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Radiation Survival Curves of Clostridium Botulinum Spores

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(Manuscript received May 10, 1961)

SUMMARY

Survival curves were obtained for massive concentrations of spores of *Clostridium botulinum* strain 12885A, when subjected to a wide range of gamma radiation doses under various conditions. The spore concentrations ranged from 37,000 to 64,800,000,000 per ampoule, with no recovery at 5 Mrad or more. Only at lower doses were observed survival counts compatible with classical hit theory. More than one hit is apparently required to inactivate the *Cl. botulinum* spore by gamma radiation. The number of hits required for inactivation is estimated at somewhere near 13. Freeze-dried spores exhibited less radioresistance in the dry state than when resuspended in neutral phosphate buffer. Spore suspensions in neutral phosphate buffer exhibited less radioresistance when irradiated under air than when irradiated under nitrogen. Spores exhibited less radioresistance suspended in phosphate buffer than in a nutrient medium (pork-pea infusion). Changes in concentration of spores over a range of 37×10^3 to 53×10^9 did not significantly affect the percent survival for a given dose except in the tail area. Regardless of spore concentration, a so-called "tailing off" of surviving spores was observed at the higher dose levels employed for varying spore concentrations. The "tailing off" phenomenon did not appear to follow the classical hit theory.

The survival or destruction of spores of *Clostridium botulinum* subjected to gamma radiation is of particular importance in determining the radiation dose required to sterilize foods (Proctor and Goldblith, 1951; Hannan, 1956; U. S. Army, Q. M. Corps, 1957). This project was planned in cooperation with the Quartermaster Food and Container Institute to parallel the classical work of Esty and Meyer (1922) in determining the heat treatment required for destruction of massive concentrations of *Cl. botulinum* (up to 60×10^9 spores per dose level).

In addition to finding the point of complete destruction, the entire survival curve was experimentally determined under various environmental conditions. Hypotheses explaining the shape of these curves were tested.

Marked differences have been observed in radioresistance of various strains of *Cl. botulinum* (Schmidt and Nank, 1960; and Wheaton *et al.*, 1961). The most radioresistant toxic strain from previous work, 12885A, was selected for this project. It also readily forms both spores and toxin.

EXPERIMENTAL PROCEDURE

Spore production. Spores were produced in the trypticase medium of Schmidt and Nank (1960). A 16-L lot of the medium (pH 7.0) was prepared and divided into 3-L flasks and bottles. A small volume of a spore suspension was heated 13 min at 176°F, and 200 ml of the bottled medium was

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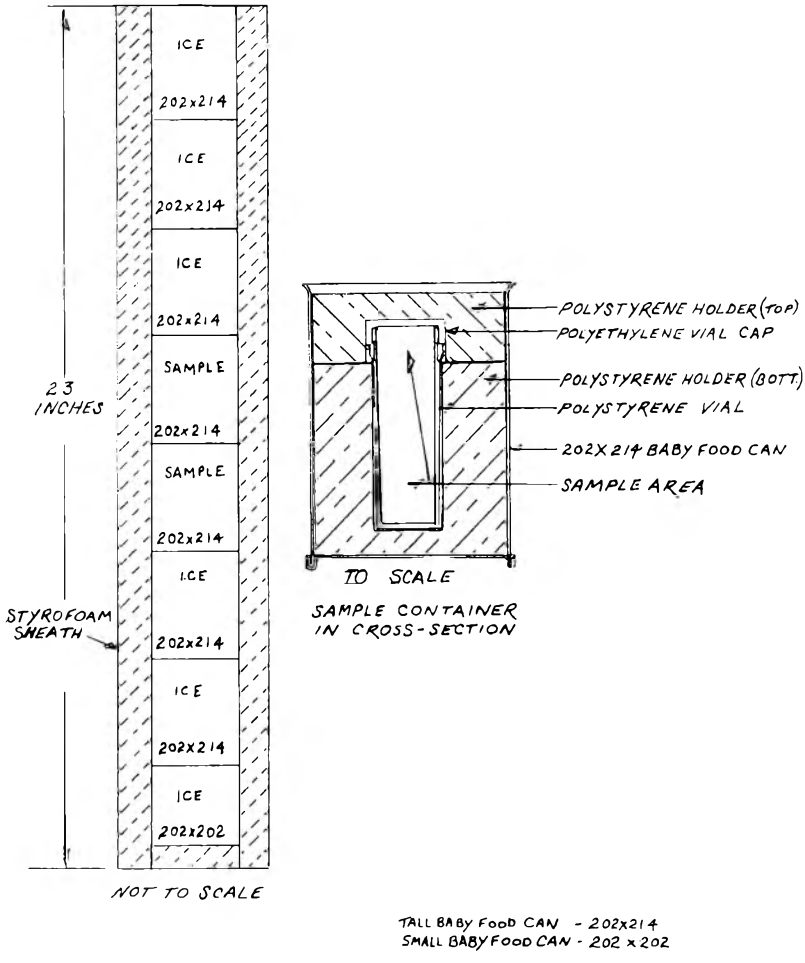

Fig. 1. Test samples and dosimetry arrangement for irradiation.

seeded. After 18 hr at 98°F these bottles were used to inoculate the 3-L flasks, which, in turn were incubated 72 hr at 85°F. These flasks were agitated 3 times daily during the 72-hr incubation period.

The spore crop was harvested by centrifugation and the spores collected in one bottle, distilled water was added, and the material was aerated 20 min. The spores were again centrifuged and the liquor discarded, with the sediment being transferred to a bottle containing glass beads. Distilled water was again added, the bottle shaken and centrifuged, with the supernatant liquid being decanted and the washing repeated once again. After the removal of this last wash water, new water was added, but only in an amount sufficient to facilitate pipetting. This stock suspension was then refrigerated at 40–44°F, after a count was made to ascertain the quantity of spores available. Using pork-pea agar as a counting medium, the spore population was calculated to be approx 15×10^{10} per ml, based on heating 13 min at 176°F. The spores were produced on two separate dates, but with the same general procedure.

Preparation of samples. Four separate irradiation experiments were conducted. In the first, the spores were suspended in pork-pea infusion (pH 6.5). Phosphate buffer (pH 7.0) prepared by neutralizing .20*M* KH_2PO_4 with .20*M* NaOH was used as the suspending medium in Experiment 2. In the third experiment, the spores were freeze-dried in a film on the inside of the vials for irradiation under three different environmental conditions: 1) dry; 2) resuspended in phosphate-buffer; and 3) resuspended in phosphate-buffer in essential absence of oxygen. The spore-containing menstrua for each of the above three experiments were prepared as follows:

Experiment 1; pork-pea infusion. Pork-pea infusion was inoculated with sufficient spores to yield

approx 60×10^6 spores per 6 ml of infusion. This was heated 13 min at 176°F and cooled, and then 6 ml was added to polystyrene vials. The vials were then shaken and agitated while freezing in dry ice and alcohol. Each vial was placed in a polystyrene holder to center it in a 202 × 214 metal can, which was then closed in air. The samples were held at −20°F until irradiated. Duplicate vials were run for each dose level, and coded "A" series and "B" series.

Experiment 2; phosphate buffer. A lower concentration of spores (37×10^6) per vial was used in this experiment. The inoculated buffer was first shaken 5 min on a paint mixer, and polystyrene vials were filled with 6 ml of the suspension without preheating the spores. Only one vial per irradiation dose was used, and the vials were frozen and placed in holders in metal cans, as before.

Experiment 3; dry atmosphere, phosphate-air, and phosphate-nitrogen. A different approach to the problem was used in this series. Approx 1×10^9 spores per vial was employed. Serum-bottle rubber stoppers were substituted for the cotton plugs before irradiation. Without any initial heating 7 ml of the spore suspension was mixed with 6.5 ml of distilled water, and 0.2 ml of the mixture was added to the polystyrene vials. The vials were then spun and a thin film frozen on the walls of the vials. The vials were placed in a centrifuge bottle which was then attached to a vacuum manifold, and the material was dried approx 2½ hr at a low temperature (0 to −10°F) under a high vacuum. All vials were held at −20°F pending the next step of preparation. One set of vials was irradiated without any further preparation. To a second set of vials was added 5 ml of phosphate buffer. The vials were then shaken and quickly frozen in dry ice and alcohol.

