[4]	Age
91F	18.6	35.85	33.87	4.92	0.58	1.28	4.90	20
91H	(17.5)[3]	35.07	29.82	8.94	0.93	1.46	5.18	20
91R	(16.6)	34.85	29.44	9.22	0.89	1.45	5.55	20
258F	20.8	35.95	32.31	5.43	0.28	1.71	3.62	22
258H	(19.0)	33.95	27.88	9.59	0.85	1.67	5.26	22
258R	(19.3)	33.84	27.81	10.18	0.92	2.03	4.42	22
94F	17.4	38.22	31.29	7.54	0.73	1.23	3.59	22
94H	(16.2)	36.39	28.54	11.02	0.87	1.36	4.42	22
94R	(16.6)	36.23	28.55	10.51	0.90	1.46	4.95	22
96F	17.7	36.46	29.85	7.64	0.78	1.77	5.79	23
96H	(16.0)	34.19	25.39	13.13	0.85	1.91	6.93	23
96R	(14.2)	34.49	25.24	13.05	0.99	2.05	6.48	23
98F	18.5	37.98	31.02	6.83	0.44	1.84	3.39	23
98H	(17.0)	36.10	28.02	10.95	1.00	1.82	3.61	23
98R	(16.8)	35.73	26.71	11.47	1.16	1.93	4.50	23
Av F	18.8	36.89	31.67	6.47	0.56	1.57	4.26	
Av H		35.14	27.93	10.73	0.90	1.64	5.08	
Av R		35.03	27.55	10.89	0.97	1.78	5.18	
Change in Heated Honey		−1.75	−3.74	+4.26	+.34	+.07	−.82	
Change in Raw Honey		−1.86	−4.12	+4.42	+.41	+.21	−.92	
% Change in Raw Honey		5.0%	13.0%	68%	73%	13.4%	22.2%	

[1] Each set of values calculated to the moisture content of corresponding cold-storage sample.

[2] The letter following sample number identifies treatment as follows: F = unheated, cold storage; H = heated, room-temperature storage; R = unheated, room-temperature storage.

[3] Moisture values in parentheses are actual values found for the samples.

[4] 100—(sugars plus water).

The mean square resulting from storage conditions was further subdivided and that of frozen samples was compared with that of the two room-temperature storage conditions. The two room-temperature storage sets (heated and unheated) were also compared with each other. A sample calculation is shown in Table 2, and Table 3 gives a summary of the mean squares and the F values obtained therefrom, for each sugar.

It can be seen from the table that the differences between the frozen samples and those stored at room temperature are significant for all sugars at the 1% probability level. It is also apparent that the differences between the average values in Table 1 for the unheated and heated samples, both stored at room temperature are, in all cases not significant, except for the higher sugar values, which are significant at the 5% probability level.

It may be concluded that when raw honey is stored for two years at temperatures ranging between 23–28° C, the following changes take place in the carbohydrate composition:

1. A decrease of free glucose (averaging 13%) and a decrease of free fructose (averaging 5.5%); an average of 18.5% of the free monosaccharide content of the honey is thus lost.

2. A marked increase of "maltose" or reducing disaccharide sugars, averaging 69% of the amount initially present.

TABLE 2

GLUCOSE

ANALYSIS OF VARIANCE

Source of Variability	S.S.	D.F.	M.S.	F
Total	72.00	14		
Storage	51.79	2	25.89	100[1]
F vs RT & H	51.43	1	51.43	198[1]
RT vs H	0.36	1	0.36	1.39
Samples	28.14	4	7.03	27.2[1]
Error	2.07	8	0.26	

[1] Exceeds .01 probability level.

3. A *relatively* large increase in sucrose content.

4. A small (13%) increase in the higher sugar content of the honey, and

5. An increase averaging 22%, in the amount of unanalyzed material (100 − sugars + water).

The heat treatment given these samples—55° C (130° F) for 30 min—had no effect on these changes, with the possible effect of reducing the extent of increase of the higher sugar values. The changes in the stored samples are in the direction of increased complexity of sugars. This might be expected from a consideration of the conditions within the sample. A high sugar concentration and a considerable acidity over a period of time would promote combination of monosacharides (reversion, [13]). The presence of an active transglucosylase enzyme (22) in the honey may also result in accumulation of oligosaccharide material; the heat treatment used was not sufficient to inactivate enzymes. Possible explanations for the changes observed are as follows:

(a) Fructose. This sugar is subject to degradation to hydroxymethylfurfural by long standing in acid solution. Conversion to non-reducing fructose anhydrides is also possible. Fructose-containing oligosaccharides may result from enzyme transfer of glucose to a fructose acceptor.

(b) Glucose. Twice as much glucose disappeared as fructose. This may reflect the specificity of the enzyme transferring glucose from oligosaccharides (honey invertase, a glucoinvertase).

(c) "Maltose." This actually represents reducing disaccharide material, including maltose, isomaltose, maltulose, turanose and nigerose (21). All of these sugars are hydrolyzed by honey α-glucosidase (18). The increase in this category of sugars accounts for most of the decrease in monosaccharides.

(d) Sucrose. Post-harvest ripening has long been known to take place in unheated honey (3). Sucrose is at or reaches a low value within a few months of removal of honey from the hive, but never disappears completely, despite (or probably because of) the presence of an active invertase. The data here show a later stage of sucrose change, where the amount present increases from a low value to approach 1%. Mold

TABLE 3

SIGNIFICANCES OF CHANGES IN HONEY COMPOSITION DUE TO STORAGE

Source of Variability	D.F.	Levulose		Dextrose		Maltose	
		M.S.	F	M.S.	F	M.S.	F
Samples	4	3.18	31.8[2]	7.03	27.0[2]	5.71	22.0[2]
Storage	2	5.46	54.6[2]	25.9	99.6[2]	31.3	120[2]
F vs R & H	1	10.90	109[2]	51.4	198[1]	62.6	241[2]
R vs H	1	.03	.0	.36	1.4	.06	.2
Error	8	.10		.26		.26	

Source of Variability	D.F.	Sucrose		Higher Sugars		Unanalyzed	
		M.S.	F	M.S.	F	M.S.	F
Samples	4	0.018	0.86	0.217	36.2[2]	3.03	17.8[2]
Storage	2	.240	11.4[2]	.061	10.2[2]	1.33	7.8[1]
F vs R & H	1	.466	22.2[2]	.073	12.2[2]	2.63	15.5[2]
R vs H	1	.013	.62	.049	8.2[1]	.02	.1
Error	8	.021		.006		.17	

[1] Exceeds 5% probability level.

[2] Exceeds 1% probability level.

enzymes have been shown to synthesize sucrose by transfructosylation during their action on sucrose (7).

(e) Higher sugars. The increase in this fraction is further evidence of reversion and transglucosylation.

(f) Unanalyzed. From the point of view of the carbohydrates, this category can contain difructose anhydrides, non-reducing disaccharides (except sucrose), and kojibiose, a very weakly-reducing disaccharide (2-O-α-D-glucosyl-D-glucose) recently discovered in honey by Watanabe and Aso (16). This sugar will not be determined in the analytical procedure used, since it has but about 6% of the reducing power of glucose against copper reagents. The increase in unanalyzed material may represent an increase in the amount of kojibiose (and possibly trehalose) in honey. Both of these compounds have been isolated from hydrol, where it is believed that they arose by reversion from glucose (15).

It is of interest to examine an analysis of a 36-year-old sample of honey and compare it with a corresponding contemporary sample.

Table 4 shows such a comparison. The 1923 sample [b] is an alsike clover-white clover honey produced at Delphos, Ohio; it had never been opened and was stored in a dark cupboard and was liquid except for a few coarse crystals at the bottom. Also shown in the table is a similar sample from the 1957 crop. It is alsike and white clover, produced at Columbia City, Indiana, by T. A. Ott. Data were calculated to the same moisture content to facilitate comparison. The differences shown in the table are all similar to those in Table 1 in trend, except that the 1957 sucrose value is higher, though the value for the aged sample (equilibrium?) is close to the average of the 2-year-old samples. In general the changes in monosaccharide and "maltose" shown occurring after 26 years of storage are similar, but larger than those found for the two-year-old samples in Table 1.

[b] Donated by C. A. Reese, Department of Entomology, Ohio State University.

TABLE 4

EFFECT OF AGE ON A CLOVER HONEY

	1957 Crop	1923 Crop	Difference	
				% of 1957
Moisture	18.2	(18.2)[1]	.....	.....
Fructose	38.25	35.05	−3.20	−8.3
Glucose	33.58	23.12	−10.29	−30.6
Maltose	5.50	16.41	+10.91	+198
Sucrose	1.68	1.04	−.64	−38.2
Higher Sugars	0.82	2.06	+1.24	+151
Undetermined	2.0	4.1	+2.1	+105

[1] Moisture content of the 1923 sample was 17.6%; data are calculated to the 18.2% shown by the 1957 sample to facilitate comparison.

Analyses of honey samples after extended storage have been previously reported by de Boer (5) and Auerbach and Bodlander (1). The analytical methods de Boer used would not detect the differences in carbohydrate composition shown here. He did not confirm the earlier conclusion of Auerbach and Bodlander that the ratio of fructose to glucose increased after storage of honey. Auerbach and Bodlander reported the analysis of 13 fourteen-year-old honey samples. Their fructose-glucose ratio ranged from 1.19 to 1.81, averaging 1.40; 10 fresh honey samples ranged from 1.06 to 1.19, averaging 1.11. These values have only relative meaning, since the analytical methods used gave no differentiation between monosaccharide and disaccharide.

The results in Tables 1 and 4 substantiate the views of Auerbach and Bodlander that the amount of free glucose decreases on storage and that the ratio of fructose to glucose increases. They ascribed this to possible enzymic condensation of glucose, which we also believe contributes.

The changes described in the sugar distribution of honey have some practical implications. With the tendency toward increasing complexity, there may be a corresponding loss of nutritive value; some of the disaccharides and higher sugars may not be digestible.

The considerable decrease in glucose content is probably responsible for the gradual liquefaction that is often noted in granulated honey samples as they stand in storage, which was mentioned by de Boer. If the glucose content of a granulated honey is near the lower limit of granulation, the

TABLE 5

EFFECT OF STORAGE ON ACIDITY OF HONEY

Sample	Free Acid		Lactone		Total Acidity	
	F [1]	R [2]	F	R	F	R
	meq/kg	*meq/kg*	*meq/kg*	*meq/kg*	*meq/kg*	*meq/kg*
91	24.04	27.07	9.87	12.39	33.92	39.46
258	20.56	24.06	6.45	7.73	27.00	31.80
92	19.85	21.66	4.90	5.32	24.35	26.98
94	15.04	15.78	2.55	2.62	17.59	18.40
96	22.28	23.90	6.17	9.21	28.45	33.11
107	23.73	24.88	2.20	2.18	25.93	27.04
97	20.82	20.13	7.00	8.08	27.82	28.21
108	22.88	24.29	1.90	4.21	24.78	28.46
109	25.24	26.45	5.83	7.68	31.05	34.13
98	25.62	26.63	8.33	10.39	33.85	37.02

[1] F = Stored at −20° C; samples 91–96 and 258, 21 months, others 24 months.

[2] R = Stored at room temperature same times as above.

changes in a year or so will reduce the glucose well below the saturation point so that the crystals will slowly dissolve.

ACIDS

Table 5 shows the free acidity, lactone content and total acidity of ten samples stored under the conditions described above. None of the samples showed visible evidence of fermentation. In Table 6 is given the analysis of variance for the free acidity, lactone and total acidity values. The average changes in each of these categories are seen to be highly significant. It has been proposed (4, 19, 24) that an enzyme producing acidity occurs in honey. If this is the case, honey samples showing high diastase number might be expected to show a correspondingly high rate of acid production. Table 7 shows these values for 10 honey samples. Also shown in the table is an analysis of variance for regression. The F value obtained, 11.5, shows a highly significant regression between the two sets of values. This is not meant to imply that the amylase enzyme system is responsible for acid production, but rather that the factors affecting amylase activity also influence the activity of the acid-producing enzyme.

DIASTASE

The amylase content of honey has long been used by Europeans as a measure of the heat treatment to which a honey has been exposed. The voluminous literature will not be reviewed here (10). Recently (6, 8) it has been proposed that diastase content alone is not a suitable criterion for the detection of overheated honey.

There appears to be relatively little information in the literature on the effect of storage of honey on its diastase content. De Boer (5), using the Gothe procedure, reported that diastase decreased gradually with age of honey, about 3 Gothe "steps" in 10 years. Schade, Marsh and Eckert (14),

TABLE 6

ANALYSIS OF VARIANCE

Source of Variability	D.F.	Free Acidity				Lactone				Total Acidity			
		S.S.	M.S.	F	S	S.S.	M.S.	F	S	S.S.	M.S.	F	S
Total	19	207.6				175.0				582.6			
Materials	9	190.6	21.17	31.0 [1]		159.2	17.69	31.1 [1]		523.9	58.2	35.9 [1]	
Storage	1	10.9	10.93	16.0 [1]		10.7	10.68	18.8 [1]		44.1	44.1	27.2 [1]	
Error	9	6.14	0.68		0.83	5.11	0.57		0.75	14.6	1.6		1.27

[1] Exceeds .01 probability level.

TABLE 7

REGRESSION OF ACID PRODUCTION BY HONEY ON DIASTASE NUMBER

Sample	Diastase No	Change in Total Acidity per Year
91	38.0	3.16
258	35.3	2.74
92	33.3	1.50
94	19.1	0.46
96	27.8	2.66
107	18.5	0.59
97	8.0	0.18
108	20.0	1.84
109	10.7	1.59
98	21.7	1.58

Analysis of Variance for Regression

Source	S.S.	D.F.	M.S.	F
Total	927.28	9		
Linear Regression	546.99	1	547	11.5 [1]
Deviations	380.29	8	47.5	

[1] Significant at .01 probability level.

using their improved procedure, reported diastase values for honey samples before and after storage for 13 to 15 months at 20° C. They reported that the diastase activity had "decreased slightly but not significantly in most cases." We have subjected their data (the 7 samples in their Table 3) to the analysis of variance, and the changes were found significant at the 1% probability level ($F = 11.7$). Their data showed an average decrease for 7 samples of 10.1% in diastase number for the approximately 14 months storage at 20° C, or 0.72% per month.

We have determined diastase number for aliquots of 20 samples of honey after storage times of 4 to 21 months at −20° C and also at laboratory room temperature. Samples were from the 1956 and 1957 crops and were frozen on receipt at the laboratory at varying times (½ to 14 months) after their extraction. Full data on samples will be included in a later publication.

The data given in Table 8 show the effect on diastase number of room temperature, dark storage for varying times, based on the reasonable assumption that no change takes place on samples stored at −20° C. This table shows an average loss of diastase value for honey stored unheated at temperatures ranging from about 23° to 28° C of 2.95% per month, which is equivalent to a half-life of 17 months.

TABLE 8

EFFECT OF STORAGE ON DIASTASE CONTENT OF HONEY

No.	Storage Time	Diastase Values		Loss	% Loss	Loss per Month
		Frozen	Room Temp			
234	21 mo.	61.2	30.9	30.3	49.5	2.36
430	20	32.6	18.6	14.0	42.9	2.16
361	20	14.6	8.11	6.5	44.5	2.23
326	19	17.6	7.23	10.4	59.1	3.11
238	17	10.6	7.59	3.01	28.3	1.66
403	13	6.74	3.97	2.77	41.1	3.16
91	13	38.0	21.8	16.2	42.6	3.28
258	13	35.3	20.8	14.5	41.1	3.16
92	13	33.3	19.0	14.3	42.9	3.30
94	13	19.1	12.9	6.2	32.5	2.50
96	13	27.8	18.4	9.4	33.8	2.60
97	13	8.00	4.42	3.58	44.7	3.44
98	13	21.7	15.8	5.9	27.2	2.09
261	13	10.3	8.40	1.90	18.4	1.41
142	13	22.4	13.2	9.2	41.1	3.16
104	9	10.8	8.15	2.65	24.5	2.72
121	8	22.6	15.9	6.7	29.6	3.70
179	8	16.7	11.4	5.3	31.7	3.96
333	8	15.2	9.38	5.8	38.1	4.76
214	4	15.2	12.8	2.4	15.8	3.95
Av	13.2	22.0	13.4		38.9	2.95

This may be compared to the 0.72% per month shown by the data of Schade *et al* (14) for a temperature probably 5–6° C lower. This at once shows the importance of low-temperature storage for honey in which diastase content must be maintained. Our data show a considerable variation in the rate of loss of diastase among the honey samples. Kiermeier and Koberlein (8) reported that the heat-sensitivity of honey diastase is related to the pH of the sample; Schade *et al* (14) are in agreement. We made an effort to relate several compositional factors to the rate of loss of diastase in storage but, as shown in Table 9, no relationship was obtained for ash, total acidity, hydrogen ion concentration, original diastase value, and moisture content. An analysis of variance for regression on the values for diastase loss vs original diastase number,

TABLE 9

CORRELATION OF DIASTASE LOSS RATE WITH OTHER FACTORS

Factor	F Value [1]
Time of Storage	12.4 [4]
Original Diastase No	2.7 [2]
Moisture Content	0.1
Total Acidity	0.5
Hydrogen Ion Conc	0.07
Ash	1.9
Total Age	7.6 [3]

[1] Calculated by analysis of variance for regression.
[2] Significant at .10 level.
[3] Exceeds .05 probability level.
[4] Exceeds .01 probability level.

for example, gave an F value of 2.66, significant at the 10% probability level. However, rate of loss was found to be correlated with storage time, with the rate for samples stored short times being significantly greater than the over-all rate for samples with longer storage periods. Analysis of variance of these data yields an F value for linear regression of 12.4, significant at the 1% probability level. A less significant relation was found between total age and rate of diastase loss. This does not provide information on the composition factors controlling rate of loss.

It may be seen from these data and also those of Schade and co-workers that storage temperature is a most important factor affecting retention of diastase in honey. Many studies relating diastase loss with degree of heating have been reported (6, 8, 10) investigating the thesis that diastatic activity is an indication of heating of honey. De Boer in his study of aging of honey did note that in general the changes occurring as honey ages are the same as those brought about by heating; he had particular reference to increase in hydroxymethylfurfural content. We have now, for the first time, evidence that over a storage period of 12–18 months, without heating, a honey may lose enough diastase to fall below the minimum values required for European acceptance as table honey.

With export practices, including storage by the producer, packer, shipper, time in transit through the Panama Canal to Europe, plus a possible 6 months in free-port storage before sampling and testing by the importer, a total elapsed time of 12–18 months from extraction to the European consumer may occur (17). Any storage temperature exceeding the average 26° C (79° F) in this study will appreciably increase diastase destruction beyond that found here. A few weeks or months of warehouse storage at 85 or 90° F or higher is commonly encountered in southwestern honey handling.

Although further studies of the effect of these relatively low temperatures on stability of honey are planned, the following practices for honey intended for export to Europe may be suggested:

1. Expeditious handling to shorten time from extractor to European customer.

2. Prompt removal from storage areas where honey is exposed to heat.

3. If long-term storage is necessary, it should be in cool or cold storage.

4. Sampling by importers for testing should be at arrival instead of after prolonged warehouse storage.

Complete analytical data on all of the honey samples used in this work will be included in a forthcoming publication on the composition of American honey.

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LITERATURE CITED

1. Auerbach, F. and E. Bodlander. 1924. Über ein neues Verfahren zur Unterscheidung von Honig und Kunsthonig. *Z. Nahr. Genussm.*, **47**, 233.
2. Austin, G. H. 1956. Maltose content of Canadian honeys and its probable effects on crystallization. *Proc. X Int. Cong. Entomol.*, Montreal, **4**, 1001.
3. Browne, C. A. 1908. Chemical analysis and composition of American honeys. Bull. 110, Bur. Chem., U. S. Dept. Agr.; Kardos, R. F. 1938. Der Rohrzuckergehalt im naturlichen Honig. *Z. Untersuch. Lebensm.*, **76**, 354.
4. Cocker, L. 1951. The enzymic production of acid in honey. *J. Sci. Food Agr.*, 2, 411.
5. de Boer, H. W. 1934. De invloed van den ouderdom op de samenstelling van honig. *Chem. Weekblad*, **31**, 482.
6. Duisberg, H. and H. Gebelein. 1958. Über die Kontrolle von Erhitzungsschaden bei Honigen. *Z. Lebensm.-Untersuch. u.-Forsch.*, **107**, 489.
7. Edelman, J. 1954. Transfer reactions catalyzed by some sucrase preparations. *Biochem. J.*, **57**, 22.
8. Kiermeier, F., and W. Köberlein. 1954. Über die Hitzeinaktivierung von Enzymen in Honig. *Z. Lebensm.-Untersuch. u-Forsch.*, **98**, 329.
9. Lochhead, A. G. 1933. Factors concerned with the fermentation of honey. *Zentr. Bakt., Parasitenk.* 2d Abt., **88**, 296; Stephen, W. A. 1946. The relationship of moisture content and yeast count in honey fermentation. *Sci. Agr.*, **26**, 258.
10. Lothrop, R. E., and H. S. Paine. 1931. Diastatic activity of some American honeys. *Ind. Eng. Chem.*, **23**, 71; Lampitt, L. H., E. B. Hughes, and H. S. Rooke. 1929. Furfural and diastase in heated honey. *The Analyst*, **54**, 381; 1930. The diastatic activity of honey. *The Analyst*. **55**, 666; Vansell, G. H. 1929. Diastase in honey. *J. Econ. Entomol.*, **22**, 926; de Boer, H. W. 1931. The behavior of diastatic ferments in honey when heated. *Bee World*, 12 (2), 13; Bartels, W. and A. Fauth. 1933. Beobachtungen bei der Untersuchung californischer Honige. *Z. Untersuch. Lebensm.*, **66**, 396.
11. *Official Methods of Analysis.* Association of Official Agricultural Chemists. 8th ed., 1955. Washington, D.C.
12. Milum, V. G. 1948. Some factors affecting the color of honey. *J. Econ. Entomol.*, **41**, 495.
13. Pigman, W. W. and R. M. Goepp, Jr. 1948. *Chemistry of the Carbohydrates.* Academic Press, New York, pp. 434, 515, 605.
14. Schade, J., G. E. Marsh and J. E. Eckert. 1958. Diastase activity and hydroxymethyl-furfural in honey and their usefulness in detecting heat alteration. *Food Research*, **23**, 446.
15. Thompson, A., K. Anno, M. L. Wolfrom and M. Inatome. 1954. Acid reversion products from D-glucose. *J. Am. Chem. Soc.*, **76**, 1309; Sato, A. and K. Aso. 1957. Kojibiose (2-O-α-D-glucopyranosyl-D-glucose): isolation and structure. *Nature*, **180**, 984.
16. Watanabe, T. and K. Aso. 1959. Isolation of kojibiose from honey. *Nature*, **183**, 1740.
17. Webster, Marvin, Agricultural Marketing Service, USDA. (Private communication).
18. White, J. W., Jr. (Unpublished data).
19. White, J. W., Jr. Enzymic production of acid in honey. Paper presented at first annual meeting of American Committee, Bee Research Association, Tampa, Fla., Jan. 1959.
20. White, J. W., Jr. 1959. Report on the analysis of honey. *J. Assoc. Offic. Agr. Chemists*, **42**, 341.
21. White, J. W., Jr., and N. Hoban. 1959. Composition of honey. IV. Identification of the disaccharides. *Arch. Biochem. Biophys.*, **80**, 386.
22. White, J. W., Jr., and J. Maher. 1953. Transglucosidation by honey invertase. *Arch. Biochem. Biophys.*, **42**, 360.
23. White, J. W., Jr., and J. Maher. 1954. Selective absorption method for determination of the sugars of honey. *J. Assoc. Offic. Agr. Chemists*, **37**, 466.
24. White, J. W., Jr., J. Petty, and R. B. Hager. 1958. The composition of honey. II. Lactone content. *J. Assoc. Offic. Agr. Chemists*, **41**, 194.

Antimicrobial Agent of Aged Surface Ripened Cheese. I. Isolation and Assay[a,b]

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SUMMARY

Two types of soft surface ripened cheese become inhibitory to microbial growth after storage at 2 to 4° C for 8 weeks. The antimicrobial agent has been concentrated 4- to 16-fold by aqueous extraction and lyophilization or drying by a current of air. The total antimicrobial activity of aged surface ripened cheese of the described types was estimated to be at least 13.6 antimicrobial units per gram of product.

The active principle inhibited growth and toxin production of type A *Clostridium botulinum* 62A, the growth of *Staphylococcus aureus* 223 and *Bacillus cereus* 800/58. Threshold subinhibitory amounts of the antimicrobial concentrates stimulated growth of these organisms.

Inhibition of *Clostridium botulinum* and molds in aged surface ripened cheese has been reported from this laboratory. Two types of surface ripened cheese became inhibitory after storage at 2° to 4° C for 8 weeks or longer, whereas prior to 8 weeks both types of cheese supported growth of *C. botulinum*. Inhibition of *C. botulinum* was estimated from the presence or absence of toxin in experimental cheese preparations which were inoculated with toxin-free spores and subsequently incubated anaerobically at 30° C. Inhibition of molds was studied by observing surface growth on cheese slices (4).

The cheese samples as used in these experiments were not suited for further investigation of the properties and identity of the antimicrobial principle of aged surface ripened cheese. This investigation was concerned with the preparation and characterization of crude concentrates from aged type I and type III cheese.[c]

MATERIALS AND METHODS

Extractions

Surface ripened cheese, type I, was obtained from the manufacturer as composite lots in polyethylene bags encased in fiberboard drums. The age of the cheese on the day of arrival at this laboratory was between 23 and 30 days. The bulk lot of type I cheese was stored at 2° to 4° C after arrival at this laboratory.

Type III surface ripened cheese was purchased from the store and subsequently kept at 2° to 4° C for the desired time period. The manufacturing date of type III cheese was stamped on the package.