In the third set, the vials were vacuumized and flushed 3 min with prepurified nitrogen. To these vials was then added sterile buffer that had been deaerated. After this the vials were intermittently vacuumized and flushed with nitrogen for 5 min. The oxygen content of headspace gas in the sample vials ranged from 3 to 6% after the above treatment.

Three vials, one each for the above three conditions (dry-air, phosphate-air, and phosphate-nitrogen), were placed in a polystyrene holder in which 3 holes had been drilled around the perimeter of the holder. The filled holder was placed in a 202 × 214 metal can and the can was then vacuumized, flushed with nitrogen, sealed, and held at −20°F until irradiated.

Experiment 4; phosphate-air. A new crop of spores was used in this experiment, and the entire irradiation exposure schedule was the same as

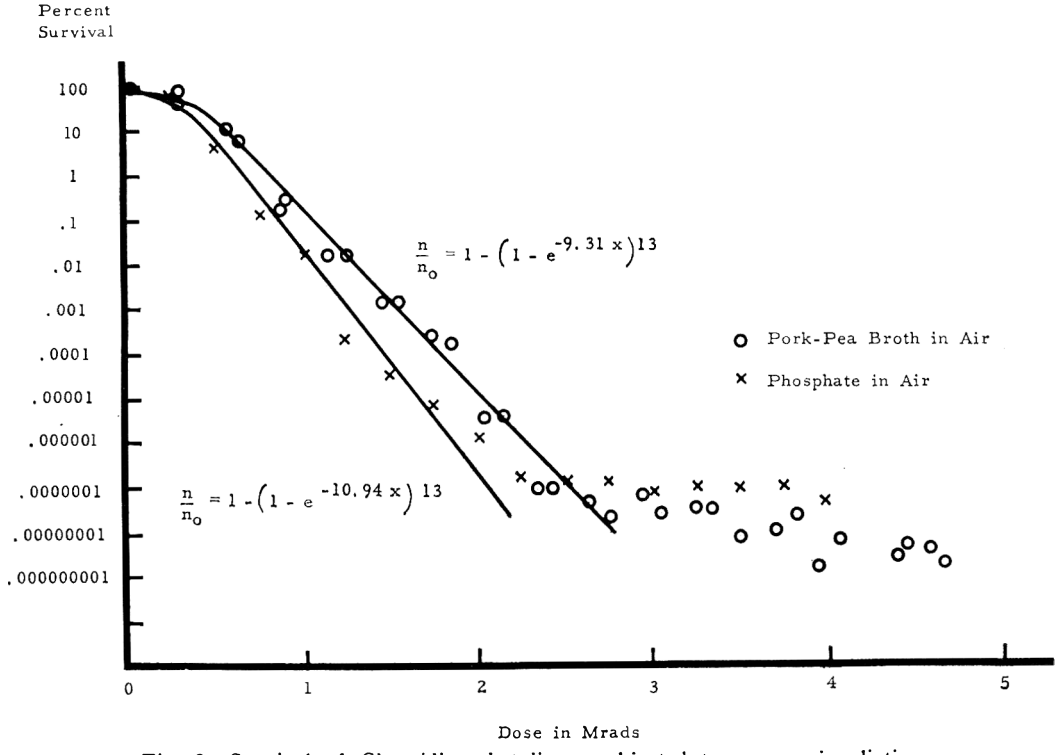

Fig. 2. Survival of *Clostridium botulinum* subjected to gamma irradiation.

Table 1. Phosphate-irradiated spore survival counts.

Code	Dose (Mrads)	Irrad. time (min.)	Count per: 1 ml	Count per: 6 ml	Log of counts
OH	0	0	6,200,000,000	37,200,000,000	10.57
S.25	0.25	15.3	4,000,000,000	24,000,000,000	10.38
S.50	0.50	27.3	240,000,000	1,440,000,000	9.16
S.75	0.75	35.9	8,000,000	48,000,000	7.68
T1.00	1.00	53.8	900		6.75
S1.00	1.00	53.8	940,000	940,900	
S1.25	1.00	74.0	11,800	70,800	4.85
S1.50	1.50	83.3	2,100	12,600	4.10
S1.75	1.75	83.3	398	2,388	3.38
T2.00	2.00	93.5	6		
S2.00	2.00	93.5	78	433	2.63
U2.00	2.00	93.5	37		
S2.25	2.25	58.7		56	1.74
S2.50	2.50	57.2	9		1.67
U2.50	2.50	57.2	2	47	
S2.75	2.75	73.3	8	48	1.68
T3.00	3.00	80	6		
S3.00	3.00	80	5	32	1.50
U3.00	3.00	80	1		
S3.25	3.29		6	36	1.55
S3.50	3.50	82	6		
U3.50	3.50	82	1	31	1.49
S3.75	3.75	85.8	6	36	1.55
T4.00	4.00	96.2	0	0	
S4.00	4.00	96.2	4	20	1.30
U4.00	4.00	96.2	0	0	
S4.25	4.25	102	0	0	
S4.50	4.48	105	0	0	
U4.50	4.48	105	0	0	
S4.75	4.73	104	0	0	
S5.00	5.00	110	0	0	
U5.00	5.00	110			

U = 1-ml aliquots removed from vial for culturing before shaking.
S = Vial shaken before removal of sample for culturing.
T = Vial top removed and rinsed with water for culturing.
OH = Heated sample to destroy vegetative cells and to activate spores.

employed previously except that three spore-population levels were 37×10^3, 43×10^6, and 53×10^6 per vial of phosphate per dose. The preparation of the vials and subsequent handling was the same as used in Experiment 3 for the phosphate-air samples.

Irradiation. All irradiation was done at the Argonne National Laboratory, with special dosimetry (Fricke) being carried out on the morning of any given pack, using polystyrene vials of the same geometry as the test samples.

All test cans were placed in a styrofoam sheath, which, in turn, was seated in the radiation urn, which rotates during the irradiation period. To maintain the samples in a frozen state, cans of frozen water were used to fill the styrofoam sheath, and this also provided for a constant location of the test cans. Fig. 1 illustrates the mechanics of handling the samples for Experiments 1 and 2. As stated previously, Experiments 3 and 4 varied from the above in that rubber stoppers were used for the vials and the polystyrene holder carried 3 vials around the perimeter instead of 1 vial in the center.

Where long exposure times and extremely hot fuel sources were involved, the cans were removed from the urns and placed in alcohol and dry ice before being returned to the urn for additional treatment.

In Experiment 4, when the irradiation dose was the same for all spore levels the three holes in the polystyrene holder were used. If, for any dose, one or more of the holes in the holder were not used, a vial of frozen water was inserted. A

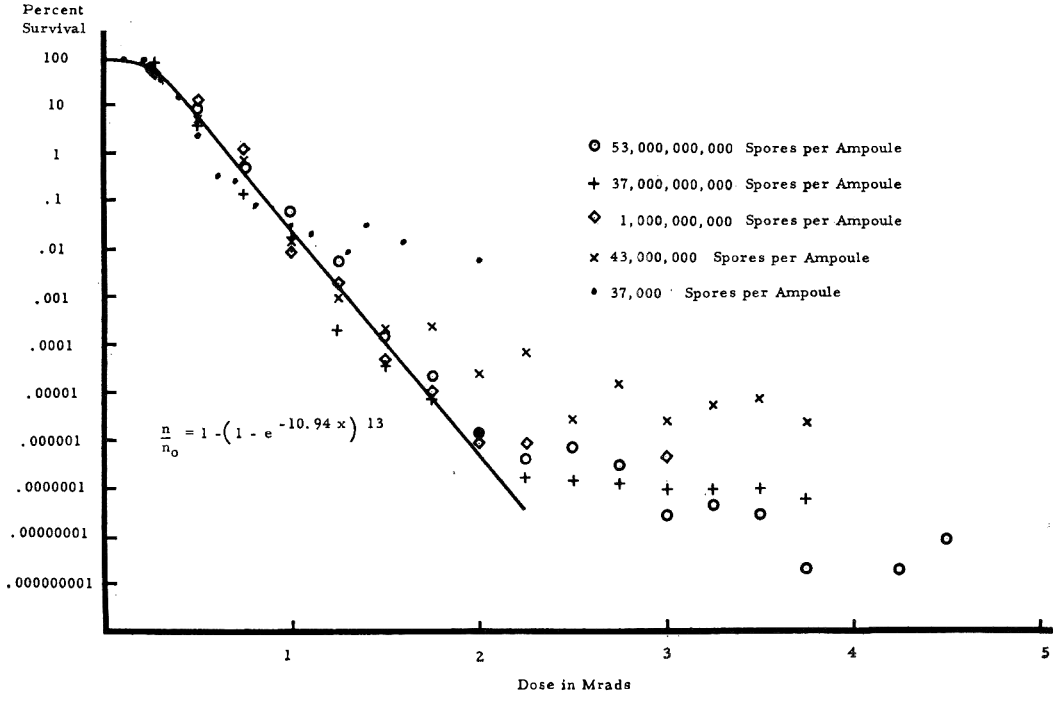

Fig. 3. Survival of *Clostridium botulinum* subjected to gamma irradiation.