The inhibitor of *C. botulinum* was isolated from thoroughly aged type I or type III surface ripened cheese by aqueous extraction. Equal parts (w/v) of aged cheese and distilled water were mixed to form a homogeneous slurry. If necessary, cakes of type III cheese were ground in a food chopper to obtain a more homogeneous mixture. The aqueous

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[b] Data reported in this paper are part of a thesis submitted by N. Grecz to the Biology Department of the Illinois Institute of Technology in partial fulfillment of the requirements for the Ph.D. degree. This project constituted a cooperative program between the Biology Department of Illinois Institute of Technology and the Food Research Institute of the University of Chicago.

[c] To avoid use of brand names, surface ripened cheese was classified types I, II, and III. The flora of type I is made up of bacteria and yeasts; II is made up of molds; and III is ripened primarily by bacteria. Type I is a soft rennet cheese, whereas type III is a semihard rennet cheese. Type I and type III develop a brown surface smear during prolonged storage.

suspension was allowed to interact overnight and was filtered by gravity. All operations were carried out at 2° to 4° C. The liquid was passed through a series of filters of decreasing porosity until a clear extract was obtained which could be sterilized by passage through a sterilizing filter sheet.[d]

At this stage the extract contained 1.8 to 2.3% sodium chloride. To avoid possible interference with the bioassay the salt was removed by dialysis to less than 0.2%. This required approximately 2 hr. The preparations were adjusted to pH 7.0 to 7.2, Seitz filtered and incorporated into the bioassay medium. Type I surface ripened cheese was used unless otherwise stated.

Cultures

The growth inhibitory activity of aqueous extracts of aged surface ripened cheese was studied on 3 different organisms: (a) *Clostridium botulinum*, (b) *Staphylococcus aureus*, and (c) *Bacillus cereus*.

Type A *Clostridium botulinum* strain 62A was obtained from K. F. Meyer, Hooper Foundation, University of California. The stock culture was transferred semiannually in Rosenow's Brain Medium, incubated for 10 days at 30° C and stored at 4° C. Spore suspensions of *C. botulinum* were produced in 5% trypticase broth at 30° C in anaerobic jars as described by Wagenaar and Dack (9). The spores were harvested by centrifugation and repeated washing with M/15 phosphate buffer (pH 7) and heat shocked at 85° C for 10 min. Counts of spores were made by seeding beef infusion agar with appropriate dilutions of the stock spore suspension in oval tubes. This agar was stratified with approximately 2 cm of 2% agar containing 0.2% sodium thioglycollate to create anaerobiosis.

Staphylococcus aureus 223, a food poisoning strain, was obtained from Miss R. David of the Food Research Institute of the University of Chicago.

Bacillus cereus 800/58 was received from Dr. S. Hauge of the Norges Veterinaerhogskole of Oslo, Norway, who isolated it from vanilla sauce which was believed to have caused an outbreak of food poisoning in a hospital in a Norwegian town (Hauge, personal communication). This strain was biochemically similar to the aerobic spore formers isolated by Hauge from an earlier food poisoning outbreak (6), except that it hydrolyzed starch rather slowly.

Assay Media and Procedures

The basic medium for testing the inhibitory activity of the cheese extracts was that of Yesair as modified by Sugiyama (8). This is commonly used for counting spores of *C. botulinum* (1). Its basic composition is beef infusion, 1000 ml; peptone, 5 g; tryptone, 1.6 g; glucose, 1.0 g; soluble starch, 1.0 g; sodium thioglycollate, 2.0 g; agar, 16 g; sufficient 1 N sodium hydroxide to adjust pH to 7.2 to 7.4. In our initial experiments this medium was modified by replacing the beef infusion broth with 0.5% of Difco bacto-beef extract paste as recommended by the Difco Laboratories, Inc. (3). The liquid portion was supplied by cheese extract samples which were assayed for growth inhibitory activity. In the case of controls, the liquid portion was supplied by distilled water. The cheese extract was sterilized by Seitz-filtration, placed into oval tubes, equilibrated at 55° to 60° C and inoculated with approximately 50 or 100 type A *C. botulinum* spores. The volume of the inoculum was kept below 0.5 ml to avoid excessive dilution. Sufficient melted 8% agar was added to give a final agar concentration of 0.8%. This medium was designated as medium A. All operations were carried out in the water bath to prevent solidification of the agar during handling.

In later experiments the bioassay of the inhibitor was simplified by lyophilization of the clear filtrates of cheese extract. The dehydrated preparations could be dissolved in sterile beef infusion broth and added to an equal quantity of melted beef infusion agar containing 3.2% agar. The medium was inoculated with type A *C. botulinum* spores, allowed to solidify and then stratified with a 1-cm layer of petrolatum to create anaerobiosis and prevent drying of the agar during incubation. Colonies were counted after 12 to 18 hr of incubation at 30° C. Counts were repeated at regular intervals for 36 to 48 hr. Tubes showing no colony formation were observed for as long as 9 months.

S. aureus was tested for growth on tryptone glucose extract agar slants containing sterile lyophilized preparations of aqueous cheese extracts. Serial two-fold dilutions of the extracts were made in melted agar which was slanted and allowed to solidify. The surface was streaked with suitable suspensions of a young culture of *S. aureus*. Following incubation at 30° C growth was estimated by visual inspection.

B. cereus was tested by the agar plate method (5) using either filter paper impregnated with the inhibitor or stainless-steel cylinders filled with inhibitor filtrates that were placed on the surface of the seeded agar. The size of the clear zone of growth inhibition around the paper or cylinders indicated antimicrobial activity.

Analytical Methods

The methods employed for pH determination,

[d] Sterilizing filter sheets, L-6, Hercules Filter Corp., Hawthorne, N. J.

NaCl analyses, and the assay of *C. botulinum* toxin were described earlier (4).

RESULTS

Aqueous extracts of aged type I cheese contained 5.3 to 6.4% of solid material as compared with 45 to 48% solids in the initial cheese. Furthermore, the initial cheese contained 3.5 to 4.5% of sodium chloride whereas the extract contained 1.8 to 2.3%. The initial pH varied between 5.6 and 6.0. No carbohydrate could be detected by the Benedict test.

Some of the differences between aqueous extracts from fresh and aged type I cheese are given in Table 1. It may be observed

TABLE 1

SOLIDS IN AQUEOUS EXTRACTS OF FRESH AND OF AGED TYPE I CHEESE

Aqueous extract of	Solids in aqueous extract		
		Solubility in 42.5% ethanol	
	Total	Soluble	Insoluble
	%	%	%
Fresh cheese (1 week)	18.40	0.77	17.63
Aged cheese (34 months)	5.83	4.95	0.88

that 18.40% of the total solids were extractable from one-week-old fresh cheese, whereas only 5.83% total solids were extracted from aged cheese. Furthermore, the percentage of the extracted solids which are soluble in 42.5% ethanol are much greater for those of aged cheese.

The aqueous extract from fresh cheese could not be easily clarified by filtration. The filtrates were always somewhat milky in appearance and the solids precipitated occasionally on standing (probably due to residual rennin activity).

BIOLOGICAL ACTIVITY

The addition of extracts of aged type I and type III cheese to the assay medium inhibited the growth of type A *C. botulinum*. Similar preparations of fresh cheese had no inhibitory effect (see Table 2). The inhibitory effect of the extracts on *C. botulinum* was two-fold: (a) the number of colonies from a given inoculum was reduced and (b) the emergence of colonies was delayed. However, once a visible colony appeared, it developed at a rate commensurate with that of the control colonies. Threshold amounts of extract added to the medium gave rise to considerably larger colonies of *C. botulinum* than those in control tubes containing no added cheese extract. Toxin production as opposed to growth was not inhibited; on the contrary, a 4- to 20-fold higher titer was present in tubes to which cheese extract was added (see Table 3). The odor of the cul-

TABLE 2

INHIBITION OF *Clostridium botulinum* BY AQUEOUS EXTRACTS OF AGED TYPE I AND TYPE III CHEESE

Sample added to Medium A	Colony count of *C. botulinum*			
	12 hr	24 hr	36 hr	48 hr
I. Extract of type I cheese				
(a) aged cheese,				
full strength	0	17	20	36
1:2 dilution	17	66	70	74
1:10 dilution	21	75	89	97
water (control)	0	56	81	85
(b) fresh cheese	17	79	....[a]	....[a]
water (control)	17	84	100	100
	24 hr	48 hr	72 hr	96 hr
II. Extract of type III cheese				
(a) aged cheese,				
full strength	0	0	0	1
1:2 dilution	13	55	56	60
water (control)	0	69	69	....
(b) fresh cheese	0	....[a]	....[a]	....[a]

[a] No counts could be made because of liquefaction, general turbidity and gas formation. Type A *C. botulinum* toxin was present as demonstrated by intraperitoneal injections into white mice. Activity of *C. botulinum* was further evident from the drop in pH and specific rancid odor in these tubes.

TABLE 3

EFFECT OF CHEESE EXTRACT ON GROWTH AND TOXIN PRODUCTION OF *Clostridium botulinum*

Medium	Colony count	Time of emergence of visible colonies	Toxin titer [a]
		(hr)	*(m.l.d./ml)*
Beef infusion agar	57	12 to 48	5×10^5
Medium A made up with water	51	36 to 72	1×10^5
Medium A made up with cheese extract [b]	9	48 to 168	20×10^5

[a] Toxin titer was determined after 1 week of incubation at 30° C.

[b] Aqueous extract of aged type I cheese.

TABLE 4

INHIBITION OF *Clostridium botulinum* BY GRADED AMOUNTS OF LYOPHILIZED EXTRACT OF AGED TYPE I CHEESE

Dehydrated powder added to assay medium [a]	Increase over conc. of solids in original cheese extract	Colony count of *C. botulinum* 12 hr	18 hr	24 hr	36 hr	48 hr
136 mg/ml	4 fold	0	0	0	0	0
68 mg/ml	2 fold	0	0	25	52	52
34 mg/ml	1 fold	0	34	69	71	71
None (control)	.....	84	131	194	194	194

[a] Assay medium was beef infusion agar (see text). Cheese extract was dialyzed for 2 hr prior to lyophilization in order to reduce NaCl from an initial 1.9 to a final 0.1%.

tures of *C. botulinum* grown in presence of cheese extract was considerably less putrid than the odor of control beef infusion cultures (both in Table 3).

Lyophilization of the aqueous extracts of aged cheese resulted in a buff colored powder which readily dissolved in aqueous solutions. This powder could be added to the assay medium in 2- and 4-fold concentration of that of the initial extract. A protocol employing 2- and 4-fold concentrations of lyophilized preparations of aged type I cheese is shown in Table 4.

Antimicrobial activity could not be related to the solid content of an aqueous extract. The solid content of extracts varied depending on the order of a repeated extraction or the age of the initial cheese (see Table 1 and Figure 1).

Repeated extraction of aged type I cheese by resuspending the cheese in distilled water and filtration resulted in elution of the inhibitory principle(s) in each successive filtrate up to the 6th extraction (see Table 5). The solid content of each successive extract gradually decreased whereas antibiotic activity remained essentially constant; this resulted in an enrichment of the antimicrobial principle(s) per unit weight of the dehydrated preparation (see Figure 1).

TABLE 5

ANTIMICROBIAL ACTIVITY OF LYOPHILIZED PREPARATIONS OBTAINED BY REPEATED AQUEOUS EXTRACTION OF AGED TYPE I CHEESE

Extraction [a]	Bioassay of inhibitory activity [b] Colony counts of *C. botulinum* 12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
1st	0	0	2	7	8	8
2nd	0	2	9	11	12	12
3rd	0	0	6	9	10	10
4th	0	1	7	10	10	10
5th	0	2	7	10	11	12
6th	0	2	8	10	10	11
Beef infusion agar (control)	5	34	40	40	....	....

[a] Successive extracts were obtained by repeated suspension of aged type I cheese in an equal amount of distilled water (w/v) and filtration by gravity at 2° to 4° C.

[b] Bioassay was done by incorporation into beef infusion agar of lyophilized preparations in 2-fold concentration as related to the initial level in the liquid extract. Heat-shocked spores of type A *Clostridium botulinum* culture 62A were used as the assay organism.

THE AMOUNT OF ANTIMICROBIAL SUBSTANCE IN AGED TYPE I CHEESE

Aged type I cheese contained at least 18.6 inhibitory units per gram of the original product.

CALCULATION

One inhibitory unit was defined as the smallest amount of antimicrobial agent which completely prevented growth of *S. aureus* 223. Ten mg/ml of the lyophilized 2nd extract in Figure 1 was necessary to inhibit *S. aureus* 223. Aged cheese contained 31 mg of extractable material (2nd extract) per gram of the original product, i.e., 3.1 inhibitory units. Since all 6 extracts in Figure 1 possessed approximately the same antibiotic activity as the second extract it may be concluded that the original type I cheese contained at least 18.6 inhibitory units per gram of cheese.

DEHYDRATED PREPARATIONS

Lyophilization of cheese extracts was com-

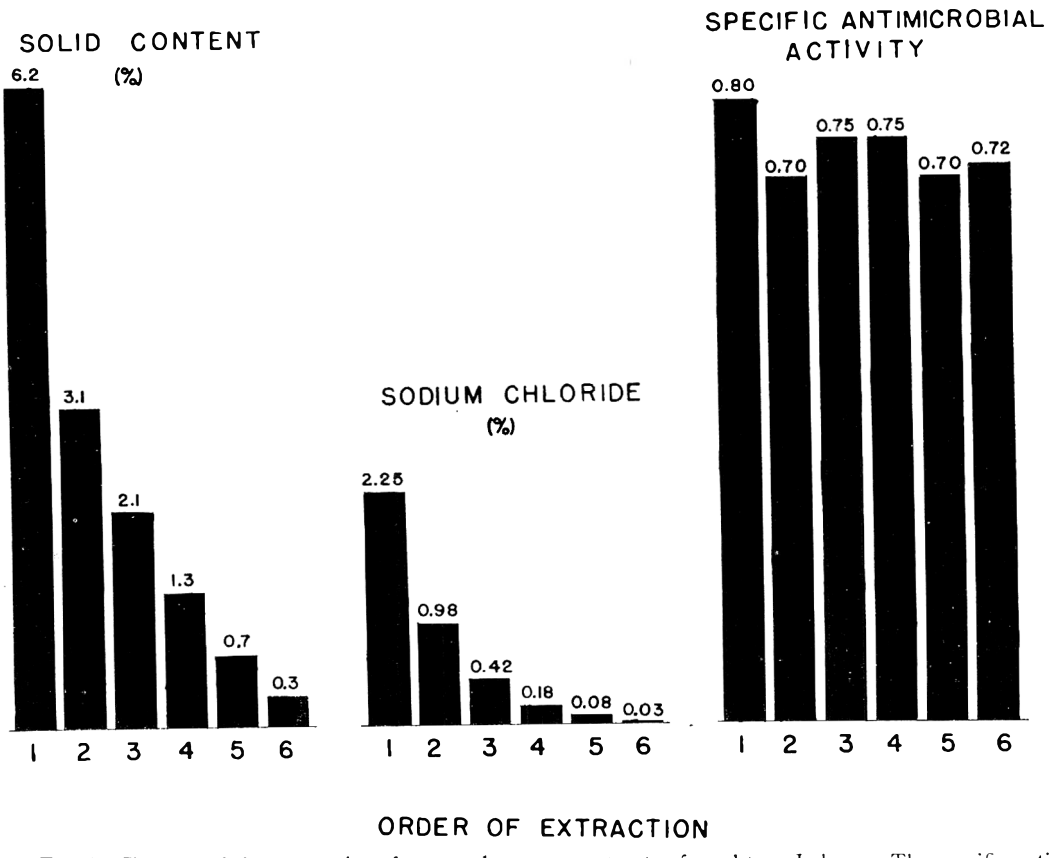

FIG. 1. Changes of the properties of repeated aqueous extracts of aged type I cheese. The specific antimicrobial activity $\alpha = \frac{\infty}{\beta}$, where ∞ is the number of colonies in the assay tube, β is the number of colonies in the control tube. Tubes were incubated at 30° C for 72 hours.

plicated by frequent melting of the samples during the lyophilization process. Some samples could not be lyophilized because of repeated failures to keep them frozen. As an alternate procedure, drying with a current of filtered air appeared not to reduce the antimicrobial potency of a sample which was dried and re-dissolved repeatedly for 3 consecutive times. Drying by air current was not complete and, therefore, it was finished *in vacuo* over calcium chloride.

THE AGAR SLANT DOUBLE DILUTION ASSAY

The inhibition of *S. aureus* strain 223 by air-dried preparations of aged type I cheese was tested by the agar slant double dilution method (see Fig 2). The growth of *S. aureus* 223 was inhibited by 25 mg/ml of the preparation. No isolated resistant colonies appeared on the inhibitory slants in contrast to controlled penicillin- and streptomycin-containing slants (controls are not shown in Fig. 2). The growth in the two tubes containing threshold amounts of cheese appeared to be stimulated as compared with higher dilutions of the antimicrobial preparations and with the control.

INHIBITION OF B. cereus 800/58 BY ANTIBIOTIC-IMPREGNATED FILTER PAPER

When cheese suspensions were filtered, liquid moved to the upper edge of the filter paper which was lining a conic funnel. As the liquid evaporated the upper rim gradually became somewhat brown in color. When the brown rim was cut off, dried, and placed on the surface of agar seeded with spores of *B. cereus* 800/58, a clear zone of growth inhibition appeared around the paper impregnated with aged cheese after a suitable

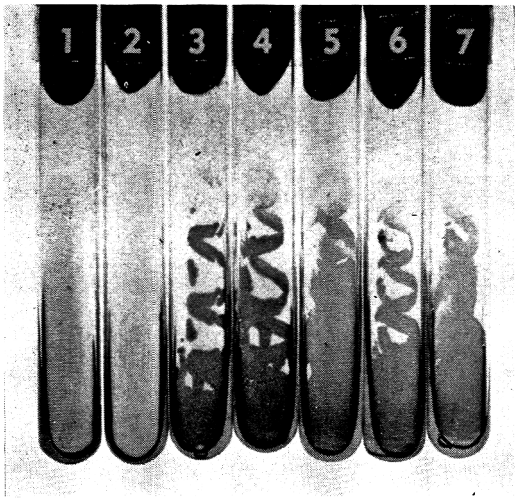

FIG. 2. Inhibition of *Staphylococcus aureus* strain 223 by the extract of aged type I cheese as tested by the agar slant method. The first tube contained 50 mg/ml of an air-dried preparation, and each of 5 subsequent tubes contained half of the amount of extract represented in the preceding tube. The 7th tube is the control containing no extract.

period of incubation. No such zone was found in similar preparations from fresh cheese (see Fig. 3). Dry filter paper segments impregnated with the substance from aged type I cheese which were stored in a screw-cap tube at 30° C retained antimicrobial activity when tested 12 months later.

DISCUSSION

The method of extraction described in the present paper was satisfactory for well ripened cheese showing a high degree of proteolysis, but was of limited value with fresh type I and type III cheese and some other types of cheese which do not undergo extensive protein breakdown during natural ripening. Extracts of these cheeses were milky suspensions which did not readily pass through filter paper. Only small quantities of filtrate were obtained after filtration in the refrigerator for 5 to 7 days.

The data obtained with antimicrobial concentrates are in general agreement with earlier studies in this laboratory (4), although it appears that the total antimicrobial potency of aged cheese is greater than was originally realized. The fact that aged cheese could be extracted repeatedly and that antimicrobial activity was present in at least 6 successive extractions from a sample of aged type I cheese, suggests that the bulk of the antimicrobial substance may have been adsorbed on some other substance in the cheese and gradually released during repeated extraction. This view was further supported by two additional observations. (a) During purification, e.g., by acetone extraction, the residue and the extract both possessed a considerably higher activity than the original cheese (unpublished results). (b) The upper rim of the filter paper through which cheese suspensions were filtered possessed a higher antimicrobial activity than filter paper discs loaded with comparable quantities of liquid extracts. This may indicate that increased activity in the filter paper rim was due to a higher degree of purification, i.e., by separation from the impurities during capillary movement on filter paper.

Similar reduction of the antimicrobial activity of some antibiotics by foreign substances has been observed by several investigators. The following compounds were

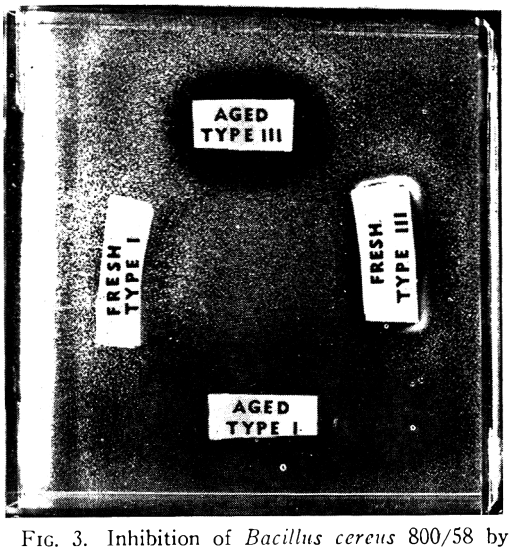

FIG. 3. Inhibition of *Bacillus cereus* 800/58 by filter paper impregnated with the antimicrobial substance of aged type I and type III cheese. The pieces of paper were cut from the upper rim of the filter paper funnel through which the cheese suspensions were filtered. The paper impregnated with extract from aged cheese was folded twice, those from fresh cheese four-fold. A noticeable zone of growth stimulation follows the clear zone of growth inhibition.

reported to reduce the activity of some antibiotics by 5 to 50%; whole blood, blood serum, some culture media, impurities in the fermentation broth, albumin, NaCl, glucose and other sugars, unsaturated fatty acids, Tween 80, and cysteine. It was proposed that antimicrobial activity was reduced because of partial reversible binding by the antagonistic substance (7).

The assay method using impregnated filter paper discs on the surface of seeded agar plates was not always satisfactory. The failure to give clear zones of inhibition around impregnated discs may have been due to: (a) interference by contaminating substances in the crude concentrates, (b) slow diffusion of the antimicrobial principle through the assay agar and (c) high adsorption affinity of paper for the antimicrobial principle. To obtain consistently clear zones of inhibition a device was developed which permitted quantitative loading of large amounts of cheese extract on small filter paper discs. This work is presently in progress and will be reported elsewhere.

Possible reduction of antimicrobial activity by diffusion should be considered when *C. botulinum* is used as assay organism. In this case the agar in the assay tube is commonly overlayed with either neutral agar or some other material to create anaerobiosis. In early experiments it was observed that the antibiotic substance diffused into the stratifying agar plug and colonies of *C. botulinum* appeared in the top portion of the assay tube. In subsequent experiments a 1-cm stratifying layer of petrolatum gave satisfactory results. This observation indicated that generally the use of stratifying agar layers in anaerobic cultures may create a micro-environment of reduced nutrient value.

The observation that the antimicrobial activity of cheese extracts was not affected by repeated drying in a current of air under normal laboratory conditions indicated that the substance was not volatile, not readily oxidized by air and not destroyed by light. Diffusion of the antimicrobial substance through 1.5% agar media indicated at least some water solubility and a small to intermediate molecular size, since large molecules (e.g., polypeptide antimicrobial agents) diffuse slowly on agar media (2).

The development of inhibitory activity in type I and type III surface ripened cheese during storage may be of practical public health significance. Holding of these two types of cheese at the manufacturing plant for 8 weeks would decrease the possibility of food poisoning. However, this practice should be considered in connection with changes in flavor which occur in cheese during storage.

LITERATURE CITED

1. Andersen, A. A. 1951. A rapid plate method of counting spores of *Clostridium botulinum*. *J. Bacteriol.*, 62, 425.
2. Bechtle, R. M., and G. H. Scherr. 1958. A new agar for *in vitro* antimicrobial sensitivity testing. *Antibiotics & Chemotherapy*, 8, 599.
3. Difco Laboratories, Inc. Difco Manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures. 9th ed., Detroit 1, Michigan (1953).
4. Grecz, N., R. O. Wagenaar, and G. M. Dack. 1959. Inhibition of *Clostridium botulinum* and molds in aged surface ripened cheese. *Appl. Microbiol.*, 7, 33.
5. Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics, a laboratory manual. New York, Medical Encylopedia, Inc.
6. Hauge, S. 1955. Food poisoning caused by aerobic spore forming bacilli. *J. Appl. Bacteriol.*, **18**, 591.
7. Spector, W. S. 1957. *Handbook of Toxicology. II. Antibiotics.* W. B. Saunders Company, Philadelphia.
8. Sugiyama, H. 1951. Studies on factors affecting the heat resistance of spores of *Clostridium botulinum*. *J. Bacteriol.*, 62, 81.
9. Wagenaar, R. O., and G. M. Dack. 1958. Factors influencing growth and toxin production in cheese inoculated with spores of *Clostridium botulinum* types A and B. I. Studies with surface ripened cheese type I. *J. Dairy Sci.*, 41, 1182.

The Effect of Added Enzymes Upon the Free Amino Groups and Organoleptic Ratings of Irradiated Beef During Storage [a,b]

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SUMMARY

The presence of catheptic enzymes in irradiated meat was shown to be responsible for an increase in free amino nitrogen and a decline in panel scores as storage progressed. Immediately following irradiation, about one-third of the free amino nitrogen was water soluble while the remainder was bound to the muscle fibers. At the end of 6 months storage, the proportion of free amino nitrogen in the heat-inactivated sample to which the inactive enzyme preparation had been added remained unchanged, but for the inactivated sample with added active cathepsins only one-third of the free amino nitrogen remained bound to the fibers, whereas, in the raw sample only 15% was bound to the fibers. The addition of catheptic enzymes resulted in the release of a higher proportion of tyrosine and tryptophane than did the naturally occurring enzymes in raw meat.

In various raw foods preserved by irradiation, it has been noted that in addition to the so-called "irradiation flavor" initially observed, a marked deterioration in flavor occurs upon storage. This deterioration is not due to bacterial decomposition. The flavor change has been attributed to the continued action of radio-resistant proteolytic enzymes (6, 7).

This study was carried out, first, to determine whether or not the presence of active natural enzymes in irradiated meat results in these undesirable flavor changes, and second, to determine the nature of such changes.