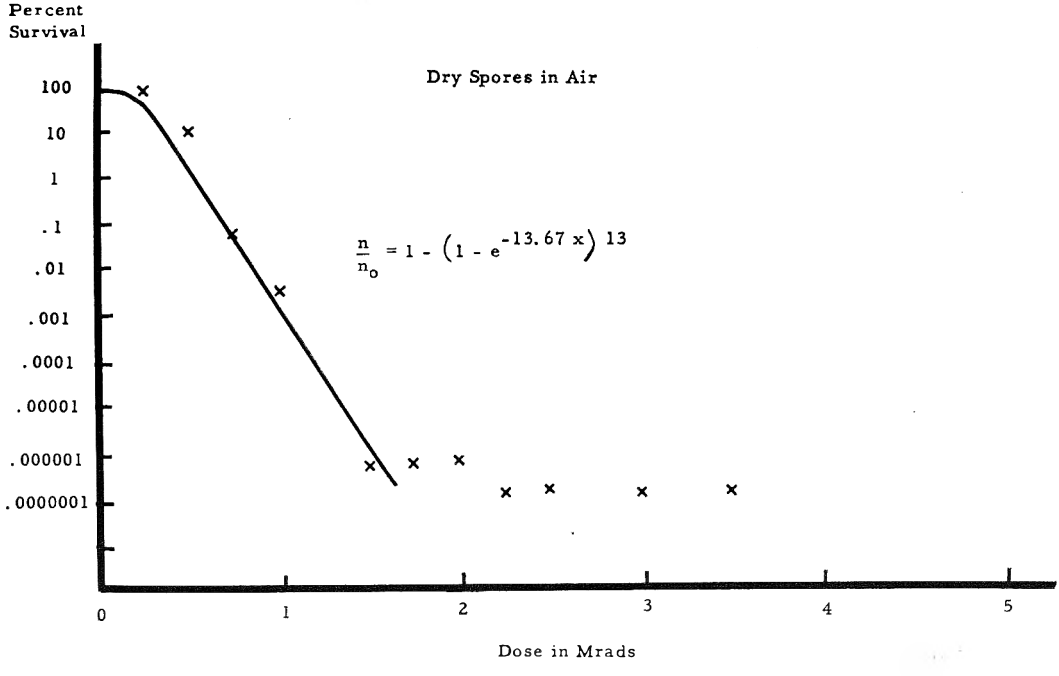

Fig. 4. Survival of *Clostridium botulinum* subjected to gamma irradiation.

thermocouple was used to measure the change in temperature during irradiation; as on previous runs, visual examination was made of the vial's contents to observe thawing.

After the irradiation had been completed, all vials were maintained frozen at −20°F until examined for recovery of viable spores.

Survival counts. Vials were prepared for examination by thawing 10–15 min at 140°F. In Experiments 1 and 4 the vials were shaken and specified volumes of material removed for either direct culturing or the preparation of serial dilutions to result in countable numbers of spores. In Experiment 2 the procedure was varied slightly in that vials subjected to 1 Mrad and above had the vial top removed and placed on an empty sterile vial, whose top in turn was placed on the irradiated vial. One-ml portions of sterile water were added to the empty vial with the irradiated top, and the top was rinsed and the rinse water cultured. Also in this experiment, 1-ml portions of the irradiated sample were removed without shaking, but merely agitated slightly with a pipette. This was done to determine the extent to which spores had been entrapped in foam formed on the side walls of the vials at the time of shaking for the freezing. After removal of this 1-ml portion the vial was shaken and the contents cultured in the routine fashion. The results are in Table 1.

For the third experiment, 5 ml of sterile buffer was added to the vials containing the spores in the dry state. The vials were shaken and the standard culturing procedure employed. With the other 2 sets (air, phosphate-nitrogen) the vials were shaken and the regular order of culturing followed.

Pork-pea infusion agar was used for all phases of this work, with the cultures being incubated at 85°F for a period up to 2 weeks. Positive cultures in the higher dose brackets that showed only a limited number of viable spores were checked for toxin formation, by intraperitoneal injection of mice.

ANALYSIS OF DATA

The hit theory for the inactivation of microorganisms by radiation (Lea, 1956) has been widely used in interpreting radiation survival curves (Atwood and Norman, 1949; Ore, 1957; and Wijsman, 1956).

A general formula that can be used to describe survival curves where more than one hit may be required for inactivation is:

$$\text{Surviving fraction} = \frac{n}{n_0} = 1 - (1 - e^{-Kx})^N$$

where n = number of surviving organisms
n_0 = initial count before irradiation
e = 2.7183
x = dose in Mrad
N = number of hits to inactivate the organism
K = constant ($Mrad^{-1}$) for describing radioresistance

This theoretical-curve formula was fitted to experimental data by a method of least squares (Pratt, 1960) using a digital computer. Points in the tail areas of the curve were not used.

Tests for difference in radioresistance were made by analysis of variance for randomized block experiments (Snedecor, 1956) using logarithms of percent survival.

RESULTS

Experiments 1 and 2. Percent survival of approx 60×10^9 spores per ampoule irradiated in pork-pea broth in Experiment 1 is compared with percent survival of approx 37×10^9 spores per ampoule irradiated in phosphate buffer in Experiment 2. These experimental points are plotted in Fig. 2, together with theoretical multi-hit curves.

No survival occurred in pork-pea suspension receiving 5 Mrad or more. No survival occurred in phosphate suspension receiving 4.25 Mrad or more.

All dose levels from 2.1 Mrad showing growth resulted in recovery of toxic cultures.

As previously reported (Morgan and Reed, 1954; Denny *et al.*, 1959), spores exhibited less radioresistance in phosphate buffer than in pork-pea broth.

Experiment 3. Percent survival of approx 1×10^9 spores per ampoule irradiated dry in air are plotted in Fig. 4. Percent survival of spores irradiated in phosphate buffer under air is plotted in Fig. 3 with a ◇, and percent survival of spores irradiated in phosphate under nitrogen is plotted in Fig. 5.

Theoretical multihit curves in Figs. 3, 4, and 5 were fitted to the corresponding data points.

Comparison of data in the body of survivor curves indicated that the freeze-dried spores (Fig. 4) were less radioresistant (at the 5% significance level) than similar spores resuspended in phosphate buffer (indicated by ◇ in Fig. 3). Dry spores of *B. subtilis* have been reported less radioresistant than moist spores (Houtermans, 1956).

Similarly the spores suspended in phosphate buffer and irradiated in air (Fig. 3) were less radioresistant (at the 1% significance level) than spores suspended in phosphate buffer and irradiated under nitrogen.

Toxicity tests made on recovery organisms from 1.75 Mrads and higher showed all to be toxic.

Experiment 4. The percent survival of the three spore concentrations 53×10^8, 43×10^8, and

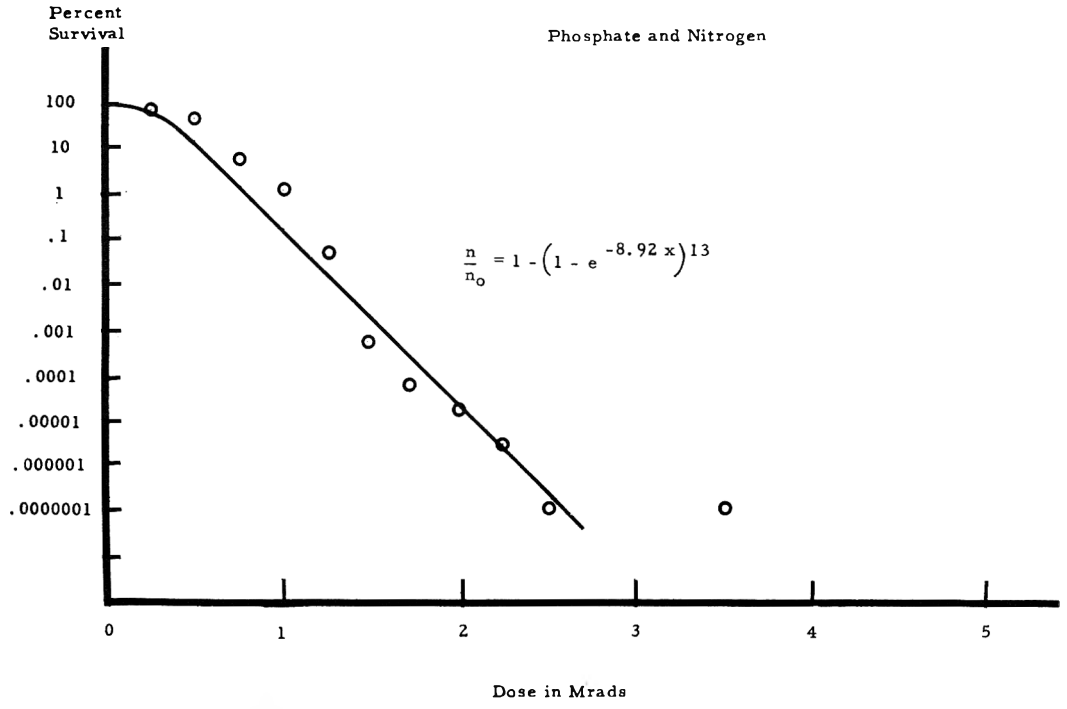

Fig. 5. Survival of *Clostridium botulinum* subjected to gamma irradiation.

37×10^3 spores per ampoule irradiated in phosphate buffer under air is plotted in Fig. 3. The percent survival of different concentrations of spores irradiated under the same conditions in Experiments 2 and 3 is also plotted in Fig. 3, with the corresponding theoretical multihit curve.

Statistical analysis of the data in the body of the curves (at lower doses) indicated no significant difference in survival rate for the five widely varying concentrations of spores: For the 53×10^9 spore concentration, no survivors were recovered at 5 Mrad; for the 43×10^6 spore concentration, no survivors were recovered at 4 Mrad; for the 37×10^3 spore concentration, no survivors were recovered at 2.25 Mrad.

Toxicity tests made on colony isolates from the high spore concentration receiving 3–4.5 Mrad, colony isolates from the intermediate spore concentration receiving 2.0–3.75 Mrad, and colony isolates from the low spore concentration receiving 1.1–2.0 Mrad indicated that all surviving colonies tested were toxic.

DISCUSSION

No survival was observed in samples receiving a 5-Mrad dose or more.

Each of the survival curves gives the appearance of being composed of two parts: 1) the body of the curve (at lower radiation doses), which is entirely consistent with hit theory; and 2) the tail of the curve (at very high radiation doses), which is not consistent with classical hit theory.

Considering first the body of the curves, all appear to be "humped" near the top rather than absolutely straight. The "humpback" curve for this organism has been previously reported by Morgan and Reed, 1954; Denny *et al.*, 1959; and Pratt *et al.*, 1959. Hit theory explains the hump as arising when more than one hit by a unit of radiation is required to inactivate a single organism.

Although the evidence is very strong that more than one hit is required for inactivation ($N > 1$), our numerical estimates of N are subject to experimental error. Based on data from every run, the least-squares estimate of the number of hits required for inactivation of spores was $N = 13$.

Let us consider next the tail areas, for without these sterility would be reached at much lower dose levels. The tail of the curves, of course, makes meaningless any extrapolation from data taken at low doses.

Thus, the tail area deserves careful attention. Since phosphate control blanks and inoculated samples receiving the highest doses were negative, and since colonies were found to produce toxin on subculture, these

recurrent tails are not easily dismissed as contamination. It first appeared that highly resistant spores in dried headspace foam might be responsible for the tail since: 1) survival counts were greater in the shaken vial (see Table 1) than when samples were drawn from the vial without shaking, and 2) appreciable counts were recovered from the cap. This explanation was discarded, however, when dry spores were found to be relatively radiosensitive.

Of course there can be no certainty that the spores were homogeneous with respect to their radioresistance. It is possible that even a very low percentage, e.g., 0.00001%, of very highly resistant spores could give rise to the data in Fig. 2. Similar reasoning would apply to all of the curves.

No study was made of whether survivors of high dose levels of irradiation would actually grow in the medium in which they were irradiated.

The body of the survival curve of *Cl. botulinum* in phosphate buffer did not seem to be affected by the concentration of spores used. The location of the tail of the curve, on the other hand, was a function of the concentration of spores. Fig. 3 demonstrates these phenomena for 5 concentrations of spores from Experiments 2, 3, and 4.

Thus, the survival of spores of *Cl. botulinum* at high dose levels (appearing as a tail on the survivor curves) remains an enigma that deserves further study.

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Antimicrobial Agent of Aged Surface Ripened Cheese. II. Sources and Properties of the Active Principle(s)[a, b]

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(Manuscript received June 27, 1961)

SUMMARY

Antibiotic activity in aged Liederkranz cheese was found to be produced by the aerobic microorganisms inhabiting its surface. *Brevibacterium linens* was the principal source of the antimicrobial agent(s). Yeast of the *Candida* type and, when present, some other microorganisms contributed minor antimicrobial activity. The antimicrobial agent(s) of aged Liederkranz cheese had the following properties: 1) dialyzable through cellulose casing, 2) absorbed on Norit A at acid pH, 3) stable in acid and alkaline solutions ranging from pH 2.0 to 12.0 at 2–4° C, 4) destroyed within 20 hr in alkaline solution at pH 12 at room temperature, 5) in aqueous solution stable to heating at 121° C for 10 min at acid pH(pH<5.0) but decreased activity at pH 5.0 to 8.5, 6) in agar medium of pH 6.8–7.0 activity remained after heating at 121° C for 45 min, 7) soluble in water, methanol, ethanol, and butanol, slightly soluble in acetone and insoluble in ethyl ether, carbon tetrachloride, chloroform and ethyl acetate, 8) inhibited growth of many gram-positive and gram-negative bacteria as well as several yeasts and molds, and 9) related to antibiotic from *B. linens* but distinct from nisin, an antibiotic commonly associated with dairy products. In a survey of market samples of cheeses to determine whether antibiotic activity was present, only surface-ripened cheeses with a brown smear made up of bacteria were found to contain antibacterial activity. Other types of cheeses which did not develop this brown smear did not possess this activity. It may be concluded that antibiotic activity is contained in the brown bacterial surface smear that develops during ripening.

INTRODUCTION

In a previous study (Grecz *et al.*, 1961) an antimicrobial agent in surface ripened cheese was reported. Since the publication of that report three questions have arisen: 1) What is the source of the agent? 2) What are its properties? 3) How prevalent is the agent in market cheeses? The present article is devoted to answering these questions.

MATERIALS AND METHODS

Pure cultures of microorganisms were isolated from the surface flora of cheese by standard plating procedures on tryptone glucose extract agar (Difco). For the isolation of yeasts, 250 μg/ml streptomycin, 250 μg/ml penicillin, and 1% glucose were added; for the isolation of *Brevibacterium linens* and other cheese halophiles, milk and 7.5% NaCl were added to the basic tryptone glucose extract agar.

[a] This study was supported in part by a grant from the National Cheese Institute and in part by a grant-in-aid from the United States Public Health Service (RG 5837).

[b] The data reported are from a Ph.D. thesis by N. Grecz at the Illinois Institute of Technology. This project constituted a cooperative program between the Biology Department of the Illinois Institute of Technology and the Food Research Institute of the University of Chicago.