[a] Journal Article 2580 Michigan Agricultural Experiment Station, East Lansing.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U.S. Army, and has been assigned number 1086 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

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PROCEDURE

EXPERIMENT 1. NATURAL MEAT ENZYMES

Press Fluid

A meat enzyme extract was prepared by grinding 10 lb of fresh beef, mixing with 3% filter pulp and pressing 170-g portions wrapped in heavy canvas in a Carver laboratory Press. The press-fluid was obtained by gradually increasing the pressure to 4,000–5,000 lb per sq in during a 30-min period. The fluid thus expressed was used as the fresh enzyme preparation.

Preparation of Meat

Thirty-six roasts of approximately 300 g each were prepared from 3 pairs of U.S. Choice beef ribs using the *Longissimus dorsi* muscles. The roasts were randomly assigned to 3 groups with 12 roasts in each group. One group was frozen, another group roasted to an internal temperature of 71° C (160° F) and injected with 20 ml of the active enzyme preparation per roast. The other group was cooked in the same manner and injected with 20 ml of the same preparation after heat inactivation of the enzymes. Inactivation was accomplished by heating the active preparation at 100° C (212° F) for 10 min. Injections were made with a hypodermic needle. Two of the fresh frozen roasts, two of the cooked active-enzyme-treated roasts, and two of the cooked inactive-enzyme-injected roasts were withdrawn and used as un-

irradiated controls. One roast from each treatment was immediately subjected to taste panel evaluation; the others were used for amino nitrogen determinations. All roasts were sealed and held in tin cans (C-enamel for pork) during irradiation and storage. The 30 remaining roasts were all irradiated at 2.79×10^6 rads with a Co^{60} source at the Fission Products Laboratory, University of Michigan. Following irradiation, one roast from each group was used for panel evaluation and another for chemical analysis. The remainder were stored at room temperature (24° C ± 3°) and one pair was removed from each treatment for panel evaluation and chemical analysis at the end of 1, 3, and 6 months of storage.

Panel Evaluation

Roasts to be evaluated by the panel were heated in a 149° C (300° F) electric oven to an internal temperature of approximately 66° C (150° F). An 18-member consumer-type panel rated each sample on the 9-point hedonic scale for acceptability as described by Peryam and Pilgrim (8).

Total Nitrogen

A modification of the Kjeldahl-Gunning method (1) was used to determine total nitrogen.

Amino Nitrogen

A 25-g sample was blenderized in 150 ml distilled water at 2° C (35° F) for 2–3 min. The resulting slurry was washed into a 250-ml volumetric flask and diluted to 250 ml with 2°C water. Free amino nitrogen was determined on 10 ml of the slurry and on 10 ml of the filtrate from the slurry by the Van Slyke nitrous acid method (1, 3). This was done in order to determine how much amino nitrogen was still bound to the fiber and how much had been freed from the fiber. Amino nitrogen is expressed as percent of total nitrogen to eliminate variation due to different protein contents in the roasts.

pH. The pH was measured on the same slurry using a Beckman Model G pH meter.

Experiment 2. Crude Cathepsin Preparation

Preparation of Meat

Four wholesale beef ribs were obtained and the *Longissimus dorsi* muscles were removed and passed once through a coarse grinding plate and twice through a fine plate. Forty cans were prepared with each containing 300 g ground beef in a polymylar bag. Fourteen of these were held in the raw state and the rest were cooked to an internal temperature of 77° C (170° F). Thirteen of the cooked samples were injected with 18 ml of an active catheptic enzyme preparation prepared from beef spleen and the remaining cooked samples were injected with 18 ml of the same preparation which had been inactivated by heating at 100° C for 10 min.

All cans were sealed and frozen. Two cans from each treatment were set aside as unirradiated controls and the remaining cans were irradiated at 2.79×10^6 rads with the Co^{60} source at the Fission Products Laboratory, University of Michigan. The controls and 2 samples of each of the irradiated treatments were immediately subjected to taste panel evaluation and chemical analysis. Storage was then begun at room temperature (24° C ± 3° C). Panel evaluation and chemical analysis were carried out after storage periods of 1, 3, and 6 months.

Panel evaluation, total nitrogen and amino nitrogen were determined as in Experiment 1.

Enzyme Preparation

Four beef spleens were chopped and frozen and a crude preparation of cathepsin was prepared by salting out with ammonium sulfate at 40 to 70% saturation (4). The final dialysate was tested for activity against a 2.5% hemoglobin substrate, reading absorbance of the trichloroacetic acid filtrate at 280 mμ on the Beckman DK-2 (9). Activity was about 7.2 hemoglobin units per can. The enzymatic activity of the raw meat was also determined by the above method taking care to extract as much of the enzymes as possible. Activity was approximately 6.4 hemoglobin units per can.

Free Amino Acid Determination

Ten grams of the ground sample were suspended in boiling water and extracted by the method of Awapara (2) and chromatographed in two dimensions with phenol-water and collidine-water solvents (5). The spots were cut out, eluted and reacted with ninhydrin. They were quantitated by spectrophotometric comparison with a chromatographed standard solution of amino acids (2).

RESULTS

Experiment 1

Results of the first experiment are summarized in Table 1. With both the raw samples and the cooked samples with the added active press fluid, panel scores declined steadily as length of storage was extended. Duplicate samples showed a marked increase in percentage of amino nitrogen as

TABLE 1

MEAN PANEL SCORES, PERCENT NITROGEN AND pH OF CONTROL AND IRRADIATED MEAT SAMPLES OVER A SIX-MONTH STORAGE PERIOD—EXPERIMENT 1

Sample	Mean score	Percent nitrogen	pH	Percent amino nitrogen	
				Suspension	Filtrate
Unirradiated control raw	6.4	3.3	5.62	7.6	2.3
Cooked + added enzyme	6.8	4.3	5.95	7.0	1.2
Cooked + inactive enzyme	6.9	3.9	5.95	7.2	2.0
Irradiated 0 days raw	5.3	3.7	5.62	5.1	1.6
Cooked + added enzyme	4.2	4.4	5.78	6.1	1.4
Cooked + inactive enzyme	4.8	4.0	5.90	6.8	1.3
Irradiated 1 month raw	3.3	3.7	5.80	10.4	5.4
Cooked + added enzyme	4.7	4.4	5.33	7.4	2.1
Cooked + inactive enzyme	6.1 [1]	4.5	5.86	5.7	1.5
Irradiated 3 months raw	3.2	3.6	6.40	14.2	10.6
Cooked + added enzyme	3.9	4.5	5.35	7.4	2.6
Cooked + inactive enzyme	5.0 [2]	3.8	5.82	7.2	1.8
Irradiated 6 months raw	2.2	3.8	6.00	16.5	14.6
Cooked + added enzyme	3.6 [3]	4.5	5.41	7.8	2.9
Cooked + inactive enzyme	5.2 [3]	4.3	5.87	6.1	1.8

[1] The cooked-inactive sample was preferred ($P = <.01$) over both the cooked-active and the raw sample. The cooked-active sample was preferred ($P = <.01$) over the raw.

[2] The cooked-inactive sample was preferred ($P = <.01$) over both the other samples.

[3] The cooked-inactive sample was preferred ($P = <.01$) over both the other samples and the cooked-active sample was preferred ($P = <.01$) over the raw.

storage became longer. Prior to storage approximately 30% of the total amino nitrogen was found in the water filtrate from the raw sample, but as storage was prolonged there was a regular increase in the proportion of amino nitrogen in the filtrate until there was a total of 88% at the end of 6 months. There was a similar increase in the proportion of water-soluble amino nitrogen during storage of the cooked samples with the added active press fluid. However, the magnitude of change was much lower than that of the raw sample with an increase from 23 to only 37%.

The cooked sample to which the inactivated press fluid was added received the highest panel score at 1, 3, and 6 months storage. The sample with the added inactivated press fluid was rated higher by the panel after 6 months storage than when tasted at 0 days. Similar samples exhibited no apparent increase in total amino nitrogen as storage was prolonged. Furthermore, no clear-cut change in the proportion of amino nitrogen in the aqueous extract was apparent.

After storing the raw sample for 6 months, crystals were present on the surface of the sample. By means of microscopic examination, the crystals were identified as tyrosine.

EXPERIMENT 2

Table 2 gives a summary of the results in the second experiment. Taste panel results showed a general decline in score as storage was extended. However, the lowest ratings were obtained for the cooked samples with added active catheptic enzymes. Again there was a steady rise in the total amino nitrogen with increasing periods of storage for both the raw and cooked samples with added active cathepsin. The water-soluble amino nitrogen showed an increase with length of storage. The proportionate amount of amino nitrogen in the water extract became considerably higher in the cooked sample with the added active preparation of cathepsin than was true for active press fluid in the first study. However, the raw sample not only exhibited a greater proportion of amino nitrogen in the aqueous extract, but it also contained more total amino nitrogen as storage progressed than did the cooked sample with added active cathepsin.

TABLE 2

MEAN PANEL SCORES, PERCENT NITROGEN AND pH OF CONTROL AND IRRADIATED MEAT SAMPLES OVER A SIX-MONTH STORAGE PERIOD—EXPERIMENT 2

Sample	Mean score	Percent nitrogen	pH	Percent amino nitrogen	
				Suspension	Filtrate
Unirradiated control raw	6.6	3.0	5.91	7.3	3.0
Cooked + added enzyme	5.6	3.2	6.12	5.5	1.8
Cooked + inactive enzyme	6.1	3.2	6.04	5.2	1.9
Irradiated 0 days raw	5.1	3.1	6.00	7.9	2.3
Cooked + added enzyme	4.2	3.1	6.04	6.8	2.0
Cooked + inactive enzyme	4.8	3.0	6.03	7.3	1.7
Irradiated 1 month raw	4.3	3.0	5.94	8.1	7.6
Cooked + added enzyme	4.4	3.0	5.76	7.6	2.6
Cooked + inactive enzyme	4.4	3.0	5.54	6.7	2.0
Irradiated 3 months raw	3.9	3.0	5.96	14.1	10.3
Cooked + added enzyme	3.6	3.0	5.16	8.4	3.5
Cooked + inactive enzyme	4.2	3.0	5.68	6.5	2.2
Irradiated 6 months raw	3.1	3.0	6.27	14.8	12.5
Cooked + added enzyme	2.1	2.9	6.01	8.6	5.7
Cooked + inactive enzyme	4.9[1]	3.1	6.04	4.5	1.9

[1] The cooked-inactive sample was preferred ($P = <.01$) over both the cooked-active and the raw samples after 6 months' storage.

The cooked samples to which the inactivated enzyme preparation was added did not change greatly in either total amino nitrogen or in water-soluble amino nitrogen as the storage period was lengthened.

Table 3 gives the level of various amino

TABLE 3

A COMPARISON OF THE FREE AMINO ACID CONTENT OF THE RAW SAMPLE AND THE COOKED + ADDED ENZYME SAMPLE AFTER SIX MONTHS' STORAGE

Amino Acid(s)	Raw sample	Cooked + added active enzyme
	$\mu M/g$	$\mu M/g$
Leucine	34.0	13.8
Phenylalanine	10.8	5.5
Valine	19.8	4.3
Arginine + Lysine	39.0	13.0
Tyrosine	19.8	20.8

acids in the raw sample and in the cooked sample with added active cathepsin. Examination of the data reveals that slightly more tyrosine and about half as much phenylalanine were found in the cooked sample with added active enzyme than were found in the raw sample. With all other amino acids studied, the cooked sample contained only about one-third as much as the raw sample. It is apparent that the added enzymes appeared to release a higher proportion of tyrosine and phenylalanine than the naturally occuring enzymes in raw meat. This was not unexpected since the method used in assaying for enzyme activity is specific for tyrosine and tryptophane (9) residues.

As in the first experiment, tyrosine crystals were found in both the raw and the cooked sample with added active cathepsin. There was no evidence of crystals in the cooked sample to which the inactive enzyme preparation was added.

DISCUSSION

Results of the first experiment show that the level of raw press fluid added to the cooked sample did not contain adequate amounts of enzymes to give as much enzymatic degradation as occurred in the raw samples. However, the increase in amino nitrogen as storage was extended with both the cooked sample containing added raw press fluid and the raw sample verified that enzymatic degradation was a serious problem in storing irradiated meat. Since the acceptability ratings tended to parallel the amount of amino nitrogen in the samples, it is obvious that the enzymes in irradiated meat must be inactivated either by heat treatment or by chemical means.

The cathepsin preparation used in the second experiment did not produce as great a percentage of amino nitrogen as the enzymes present in the raw meat. However, the cathepsin preparation did produce a greater amount of tyrosine and proportionately more phenylalanine in relation to the other amino acids than the natural enzymes in raw meat. Since the panel score was lower for the cathepsin-treated samples, it is possible that the tyrosine and perhaps the tryptophane contents may be major contributors to the poor acceptability of raw irradiated beef after storage.

The possible relationship between panel scores and the amounts of tyrosine and/or tryptophane would suggest that a selective inhibitor for blocking the enzymatic hydrolysis that yields these two compounds may be used in preventing some of the flavor problems in raw irradiated meat during storage. Perhaps the most effective way of doing this would be blocking the cathepsin C fraction, which can be accomplished with ascorbic acid or $MnSO_4$ (9).