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แผนกห้องสมุด กรมวิทยาศาสตร์

Isolates from aged Liederkranz cheese were grown for antibiotic production in the following broth (composition expressed as percentage): corn-steep liquor (52% solids), 3; yeast extract, 0.3; peptone, 0.5. The corn-steep liquor was dissolved in distilled water, adjusted to pH 7 with 5*N* NaOH, and autoclaved 30 min at 15 lb steam pressure. A heavy precipitate that formed was removed by filtration through adsorbent cotton, and the solution was further clarified by addition of 0.2% Norit A and repeated filtration. After all precipitate was removed, peptone and yeast extract were added. The medium was once more adjusted to pH 7 and filtered when necessary. The clear broth, in 50- and 25-ml quantities, was transferred to 250- and 125-ml Erlenmeyer flasks and sterilized by autoclaving 15 min at 121°C. This medium supported good growth of virtually all microorganisms tested.

Isolates were washed from agar slants at the peak of their development, which was usually after 48 hr of incubation at 22–25°C, and inoculated into the corn-steep liquor broth. The broth cultures were incubated at 22–25°C on a shaker (125 rpm) in duplicate flasks. The clear centrifugates were tested for antibiotic production after 3, 5, 7, and 10 days of incubation.

Aliquots of 0.25 ml of the culture centrifugates were dried on filter-paper discs [Carl Schleicher & Schuell Co., Keene, N. H., No. 740-E paper, ¼ in. (6.35 mm) in diameter] with the aid of a special loading pipette, namely a 1-ml pipette, the tip of which was drawn out to a capillary and bent upward. When the loading pipette was filled with the desired amount of liquid, a paper disc was affixed at the capillary end. The paper disc was allowed to remain on the loading device until all the liquid was consumed and the discs were completely dry.

The paper discs were then placed on an agar sheet of uniform thickness seeded with spores of *Bacillus cereus* 800/58 (approx. 10^4 spores per ml of agar). The assay plates were incubated overnight at 22–25°C in an atmosphere saturated with moisture. The size of the clear zone of growth inhibition around a paper disc was taken as an index of antimicrobial activity. The zones of growth inhibition were read with a modified Quebec colony counter (Grecz, 1961).

Survey of market cheese for antibiotic activity. Cheese samples were purchased from local stores and a clear extract was prepared as follows: One weight of cheese (approx. 50 g) was triturated in a mortar with one weight of distilled water. The suspension was poured into a Visking dialysis bag; the ends of the bag were carefully tied in order to prevent leakage. The dialysis bag was then placed into a glass beaker and covered with two weights of distilled water. The sample was allowed to equilibrate two days at 2–4°C. Antibiotic activity of clear dialysates was assayed by the method described above, using *B. cereus* 800/58 as a test organism.

In determining the properties of the antimicrobial agent(s) of aged Liederkranz cheese, aqueous cheese extracts (Grecz *et al.*, 1961) were used.

RESULTS

Source of antimicrobial activity of aged Liederkranz cheese. In a series of preliminary experiments (see Figs. 1 and 2) sterile extract from fresh cheese was inoculated with the mixed surface flora of Liederkranz cheese in order to simulate the natural process of microbiological aging of surface ripened cheese. The surface flora from fresh cheese was compared with that of aged cheese since the predominant microbial flora of Limburger-type cheese is known to vary in a definite sequence during the progress of ripening and aging (Kelly, 1937).

Incubation of the aqueous Liederkranz cheese extract inoculated with the mixed microbial surface flora of Liederkranz cheese (Figs. 1 and 2) resulted in antimicrobial activity. By contrast, the antimicrobial activity of the uninoculated control sample, incubated under similar conditions, decreased appreciably. The samples incubated at 22–25°C without shaking yielded basically the same data as those incubated with shaking except that the antimicrobial activity formed more

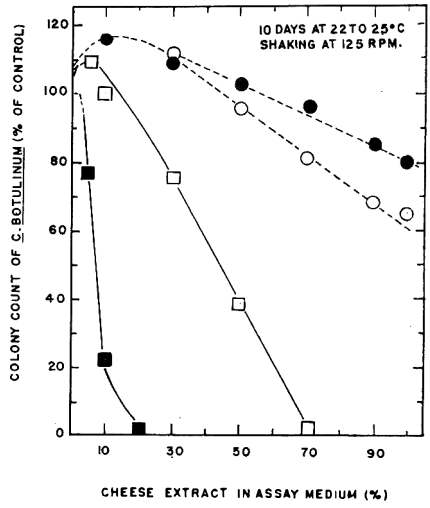

Fig. 1. Antimicrobial activity of extracts of Liederkranz cheese after shaking (125 rpm) for 10 days at 22–25°C. Sterile filtrate of fresh Liederkranz cheese: ○ - - - ○ at the start of the experiment, ● - - - ● after incubation for 10 days. Cheese extract inoculated with mixed surface flora of Liederkranz cheese: □ - - - □ from 2-week-old cheese, ■ - - - ■ from 6-week-old cheese.

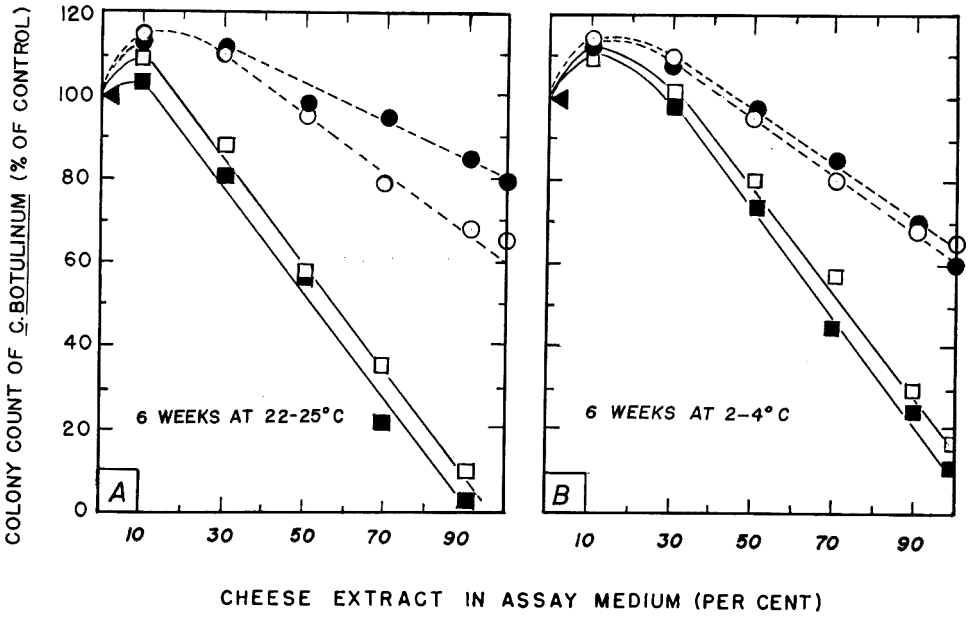

Fig. 2. Assay of the antimicrobial activity of extracts of fresh cheese inoculated with the mixed surface flora of Liederkranz cheese. The flasks were incubated for 6 weeks without shaking at 22–25°C(A) and 2–4°C(B). Sterile filtrate of fresh Liederkranz cheese: ○ - - - ○ at the start of the experiment, ● - - - ● after incubation for 10 days. Cheese extract inoculated with mixed surface flora of Liederkranz cheese: □ - - - □ from 2-week-old cheese, ■ - - - ■ from 6-week-old cheese.