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LITERATURE CITED

1. Association of Official Agricultural Chemists. 1955. *Official Methods of Analysis.* 8th ed.
2. Awapara, J. 1949. Application of paper chromatography to the estimation of some free amino acids in tissues of the rat. *J. Biol. Chem.,* **178**, 113.
3. Davis, N. C., and E. L. Smith. 1955. Assay of proteolytic enzymes. *Methods of Biochemical Analysis.* **2**, p. 237. Interscience Publishers, New York, N. Y.
4. de la Haba, G. L., P. S. Cammarata, and J. S. Fruton. 1955. Cathepsin C from beef spleen. *Methods in Enzymology,* **2**, 64. Academic Press, New York, N. Y.
5. Dent, C. E. 1948. A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-"collidine" filter paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.,* **43**, 169.
6. Drake, M. P., W. P. Giffee, Jr., R. Ryer, III, and H. Harriman. 1957. Proteolytic activity in irradiation-sterilized meat. *Science,* **125** (3236), 23.
7. McArdle, F. J., and N. W. Desrosier. 1955. Influences of ionizing radiations on the protein components of selected foods. *Food Technol.,* **9**, 527.
8. Peryam, D. R., and F. J. Pilgrim. 1957. Studies in food science and technology. 1. The methodology of sensory testing—the hedonic scale method. *Food Technol.,* **11** (9), Insert p. 9.
9. Tallan, H. H., M. E. Jones, and J. S. Fruton. 1953. On the proteolytic enzymes of animal tissues. X. Beef spleen cathepsin C. *J. Biol. Chem.,* **194**, 793.

Influence of Salt (NaCl) on Pectinolytic Softening of Cucumbers [a]

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SUMMARY

Experimental packs of pasteurized cucumbers were treated with pectinase from three sources under controlled conditions with respect to temperature, pH, acidity, salt concentration, and absence of microbial development. The enzyme-treated lots revealed that as the salt content of the cucumbers increased, their firmness likewise increased according to a first-order reaction. Based on cucumber softening data obtained by use of the three pectinases, tabulated information is presented which permits an estimate of the relative degree of softening that may be expected in curing brines at different salt concentrations.

In the commercial brining of cucumbers for salt-stock pickles, salt (NaCl) concentration is most important for determining the nature of the fermentation and for preserving the stock for long periods of time. The initial salt concentrations may range from 6.5 to 10% depending on the individual pickle plant, but the final concentration is about the same for all plants: 16% salt at the end of the fermentation period (11). The influence of the initial salt concentration on the amount of total acid formed in the fermenting brine, along with the corresponding pH values, has been established (15). Etchells and Jones (12) demonstrated that the use of a low salt brine (about 5% NaCl) gave rapid formation of a high amount of acid and a low pH, whereas, the use of increasingly higher brine strengths (10% NaCl and above) gave slower rates of acid formation, together with increasing rates of gas evolution and resultant bloater spoilage (hollow cucumbers).

Acid-forming bacteria and yeasts are the principal groups of organisms usually found during the active fermentation of cucumbers. Their population changes and identification in commercial brines have been studied extensively by Etchells and associates (4, 8–10, 12, 13). Brining procedures at commercial plants which use high initial salt concentrations were reported (4) to favor large populations of fermentative yeast species and to be chiefly responsible for the bloater formation.

In 1958, Etchells *et al* (7) reviewed the earlier investigations as well as the more recent studies dealing with the nature of softening-type spoilage of cucumber salt-stock. It has been established (1, 2, 5–7) that softening is caused by the pectin-splitting fungal enzyme pectinase (complex enzyme system of which one component of the pectic enzyme mixture is polygalacturonase); cellulase is also associated with softening in the commercial brines. These studies definitely implicated filamentous fungi as the actual causative agent responsible for softening of cucumbers under commercial conditions. Further, the hydrolytic enzymes pectinase and cellulase were reported to be introduced into the brines chiefly by way of fungus-laden flowers that remain attached to the cucumbers and to a lesser extent by the cucumber fruit. The enzymes are produced in the flowers prior to entering the brine and not by fungal growth during the fermentation.

Mechanical removal of flowers from cu-

[a] This study was carried out under a cooperative project with the Departments of Animal Industry and Horticulture of the North Carolina Agricultural Experiment Station.

[b] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

cumbers or draining off the brine after 36 hours are procedures recommended (6) and used by the pickle industry to reduce the softening enzymes in the brines.

In 1950, Bell *et al* (1) reported the effect of 0, 5, 10, 15 and 20% sodium chloride solutions on the viscosity of pectin and on the activity of 0.20 and 2.00 μg/ml of polygalacturonase upon pectin. In those studies the method of assay caused a fivefold dilution of the salt-enzyme solutions; so the final salt concentrations above were lower, and salt influence on enzyme action reversible. They reported that increasing the salt concentration levels raised the relative viscosity of the pectin solution and reduced the enzyme activity as measured by percent loss in viscosity. They concluded that high concentrations of salt did not completely denature polygalacturonase.

It has been generally believed by experienced commercial operators in the pickle industry that the use of low-salt-brining procedures for cucumbers will result in more softening losses than the use of higher brine strengths (11, 12). Scientific reports are not available to substantiate these beliefs; therefore, the present investigation was undertaken to study the influence of salt concentration on the pectinolytic softening of cucumbers. The tests were conducted in the laboratory where controlled conditions could be maintained.

MATERIALS AND METHODS

Experimental Cucumbers

Fresh-pack (pasteurized) cucumbers were made during the 1957 and 1958 growing season at a commercial plant located in Ayden, North Carolina. These experimental packs consisted of 12 jars (1 quart capacity) each of the individual salt treatments calculated to equalize at 0, 4, 8, 12, 16 and 20% NaCl for the 1957 pack; and, at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16 and 20% NaCl for the 1958 pack. The experimental packs were prepared by first weighing the salt and placing it into each jar, then packing the jars with 14 to 16 washed cucumbers, Model variety, 1 to 1⅛ inches in diameter. For the 1957 pack, a cover liquor containing 19 ml of 85% lactic acid per gallon of water was poured onto the cucumbers to approximately one-half inch from the top of the jar; for the 1958 pack, the acid was increased to 23 ml per gallon. These jars were then sealed, coded, and pasteurized in a continuous commercial unit using steam which gave an internal product temperature of 165° F; the jars were promptly cooled with water to less than 100° F and cased according to code numbers. The final equalization of the lactic acid was in a range of 0.20 to 0.24% and the brine pH was 3.8 to 4.1; the 1958 pack was slightly lower in pH and higher in acidity than the 1957 pack, but it was in the given range. A few jars spoiled in the 0 to 4% salt treatments at pH 4.0 but a sufficient number of jars in these particular lots were free of spoilage to conduct the enzyme tests.

Pectinase Enzyme Sources

A commercial concentrated pectinase enzyme 46 AP (Lot No. 32) of fungal origin, was used.[c] One percent aqueous solution of this enzyme was sterilized by Seitz filtration and decimal dilutions prepared so that one ml would give the desired concentration per treatment (Table 1).

Purified polygalacturonase (PG), prepared from fungal pectinase at the Western Utilization Research and Development Division, USDA, Albany, California (16), was kindly supplied by E. F. Jansen of that laboratory. The purified enzyme was used in the 1958 and 1959 experiments at 1.0 ppm and 0.1 ppm respectively.

Four fungi, previously isolated and studied (7), were also used as pectinase sources. The fungi were grown on the surface of White's mineral broth plus pectin (7) and after two weeks incubation at 28° C, the clear filtrate was separated from the cells, diluted with 9 parts of water, and 1 ml of the 1:10 dilution added per jar of cucumbers (Table 2). Ten drops of toluene were added to each jar as a preservative.

Measurement of Cucumber Firmness

The USDA Fruit Pressure Tester (17) was used to measure cucumber firmness (2). For this study, each pressure test value represents the average of 10 cucumbers, each with a single center punch and recorded to the nearest pound resistance to the 5/16-inch tip of the instrument. Firmness values of less than 3 lb are not measurable with this tester. Adjective "Firmness Ratings" for salt-stock cucumbers of 1 to 1⅛ inches diameter assigned to pressure test values have been reported (2) as follows: *Very firm*, 18 lb and above; *Firm*, 14 through 17 lb; *Inferior*, 11 through 13 lb; *Soft*, 5 through 10 lb; *Mushy*, 4 lb and below. This information is given to assist the reader in interpreting the data in Tables 1 and 2.

[c] Supplied by Rohm and Haas Co., Philadelphia, Penna.

TABLE 1

INFLUENCE OF PECTINASE ENZYME AND SODIUM CHLORIDE CONCENTRATIONS ON CUCUMBER FIRMNESS

Enzyme	Enzyme concentration	Cucumber firmness [1] Sodium chloride concentration in %											
		0	1	2	3	4	5	6	8	10	12	16	20
	ppm	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*
(1958 Experiment)													
Control	0	14.4	.....	.....	.....	14.4	.....	.....	14.7	.....	14.6	14.3	14.6
46AP	0.01	<3	.....	.....	.....	9.5	.....	.....	10.5	.....	12.5	12.7	13.8
46AP	0.10	<3	.....	.....	.....	9.9	.....	.....	12.4	.....	13.1	13.3	13.4
46AP	1.00	<3	.....	.....	.....	4.2	.....	.....	8.2	.....	8.9	10.6	11.7
46AP	10.00	<3	.....	.....	.....	<3	.....	.....	5.0	.....	7.0	9.5	10.5
46AP	10.00 [2]	<3	.....	.....	.....	6.9	.....	.....	8.6	.....	11.2	11.7	12.7
PG	1.00	<3	.....	.....	.....	<3	.....	.....	5.6	.....	8.1	10.5	9.6
PG	1.00 [2]	<3	.....	.....	.....	7.3	.....	.....	10.1	.....	11.6	13.2	13.0
(1959 Experiment)													
Control	0	17.0	15.9	16.9	16.4	16.0	16.1	16.6	16.6	17.1	16.8	17.0	16.5
46AP	0.01	7.9	.....	13.7	14.1	12.9	14.7	.....	14.3	15.2	15.2	14.4	15.5
46AP	0.10	3.8	5.8	7.1	9.2	11.9	.....	.....	13.6	14.0	14.7	12.0	14.1
46AP	1.00	<3	<3	3.3	3.9	6.8	12.4	9.3	10.9	13.2	12.7	13.0	13.8
46AP	10.00	<3	<3	<3	<3	<3	3.3	3.7	5.6	8.4	10.1	11.5	12.2
46AP	10.00 [2]	<3	<3	4.8	6.8	10.8	10.2	11.8	11.8	14.5	14.5	14.8	15.7
PG	0.10	<3	<3	4.2	4.2	9.7	9.9	11.5	12.2	13.8	15.6	15.1	15.9

[1] Pressure test readings were taken after 1-month incubation at 30° C except where 7 days is indicated. Value of <3 = too soft to pressure test and the cucumbers ranged from very soft to mushy. Firmness values for controls averaged 14.5 lb in 1958 and 16.6 lb in 1959.

[2] Incubated 7 days.

RESULTS AND DISCUSSION

Softening Activity of Pectinase 46 AP and PG

Experimental cucumber packs (one-quart jars) with six levels of salt (0, 4, 8, 12, 16 and 20%) were treated in 1958 with increasing concentrations of 46 AP pectinase calculated to equalize at 0.01, 0.1, 1.0 and 10.0 ppm. An additional salt series was treated with purified polygalacturonase calculated to equalize at 1 ppm. The experimental lots of cucumbers together with a matched control salt series (without added enzyme) were incubated at 30° C. Cucumber firmness in pounds was recorded for the 10 ppm level of 46 AP enzyme and 1 ppm level of the PG enzyme after 1-week incubation and for all treatments after one-month incubation (Table 1, top-half). The firmness values for the control cucumber samples ranged from 14.3 to 14.7 (av 14.5); no change in firmness was demonstrated by increasing the salt concentration alone. For the added enzyme treatments, the cucumbers from the higher salt levels gave higher firmness readings. Cucumber firmness for all enzyme treatments without salt was less than 3 lb. Increasing levels of 46 AP pectinase decreased the firmness of the cucumbers within each salt concentration. An increase of incubation time for the two enzyme treatments (10 ppm of 46 AP and 1.0 ppm of PG) gave decreasing firmness of the cucumbers within each salt treatment. In comparing the action of pectinases from 2 sources, 1 ppm PG reduced cucumber firmness in the same order of magnitude with the different salt levels as did 10 ppm 46 AP and at about the same rate.

A second experiment was conducted in 1959 with double the number of salt concentration levels used in the experiment just described. The 46 AP enzyme was used at the same concentrations but the PG enzyme level was reduced to 0.1 ppm. Cucumber firmness values as shown in the lower part of Table 1 are the results of these tests.

In general, the results obtained for the second experiment were in agreement with those from the first. It was again demonstrated that the use of increasingly higher salt concentrations gave correspondingly higher values for cucumber firmness. Also, an increase in enzyme concentration resulted in a decrease in cucumber firmness at all levels of salt concentration.

The action of 1 ppm PG enzyme on the firmness of cucumbers at different salt strengths calculated from the data in Table 1, is presented in Figure 1. The rate of

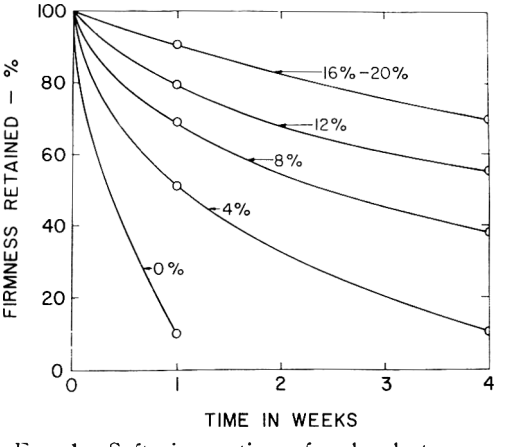

Fig. 1. Softening action of polygalacturonase enzyme (1 ppm) on cucumbers at different salt concentrations (in %).

enzyme action was very rapid at the lower salt levels and a higher degree of cucumber firmness was retained at the higher salt concentrations after 4 weeks' storage. However, it is emphasized that even with 16 to 20% salt the cucumbers lost 30% of their initial firmness in 4 weeks.

Softening Activity of Pectinase from Cell-Free Fungal Filtrates

Four fungi—*Fusarium roseum, F. oxysporum, F. solani* and *Ascochyta cucumis*—were among the 10 most frequently isolated species from the cucumber plant (*Cucumis sativus* L.) and represented 27% of 1032 fungus isolations obtained by Etchells *et al* (7). The 10 most frequently isolated species were shown (7) to produce pectinase and to soften cucumber tissue in a 2.5% salt brine acidified to pH 3.7. In the current study, 1-ml amounts of a 1:10 dilution of the cell-free filtrate from 14-day growth in broth from each of the above-named species were

added to a series of jars of cucumbers containing 0, 4, 8, 12, 16 and 20% salt. The final concentration of fungal filtrate per quart jar of cucumbers and brine was approximately 100 ppm.

The firmness values for the fungal filtrate experiment after 1-month incubation at 30° C are presented in Table 2. The filtrates of the 4 species reduced cucumber firmness to a greater extent in the lower salt levels than in the higher. These results were essentially the same as those from the pectinase enzyme experiments. If the data for the action of the fungi filtrates on cucumber firmness (Table 2) are plotted together with those for 10 ppm of pectinase 46 AP and 1 ppm PG, it is apparent that there is little difference in the softening behavior of the 3 enzyme sources toward cucumber tissue (Fig. 2). Further, the cucumbers retained more firmness as the salt strength was increased. If the log of the salt concentration is plotted against percent cucumber firmness retained, a straight line is obtained. This suggests that pectinolytic softening is a first-order reaction. Such data for pectinase 46 AP and the fungal filtrates are given in Figure 3.

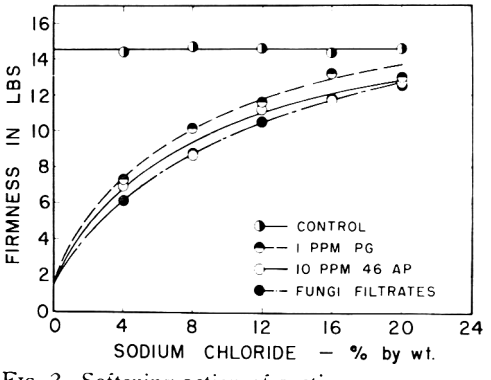

FIG. 2. Softening action of pectinase enzymes on cucumbers at different salt concentrations (in %).

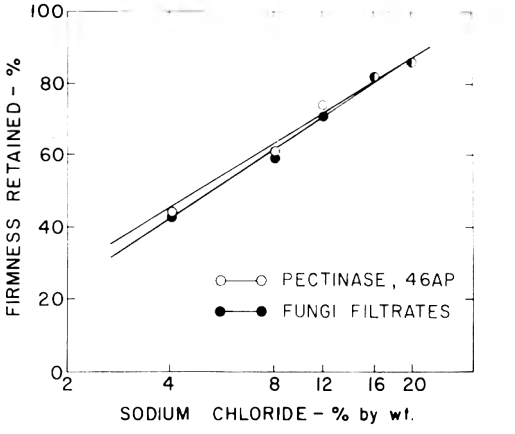

FIG. 3. Relationship of salt to pectinase activity on cucumber firmness.

SALTING-OUT ENZYMES

Enzymes have the chemical structure and general characteristics assigned to proteins. In the present study different sources of the pectinase enzymes were exposed to increasing concentrations of sodium chloride. Thus, the mention of a few general characteristics of enzymes, particularly their relation to several salts, is in order to form a basis of

TABLE 2

INFLUENCE OF CELL-FREE FUNGAL FILTRATES AND SODIUM CHLORIDE CONCENTRATIONS ON CUCUMBER FIRMNESS

Fungal filtrate added [1]		Cucumber firmness [2]					
		Sodium chloride concentration in %					
		0	4	8	12	16	20
species	*no.*	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*
Fusarium							
F. roseum	C-653	.....	9.0	9.8	11.3	12.3	12.9
F. oxysporum	C-214	<3	4.1	8.5	11.4	12.7	12.2
F. solani	C-775	.....	4.4	7.7	9.6	11.3	12.8
Ascochyta cucumis	C-1048	.....	6.7	8.9	9.2	10.7	12.2
Average		<3	6.1	8.7	10.4	11.8	12.5
Control		14.4	14.4	14.7	14.6	14.3	14.6

[1] Cell-free filtrate from 14-day-old growth in White's mineral broth plus pectin. One ml of a 1:10 dilution of the filtrate added to each quart jar of cucumbers.

[2] Pressure test readings were taken after 1-month incubation at 30° C.

explanation as to the influence of sodium chloride on enzymatic softening of cucumber tissue presented in this paper.

Pallmann *et al* (19) observed that a number of chloride solutions activated pectinase; maximum activity was obtained at 3.0 milliequivalents (0.017% NaCl) regardless of whether sodium or potassium chloride was used. The presence of small amounts of salt is necessary for the solution of proteins or enzymes in water. However, when increasingly larger quantities of very soluble salts, such as $(NH_4)_2SO_4$ and Na_2SO_4, and to a lesser extent NaCl, $MgSO_4$ and K_3PO_4, are added to enzyme solutions, the solubility of the enzyme decreases (3, 18, 20). At some definite salt concentration, the enzyme is almost completely precipitated from solution, and this technique is used for enzyme and protein isolation studies.

In general, precipitation of an enzyme by salt is most complete at or near the isoelectric pH of the specific enzyme. This would be at a pH value where the positive charges would equal the negative with a net charge of zero. Under such conditions, the protein or enzyme would not migrate in an electric field. Dixon and Webb (3) give the following general equation for enzyme solubility in concentrated salt solutions: $\log S = B\text{-}KI$, where S is solubility, I is the ionic strength, B and K constants. B depends greatly on temperature and pH, but K is independent of these factors. This equation tends to explain the log relationship of salt concentration to per cent firmness of cucumbers as reported in Figure 3 herein.

TABLE 3

RELATIVE PECTINOLYTIC SOFTENING OF CUCUMBERS AS INFLUENCED BY INCREASING SALT (NaCl) CONCENTRATIONS

Salt concentration		Relative [1] softening activity
salometer	by wt	
degrees	%	%
<5	<1.3	>90
6	1.6	82
8	2.1	76
10	2.6	70
12	3.2	65
14	3.7	60
16	4.2	56
18	4.8	53
20	5.3	50
22	5.8	47
24	6.4	44
26	6.9	42
28	7.4	40
30	8.0	38
32	8.5	36
34	9.0	34
36	9.5	32
38	10.1	30
40	10.6	29
42	11.1	28
44	11.7	26
46	12.2	25
48	12.7	24
50	13.2	23
52	13.8	22
54	14.3	21
56	14.8	20
58	15.4	19
60	15.9	18
65	17.2	15
70	18.6	12
>75	>19.8	<10

[1] Expressed as relative % loss of cucumber firmness to be expected at different salt strengths by the action of a constant pectinase level when compared to non-enzyme-treated controls.

RELATIVE PROTECTION OF CUCUMBER FIRMNESS AGAINST SOFTENING ACTIVITY OF PECTINASE

In order to develop a relative rate of pectinolytic softening of cucumbers, an overall cucumber firmness average at each salt treatment was calculated; this required consideration of the data in Tables 1 and 2. The averages were made in terms of percent retained firmness of the cucumbers and plotted against the log of salt concentration expressed as percent saturation of NaCl (degrees salometer). Table 3 was prepared from this graph and is proposed to be used as a guide to estimate the protective action of various percentages of salt against softening enzyme activity. For example, if the pectinase level was sufficiently high (= to 1 ppm 46 AP) and remained constant in 38° and 20° salometer fermentations, then the relative degree of pectinolytic softening expected would be 30 and 50%, respectively, of original cucumber firmness, indicating that cucumbers brined at the lower brine strength would be about 20% softer.

A commercial cucumber fermentation usually does not have a fixed concentration of salt other than at the time the vats are filled, headed and covered with brine. Brining procedures, as a rule, provide for a gradual increase in brine strength during the first 4 to 6 weeks after filling the vats. It would be difficult indeed to predict the softening rate under such conditions other than to point out that the results of these experiments indicate a decrease of pectinolytic softening would be expected where higher initial brine strengths were employed. However, if brining procedures were modified to substantially increase the initial cover brine, such practice would, as mentioned in the introduction, be at the risk of increasing bloater formation (hollow cucumbers) resulting from a more gaseous fermentation.

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LITERATURE CITED

1. Bell, T. A., J. L. Etchells, and I. D. Jones. 1950. Softening of commercial cucumber salt-stock in relation to polygalacturonase activity. *Food Technol.*, **4**, 157.
2. Bell, T. A., J. L. Etchells, and I. D. Jones. 1955. A method for testing cucumber salt-stock brine for softening activity. U. S. Dept. Agr., *ARS-72-5*, 15 pp.
3. Dixon, M., and E. C. Webb. 1958. *Enzymes*. Academic Press, Inc., New York, N. Y.
4. Etchells, J. L., and T. A. Bell. 1950. Classification of yeasts from the fermentation of commercially brined cucumbers. *Farlowia*, **4**, 87.
5. Etchells, J. L., T. A. Bell, and I. D. Jones. 1955. Studies on the origin of pectinolytic and cellulolytic enzymes in commercial cucumber fermentations. *Food Technol.*, **9** (3), 14.
6. Etchells, J. L., T. A. Bell, and I. D. Jones. 1955. Cucumber blossoms in salt-stock mean soft pickles. *Research and Farming*, (N. C. Agr. Expt. Sta.), **13** (1–4), 14.
7. Etchells, J. L., T. A. Bell, R. J. Monroe, P. M. Masley, and A. L. Demain. 1958. Populations and softening enzyme activity of filamentous fungi on flowers, ovaries, and fruit of pickling cucumbers. *Appl. Microbiol.*, **6**, 427.
8. Etchells, J. L., F. W. Fabian, and I. D. Jones. 1945. The *Aerobacter* fermentation of cucumbers during salting. Mich. Agr. Expt. Sta. *Tech Bull. 200.*
9. Etchells, J. L., and I. D. Jones. 1943. Bacteriological changes in cucumber fermentation. *Food Inds.*, **15** (2), 54.
10. Etchells, J. L., I. D. Jones, and W. M. Lewis. 1947. Bacteriological changes during the fermentation of certain brined and salted vegetables. U. S. Dept. Agr. *Tech Bull. 947.*
11. Etchells, J. L., I. D. Jones, and T. A. Bell. 1950-51. Advances in cucumber pickling. *Yearbook of Agr.* (U. S. Dept. Agr.) 229. U. S. Government Printing Office, Washington, D. C.
12. Etchells, J. L., and I. D. Jones. 1951. Progress in pickle research. *Glass Packer*, **30** (4), 264; (5) 358.
13. Fabian, F. W., C. S. Bryan, and J. L. Etchells. 1932. Experimental work on cucumber fermentation. Mich. Agr. Expt. Sta. *Tech. Bull. 126.*
14. Fabian, F. W., and L. J. Wickerham. 1935. Experimental work on cucumber fermentation. VIII. Genuine dill pickles—A biochemical and bacteriological study of the curing process. Mich. Agr. Expt. Sta. *Tech. Bull. 146.*
15. Jones, I. D. 1940. Salting of cucumbers: influence of brine salinity on acid formation. *Ind. Eng. Chem.*, **32**, 858.
16. Lineweaver, H., R. Jang, and E. F. Jansen. 1949. Specificity and purification of polygalacturonase. *Arch. Biochem.*, **20**, 137.
17. Magness, J. R., and G. F. Taylor. 1925. An improved type of pressure tester for the determination of fruit maturity. U. S. Dept. Agr. Dept. Circ. 350.
18. Neilands, J. B., and P. K. Stumpf. 1955. *Outlines of Enzyme Chemistry*. John Wiley and Sons, Inc., New York, N. Y.
19. Pallmann, H., J. Matus, H. Deuel, and F. Weber. 1946. Über die aktivität der pektinase. *Rec. trav. chim. Pays-Bas*, **65**, 633.
20. West, E. S., and W. R. Todd. 1955. *Textbook of Biochemistry*. 2nd ed. The Macmillan Co., New York, N.Y.