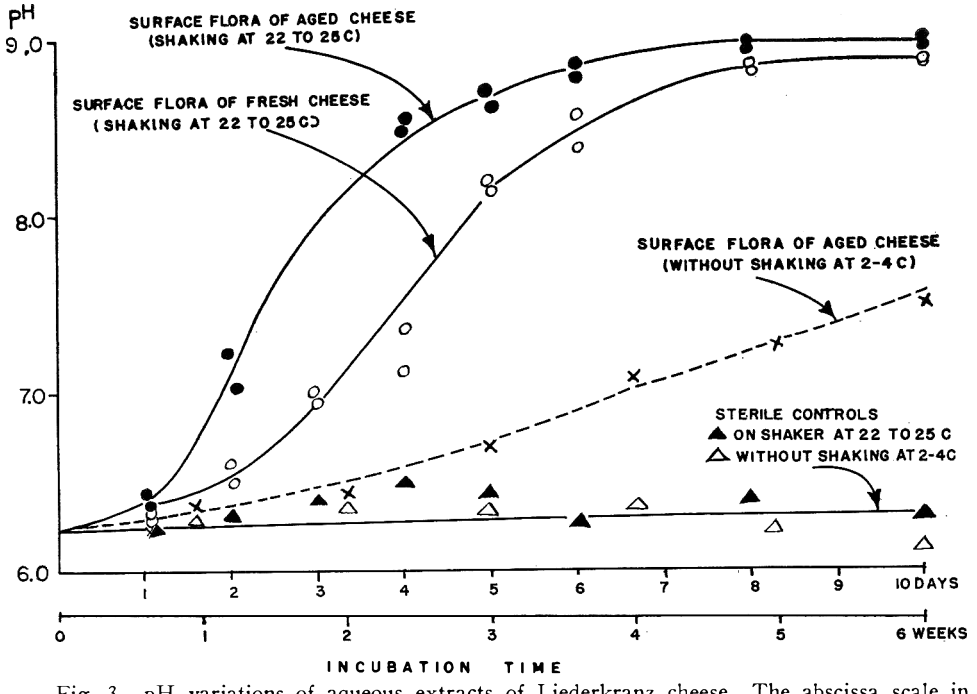

Fig. 3. pH variations of aqueous extracts of Liederkranz cheese. The abscissa scale in days pertains to shake cultures, whereas that in weeks pertains to cultures not shaken.

rapidly in shaken cultures (10 days at 22–25°C in a shaken culture as opposed to 6 weeks in the stationary culture). The pH changes of the samples indicated that a rise in pH was always commensurate with a rise in antimicrobial activity (Fig. 3).

The data pointed to an aerobic process as the source of antimicrobial activity (Fig. 4) since no activity was produced in cheese cultures incubated with shaking in an atmosphere of nitrogen. It is indicated, on the basis of these model system experiments, that the antimicrobial principle(s) of Liederkranz cheese was produced by aerobic microorganisms in the surface flora of this cheese.

Screening for antibiotic-producing microorganisms. Six varieties of cheese that had developed a brown surface smear during ripening and which showed consistent antimicrobial activity were selected for screening of their surface microorganisms for antibiotic production. The cheeses studied were Harzkaese (German), Liederkranz (USA), Limburger (USA), St. Mang (German), Tilsiter (German) and Trappist (USA).

A striking similarity in the composition of the surface flora of these six varieties of cheese was observed. The differences appeared quantitative rather than qualitative. The antimicrobial activities of the major colonial types isolated are summarized in Table 1. It will be noted that out of a total of 158 cultures tested 95 possessed discernible antimicrobial activity. The groups of microorganisms which did not develop antimicrobial activity were not further identified.

Orange chromogenic colonies (groups 1 and 2) (Table 1) were identified on the basis of their yellow orange pigmentation, short-rod morphology, gram-positive staining, catalase production, growth in milk, and, finally, characteristic color reactions with bases and acids as described by Grecz and Dack (1961). The colonies were identified as typical *B. linens* except as noted in Table 1.

Table 1. Antimicrobial activity of representative isolates from six varieties of surface-ripened cheese.

Groups of microorganisms [a]	Strains examined			Clear zone of growth inhibition of *B. cereus* 800/58	
	Total	Antibiotically active	Per cent antibiotically active	Range (mm)	Average (mm)
1. Orange chromogens					
a) Characteristic smooth *B. linens type*	35	34 [b]	97	1.0–2.5	1.6
b) Pale, translucent *B. linens* type with tough, leathery slime	37	36 [b]	97	0.25–3.5	1.2
c) Not characteristic *B. linens* type [c]	3	3	100	0.5–2.5	1.5
d) Bright orange, dry, granular type	5 [d]	0	0	...	0.0
2. Orange-yellow, wrinkled tough cultures with leathery slime	9	9	100	0.5–2.6	1.7
3. Yellow chromogens	9	1	11	0.0–1.5	0.15
4. Red chromogens	5	0	0	...	0.0
5. Cream-to-Gray colonies	23	6	26	0.4–0.9	0.1
6. Yeasts of the *Candida* type (mostly gray, some pink)	26	4	15	1.5–2.0	0.3
7. Molds	4	0	0	...	0.0
8. Streptomycetes	2	2	100	1.1–1.6	1.4
Total	158	95	60.1	0.0–3.5	

[a] Only representative microorganisms were tested.

[b] The culture possessing no antimicrobial activity showed only weak growth in laboratory broth cultures.

[c] Three cultures with orange pigmentation that gave no characteristic red color with NaOH. These cultures had pigment components probably quantitatively different from those of typical *B. linens* organisms, but otherwise belonged to group 1a in this table.

[d] Many microorganisms belonging to this group were isolated, but not tested for antimicrobial activity.

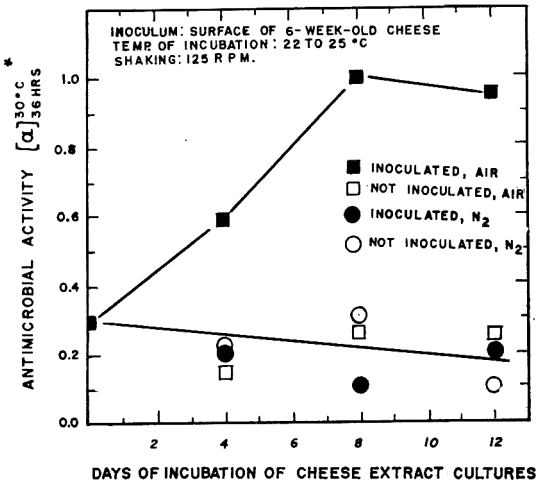

Fig. 4. Effect of air and nitrogen atmosphere on the development of antimicrobial activity in extracts of Liederkranz cheese. Anaerobic conditions were established by bubbling nitrogen through flasks for 30 min and sealing with rubber stoppers and parafilm.

* The term antimicrobial activity [a] was defined as the specific antibiotic activity of the sample. $[a]^{30°C}_{36\ hr} = 1 - \frac{\alpha}{\beta}$; where α was the colony count in the bioassay tube containing the indicated fraction, and β was the count in the control tube containing the assay medium alone; 30°C and 36 hr indicate temperature and time of incubation.

Almost all colonies that were slimy orange and orange-yellow possessed antimicrobial activity (Table 1; groups 1a, 1b, 1c and 2). Poor growth in the test medium accounted for the exceptions.

Orange-colored colonies of dry, rough, granular appearance were antibiotically inactive (Table 1, 1d). These organisms were closely related to the general *B. linens* type except for the rough appearance and lack of antimicrobial activity. In several respects the rough colonies resembled *Brevibacterium erythrogenes*. They were somewhat longer than the regular *B. linens* type; in milk they produce a precipitate of casein, leaving a clear milk serum. On rare occasions this serum became blood red, but usually it remained colorless. The typical smooth colonies of *B. linens* produced a general browning of the milk (Breed *et al.*, 1957) but no coagulation. Both types gave typical color reactions with strong bases and acids (Grecz and Dack, 1961).

Yellow strains (group 3). Although one yellow strain gave appreciable antimicrobial activity in several tests, 89% of the yellow strains were entirely inactive (Table 1, group 3). The group was composed of short gram-positive rods.

Red colonies (group 4). The microorganisms producing red colonies were small gram-positive cocci; they possessed no antimicrobial activity (Table 1, group 4).

Gray-to-cream colonies (group 5). Twenty-six per cent of the gray-to-cream colonies possessed low to medium-strong antimicrobial activity (Table 1, group 5). Some of these strains were derived from characteristic *B. linens* type cultures, which had lost the ability to produce orange pigmentation after repeated subcultures. The cream-to-gray colonies were either short gram-positive rods (11 strains, including the six antibiotically active cultures) or minute gram-positive cocci (12 strains). All were salt-tolerant and catalase-positive. The antibiotically active cultures appeared to be pure strains.

Yeasts (group 6). Several yeasts (15% of the total number tested) produced appreciable activity that could be confirmed in repeated tests. The majority of yeasts, however, were antimicrobially inactive (Table 1, group 6). The yeast isolates were made up of single oval budding cells (5 strains), double and single fission-type cells (8 strains) and mycelial budding forms of the general *Candida* type (13 strains). The four antibiotically active cultures belonged to the mycelial type. All strains were salt-tolerant film yeasts and gave an acid reaction in litmus after 4–5 days. In sterile milk, a viscous clear fluid was formed in 7–15 days, which was either clear or pale rose in color. On tryptone glucose extract agar the colonies were gray, dry, and wrinkled, except for one culture of oval budding yeasts, which formed a slimy, pale-rose colony. The production of antibiotic by yeasts isolated from cheese is of interest, since it has been recently reported that many *Candida* yeasts are antibiotically active (Emmanouilidou-Arseni and Soultani, 1960).