The Complementary Effects of Thermal Energy and Ionizing Energy on Peroxidase Activity in Green Beans [a,b]

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SUMMARY

The work herein presented demonstrates clearly that ionizing energy, in relatively large amounts, has a measurable effect on peroxidase "in situ." However, even a relatively small amount of heat can accomplish the same degree of destruction. There appears to be no advantage to be gained in the order of application of the two types of energy. Thus, for any practical purposes, insofar as enzymes are concerned, there appears to be no practical advantage to be gained by the application of ionizing energy to plant materials which are to be heat-blanched. Conversely, if plant materials are to be sterilized by radiation treatment, a heat blanch is still required, the degree of which is not markedly affected by sterilizing doses of ionizing radiations.

It is well established that ionizing energy, via indirect action, inactivates enzyme systems in dilute solution fairly readily. However, enzyme systems in their natural environment, "in situ," are far more resistant, being protected from free radicals and activated molecule reactions by the many other solutes that are present in foods, which are complex systems (1). Since it is well established that enzymes in natural food materials must be inactivated prior to further processing in order to store well, and since ionizing energy has little effect on enzyme systems, "in situ," one of us, in 1951, suggested the possible use of electronic heating for enzyme inactivation in conjunction with ionizing energy (1).

Since 1951, we have conducted several preliminary studies on the subject of the complementary effects of thermal and ionizing energy on enzyme systems "in situ." Preliminary unpublished data derived from these studies indicated the desirability of a more comprehensive study on an enzyme system "in situ." The present paper reports such a study; namely, the effects of the two forms of energy on peroxidase in green beans.

Materials and Experimental Procedures

A large quantity of fresh green beans obtained from a local farm were thoroughly washed and snapped. They were then ground in a meat grinder, blended to a fine uniform slurry, thoroughly mixed, packaged and frozen.

Suitable quantities were removed from the freezer as needed, thawed, and 30-g portions transferred into 4 x 9-inch polyethylene packets, rolled to a thickness of 2.5 mm, heat-sealed, and held at 36° F to 40° F until given a heat and/or irradiation treatment. Samples were held no longer than 4 hr.

Following treatment, the samples were assayed for peroxidase activity by a modification of the method of Sumner and Gjessing (2).

Samples were exposed to 800 KVP electrons from a Resonant Transformer either prior to or following heating in a water bath thermostatically controlled at 140° F. Immediately following heating, the samples were immersed in ice water. The thickness of 2.5 mm of sample is sufficiently small to permit a fairly uniform radiation dose, as well as a fairly rapid heat transfer.

Doses of 0.5, 1.0, 2.0, and 3.0 megarep were used. Heating was for periods of 3, 6, 9, and 15 min at 140° F.

Two runs were made for each variable and 4 replicates made for each run. For example, with a

[a] Presented at the Twentieth Annual Meeting of the Institute of Food Technologists, May 17, 1960, San Francisco, California.

[b] Contribution No 402, Department of Nutrition, Food Science and Technology, M.I.T.

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pre-irradiation dose of 0.5 megarep, 4 control samples were assayed, as well as 4 samples heated following irradiation for 3 min, 4 at 6 min, 4 at 9 min, and 4 at 15 min as well as corresponding non-irradiated controls at all heating periods. Each run, such as that described, was repeated twice. Average values are presented.

In order to point up the quantity of data presented herewith in summary form, some idea of the numbers of samples assayed may be helpful. Some 200 samples were assayed in a combination of heating and irradiation.

Results and Discussion

The data obtained are summarized in Table 1 in terms of either irradiating first or heating first. Each figure represents the average of 8 assays.

Figure 1 shows the retention of peroxidase as a function of radiation dose for various levels of heating. Similar patterns of retention were obtained independently of the order of application of the two types of energy.

Figure 2 presents data on the retention of peroxidase as a function of heating time for different irradiation doses. Again, the data indicate no marked effect in the order of application.

An inspection of the data presented in Figures 1 and 2 indicated, qualitatively, no

TABLE 1

Average Retention of Peroxidase in Green Beans Treated by Thermal and Ionizing Energy

Heat First	Dose (Rep × 10^{-6})				
Heating Time	0	0.5	1.0	2.0	3.0
(min.)					
0	100	94.2	85.8	77.8	75.2
3	92.6	86.2	73.2	63.5	59.8
6	82.5	74.0	62.8	54.7	49.6
9	69.0	59.1	53.0	45.3	40.9
15	44.5	36.8	33.1	26.3	23.6
Irradiate First					
0	100	96.6	92.9	86.8	83.6
3	89.8	84.8	75.9	67.2	62.0
6	75.2	69.7	64.0	56.2	49.8
9	61.2	56.2	51.2	44.5	40.2
15	39.4	34.7	32.6	27.0	23.7

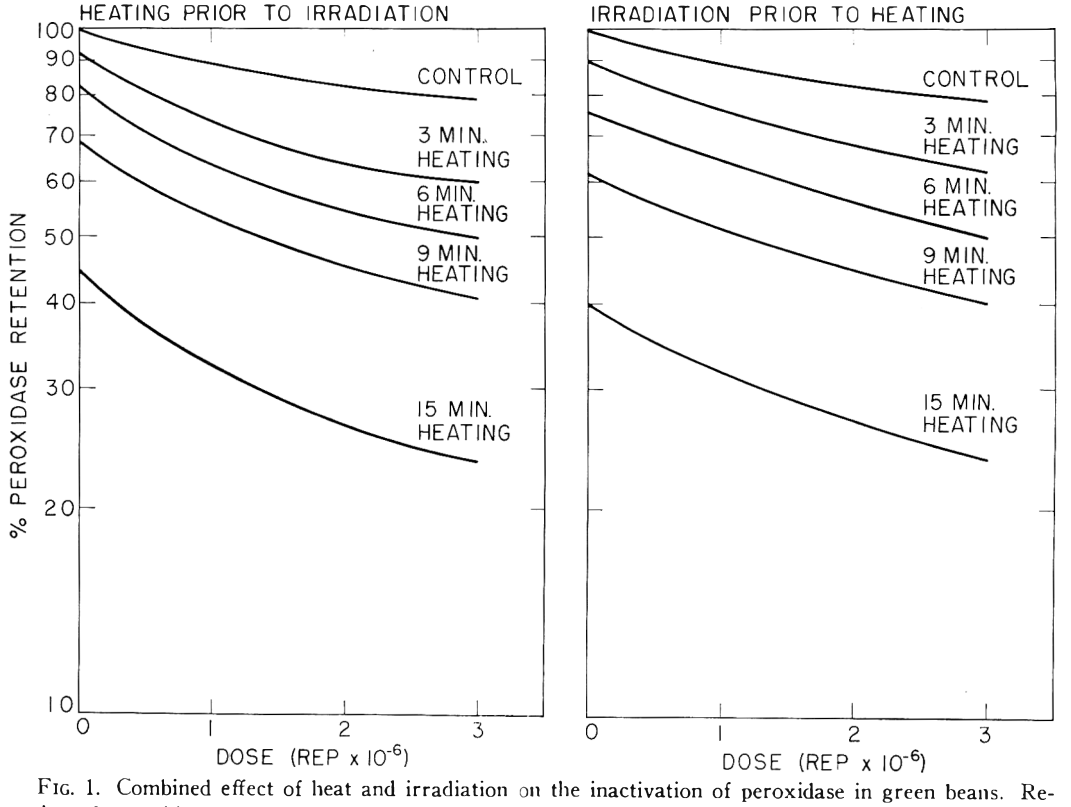

Fig. 1. Combined effect of heat and irradiation on the inactivation of peroxidase in green beans. Retention of peroxidase as a function of radiation dose.

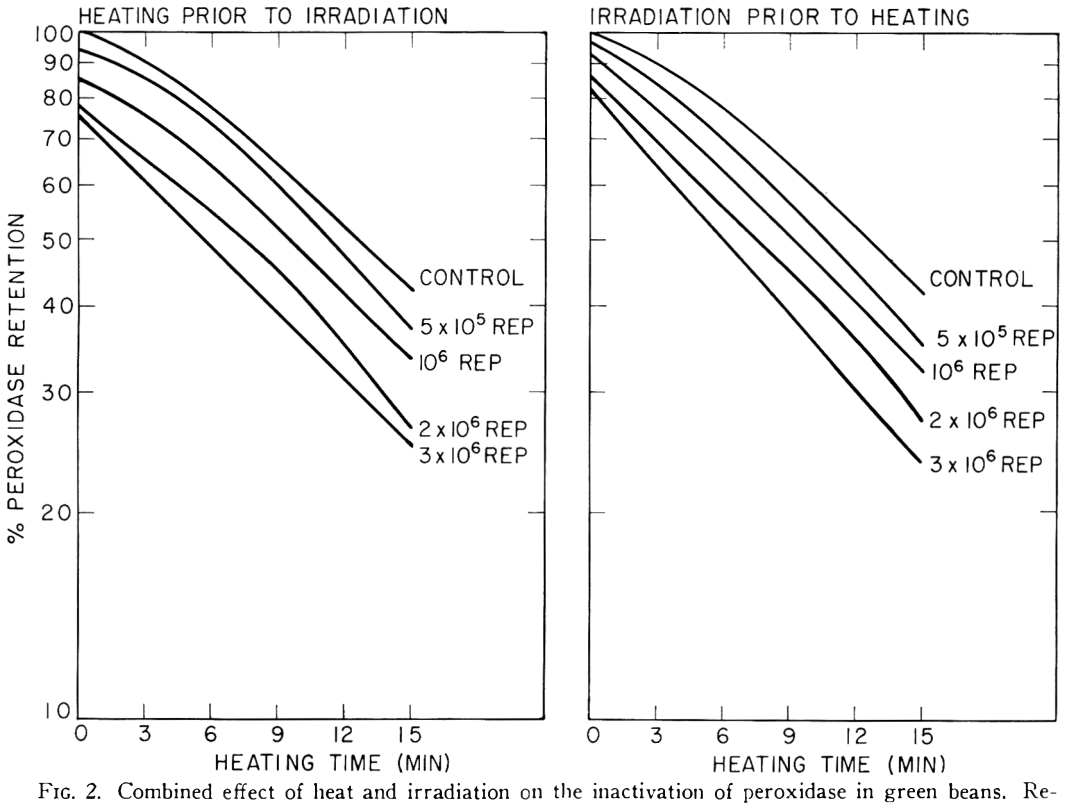

FIG. 2. Combined effect of heat and irradiation on the inactivation of peroxidase in green beans. Retention of peroxidase as a function of heating time.

marked effect due to the order of application of the two types of energy.

Because of the observed deviation from linearity (non-first order reaction), a direct comparison of the slopes of the retention curves could not be made.

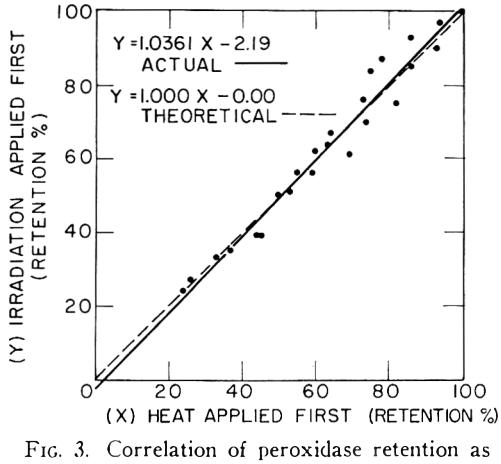

FIG. 3. Correlation of peroxidase retention as a function of order of application of ionizing energy and thermal energy.

Instead, the data in Table 1 were then plotted (Fig. 3) using, as the X-coordinate, the retention of peroxidase for a given quantity of energy, with heating administered prior to irradiation; and using as the Y-axis, the retention for the same quantity of energy with irradiation applied prior to heating.

A regression analysis on the resulting correlation curve was then made.

The hypothesis that there is no difference due to the order of application of the two types of energy corresponds to the theoretical line in Figure 3, (slope 45°).

Visual inspection indicated that the initial hypothesis was true. The difference between the actual (1.0361) regression coefficient and the predicted one (1.000) was shown statistically ($p \cong 0.38$) to be insignificant at any meaningful level, using the following:

$$t = \frac{b - \beta_0}{\sqrt{\frac{\Sigma e^2}{n-2}}} \sqrt{\Sigma (X - \bar{X})^2}$$

where: β_o = hypothetical slope (45°)

b = errors present due to unknown, uncontrollable variables (see below)

n = number of observations

$$\sum e^2 = \sum_{i=1}^{n} [Y_i - a - bX_i]^2$$

where: a = intercept on Y-axis

b = slope of regression line

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LITERATURE CITED

1. Proctor, B. E. and S. A. Goldblith. 1951. Food processing with ionizing radiations. *Food Technol.*, **5**, 376.
2. Sumner, J. B., and E. C. Gjessing. 1943. A method for the determination of peroxidase activity. *Arch. Biochem.* **2**, 291–293.