Molds and streptomycetes (groups 7 and 8). Molds and streptomycetes were rarely found; the few isolates may be accidental contaminants of the cheese. Selective plates from which bacteria were excluded by addition of penicillin and streptomycin developed yeasts but not molds or streptomycetes. The molds (Table 1, group 7) produced no discernible *in vitro* activity, and therefore were ruled out as possible causal agents of the antimicrobial activity. On the other hand, the two streptomycetes (Table 1, group 8) possessed moderate antibiotic activity. Since they were rarely found (less than 0.4% of 500 isolates, including those not tested for antimicrobial activity), they also may be dismissed as contributing to the natural antimicrobial activity of aged surface-ripened cheese.

In summary, it may be concluded that the major part of the antimicrobial activity of aged

Liederkranz- and Limburger-type cheese was due to microorganisms of *B. linens* type in the surface flora. Further antibiotic activity may come from other organisms, particularly yeasts of the *Candida* type and other organisms which are unable to predominate in the natural flora of aged cheese. Although some yeasts have produced appreciable antibiotic activity in broth cultures, their role in the natural antibiosis of aged cheese was questioned because the antibiotic in cheese appears during the late ripening stages when yeasts are known to disappear from the cheese flora.

The growth of natural yeasts occurring predominantly on fresh Limburger-type cheese (Kelly, 1937) may be later inhibited by the substances produced by bacteria predominating in aged cheese. Therefore, 15 typical isolates of mycelial budding and fission film-forming yeasts from Limburger cheese were tested against the extract from aged Liederkranz cheese. Incorporation of 50–100 mg/ml of the lyophilized extract into the culture medium completely eliminated yeast growth, 12.5–25.0 mg/ml retarded the growth appreciably, whereas 6.2–12.5 mg/ml exerted a noticeable stimulatory effect.

Properties of the antimicrobial agent(s) of aged Liederkranz cheese. The antimicrobial agent(s) was dialyzable through Visking cellulose casing. It was adsorbed on Norit A at pH 5.8–6.3 but not at pH 9.5. The antibiotic agent(s) could be eluted from Norit A with 40% ethanol, yielding a 2.4-fold purification of the initial aqueous extract.

During heating at 121°C for 10 min in aqueous solutions the antimicrobial agent was most stable in the acid pH range (pH < 5.0), but showed a decrease in activity at pH 5.0–8.5. When heated in beef infusion agar (6.8–7.0) the preparation remained inhibitory to the growth of *Clostridium botulinum* after 45 min at 121°C.

Treatment with hydrochloric acid and sodium hydroxide, at pH 2.0 to 12.0 for 20 hr had no apparent effect on the activity of the antimicrobial agent(s) at 2–4°C, although at room temperature 20 hr at pH 12.0 caused a complete loss of activity.

The antimicrobial agent was soluble in water, ethanol, methanol, and butanol, slightly soluble in acetone and insoluble in ethyl ether (pH 2.0), carbon tetrachloride, ethyl acetate and chloroform. Acetone extraction of dehydrated preparations of aged Liederkranz cheese yielded approximately 3.6% of highly inhibitory extract (see Fig. 5).

Biological activity. The biological activity of crude lyophilized preparations was established by the agar slant dilution method. One antibiotic unit was defined as the amount causing complete inhibition of growth of *Staphylococcus aureus* 223 for 24 hr at 30°C. The actual antibiotic potency of crude lyophilized powders varied somewhat from preparation to preparation; one unit was equal to 12.5–50.0 mg of powder per ml of agar media at pH 7.

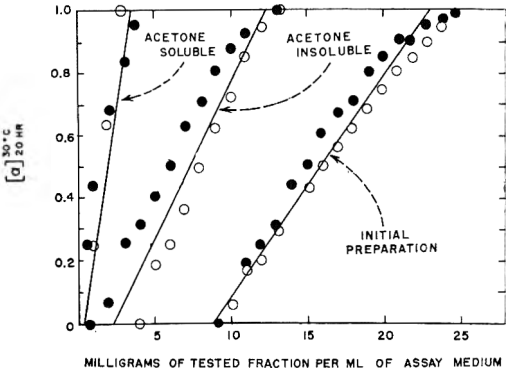

Fig. 5. Specific antimicrobial activity of fractions obtained by acetone extraction of lyophilized Liederkranz cheese extract.

[a] was defined as the specific antibiotic activity of the sample. $[a]^{\frac{30°C}{20\ hr}} = 1 - \frac{\alpha}{\beta}$; where α was the colony count in the bioassay tube containing the indicated fraction and β was the count in the control tube containing the assay medium alone; 30°C and 20 hr indicate temperature and time of incubation.

The *in vitro* biological activity of crude antibiotic preparations of aged Liederkranz cheese (expressed in units per ml of assay medium) causing complete growth inhibition of the re-

Table 2. pH dependence of the activity of the antimicrobial agent(s) of aged Liederkranz cheese.

	Growth of the test oragnism [a]			
	B. cereus 800/58		*Candida albicans* 10231	
pH	No antibiotic added	Antibiotic added [b]	No antibiotic added	Antibiotic added [b]
5.0	—[c]	—	++++	—
5.5	+	—	++++	—
6.0	+++	—	++++	+++
6.5	++++	+	++++	+++
7.0	++++	+	++++	+++
7.5	++++	+	++++	++
8.0	++++	—	++++	—
8.5	++++	—	++++	—

[a] Results were similar with *Staphylococcus aureus* 223, *Saccharomyces cerevisiae* Y977, and a yeast isolate of the *Candida* type from Liederkranz cheese.

[b] These tubes received 25 mg/ml of lyophilized aqueous extract of aged Liederkranz cheese.

[c] Growth estimated as ++++ abundant, +++ good, ++ retarded, + faint, and — no growth.

spective microorganisms was as follows: *Bacillus cereus*, 0.5; *Escherichia coli*, 2; *Clostridium botulinum*, 1; *Salmonella typhimurium*, *S. typhi*, *S. pullorum*, 1-2; *Mycobacterium avium*, *M. tuberculosis*, *M. smegmatis*, 0.5–1; *Staphylococcus aureus* (6 strains other than strain 223), 2; *Neurospora crassa*, 1; *Penicillium roquefortii*, 4; *P. italicum*, 2; *Rhizopus nigricans*, 2; *Saccharomyces cerevisiae*, 2; *Candida albicans*, 2; *Hansenula anomala*, *H. saturnus*, *H. capsulata*, 2; *Torulopsis holmii*, 2; *Streptomyces lavendulae*, 0.5.

pH effect. The antimicrobial agent(s) of aged Liederkranz cheese was most active at pH 5.0–6.0 and pH 7.5–8.5 (Table 2). At the neutral pH range (pH 6.5–7.0) the activity of the antibiotic was appreciably reduced.

Relation to antibiotic from Brevibacterium linens. The following properties of the antibiotic of *B. linens* were found to be the same as those of the antibiotic agent(s) extracted from aged Liederkranz cheese: dialysis through Visking cellulose casing, adsorption on Norit A, solubility in solvents, stability to heating, stability to pH, and the antimicrobial spectrum.

These similarities suggested a close relationship between the antibiotic from aged cheese and that produced by *B. linens*. However, the final proof for the identity of the two active agents can be established only after chemical characterization of the compounds involved.

Relation to antibiotic nisin. Since nisin is commonly associated with dairy products, nisin was compared with the antibiotic of aged Liederkranz cheese.

Antibiotic assays revealed that 100,000 units/ml of nisin (Producers Creamery Co., Springfield, Mo.) failed to inhibit the growth of several yeasts, molds, and gram-negative bacteria, *viz.*, *Candida albicans* 10231, *Saccharomyces cerevisiae* Y977, *Torulopsis holmii* Y1507, *Penicillium expansum*, *Aerobacter aerogenes*, and *Escherichia coli*. On the other hand, only 1,000 units/ml of nisin was required to arrest the growth of two gram-positive bacteria, *viz.*, *Bacillus cereus* 800/58 and *Clostridium botulinum* 62A. These data indicate that nisin and the antibiotic of aged Liederkranz cheese possessed completely different antimicrobial spectra.

Survey of market cheese for antimicrobial activity. Results of a typical assay of the antibiotic activity of clear dialysates of market samples of cheese are given in Fig. 6.

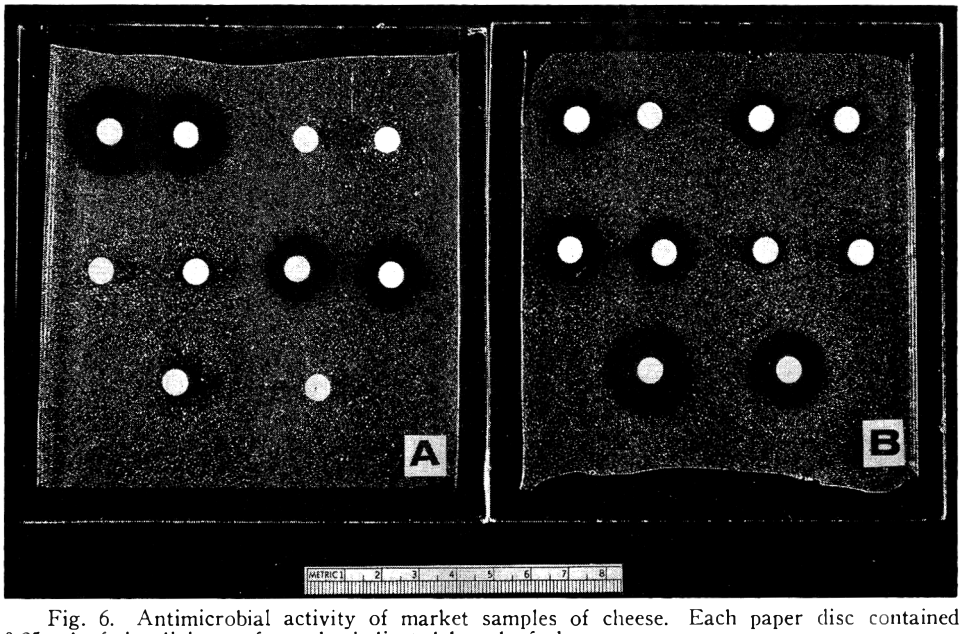

Fig. 6. Antimicrobial activity of market samples of cheese. Each paper disc contained 0.25 ml of the dialysate from the indicated brand of cheese.

Plate A. 1) St. Mang Limburger, well ripened, surface; 2) St. Mang Limburger, well ripened, interior; 3) Mozzarella; 4) Provolone; 5) Port Salut, fresh, sliced, surface; 6) Port Salut, fresh, sliced, interior; 7) Trappist, well ripened, surface; 8) Trappist, well ripened, interior; 9) Cheddar; 10) Swiss.

Plate B. 1) Kraft Limburger, medium ripened, surface; 2) Kraft Limburger, medium ripened, interior; 3) Brick, medium ripened, surface; 4) Brick, medium ripened, interior; 5) Tilsiter, well ripened, surface; 6) Tilsiter, well ripened, interior; 7) Borden Camembert, fresh, surface; 8) Borden Camembert, fresh, interior; 9) Liederkranz, well ripened, surface; 10) Liederkranz, well ripened, interior.

In addition to the samples listed in Fig. 6 the following brands of market cheese contained antimicrobial activity: Beerkaese, Muenster, Romadour, Harzkaese (surface only); no activity was detected in Ski Queen, Harz (interior); cottage cheese, and cream cheese.

From these results it was concluded that antimicrobial activity was present only in those brands of cheese that develop a brown bacterial surface smear during ripening. Well ripened cheese had a higher activity than fresh cheese, and the activity was concentrated in the surface portion of the cake, especially when a rich-brown surface smear was present.

DISCUSSION

Antimicrobial agent(s) in aged surface-ripened cheese appeared to be adsorbed on substances in the cheese (Grecz *et al.*, 1961). Such an adsorption may affect the apparent properties of the antibiotic activity, e.g., resistance to heating, rate of dialysis, extractibility by solvents. In turn, adsorption may lead to a reduction of the antibiotic activity in the assay substrate.

The gradual accumulation of the antibiotic agent(s) in aged surface-ripened cheese suggests that natural antibiosis may play a role in the ripening process of these types of cheese. For example, the overgrowth of the surface flora of Limburger cheese by *Brevibacterium linens*-type organisms (Kelly, 1937) may be conveniently explained as the antibiotic production by this organism. The exact mechanism by which *B. linens* is able to predominate in the flora on the surface of Limburger and related types of cheese during the late stages of ripening has never been satisfactorily explained.

It is of interest that Professor Victor C. Vaughan made the observation as long ago as 1884 that old foul-smelling cheeses such as Limburger and Schweizer, are alkaline in reaction and that poisoning never results from their use. The reason is only now becoming clear. The present investigation would suggest that an added safety factor in the prevention of food poisoning may be obtained by allowing surface-ripened cheeses which contain *Brevibacterium linens* to age before use.

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Spectrophotometric Estimation of Metmyoglobin in Frozen Meat Extracts [a]

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SUMMARY

Frozen meat-water extracts stored under lights exhibit a pattern of metmyoglobin formation similar to that in frozen steaks. The spectrophotometric method of analysis was satisfactory for estimating the percent metmyoglobin in the solutions. Fluorescent lights significantly increased the formation of metmyoglobin in the solutions, similar to that reported for frozen steaks. Dialyzing the extract inhibited the increase of metmyoglobin in the frozen solution when stored under light. Addition of copper, manganese, or calcium ions in the form of the chloride salts to the dialyzed solution did not increase rate of metmyoglobin formation appreciably. The addition of chloride salts of magnesium and iron contributed to an increase in the rate of metmyoglobin formation. When the dialysate was concentrated and added to the dialyzed residue there was a significant increase in the rate of metmyoglobin formation.

Desirable color, while not necessarily affecting palatability or nutritive value of meat, is one of the primary quality attributes demanded by the consumer. The oxidative states of myoglobin are directly associated with meat color. Reduced myoglobin is the dark red-purple color of freshly cut meat; the oxygenated form, oxymyoglobin, is the bright desirable color; and the oxidized form, metmyoglobin, is the undesirable brownish color (Brooks, 1937; Grant, 1955).

The practice of merchandising frozen meat in transparent wrappers under display-case illumination has been accompanied by several technical problems (Townsend and Bratzler, 1958). Such things as storage temperature (Ramsbottom, 1947), rate of freezing (Robertson, 1950; Ramsbottom and Koonz, 1941), and display lights (Naumann *et al.*, 1957) may harm the quality of the meat product.

Townsend and Bratzler (1958) found that fluorescent lights caused an increase in the rate of metmyoglobin formation and that this was correlated with a deterioration in the lean meat color. Primarily responsible for the color degradation was light between wavelengths of 560 mμ and 630 mμ, in the yellow and orange portion of the visible spectrum. It was concluded that the increase in color degradation was due to a photodynamic phenomenon that accelerated the oxidation of the muscle pigment myoglobin to metmyoglobin.

This report is concerned with the adaptation of frozen muscle-water extracts to spectrophometric analysis of metmyoglobin. The effects on the frozen extracts of fluorescent illumination, dialysis, addition of the concentrated dialysate, and the addition of various metal ions were studied in an attempt to elucidate the mechanism of the light-catalyzed color degradation of fresh-frozen meat products.

EXPERIMENTAL PROCEDURE

Portions of beef rounds were frozen at −20°F prior to preparation of the extracts. Slices of the frozen round were removed, trimmed of all external fat, connective tissue, and the outside layer of lean. The samples were cut into small cubes, weighed to the nearest 0.1 g, and placed in a chilled Waring blender. Distilled water was

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added 8:1 and the mixture blended 2 min. The slurry was centrifuged 30 min at 2500 rpm at 32°F, and the supernatant decanted through absorbent cotton to remove the accumulated fat. The clear solution was filtered through No. 42 Whatman filter paper in a chilled Buchner funnel. This filtration was the only step in the preparation that was performed at a temperature above 34°F. The filtered solution was frozen either immediately or after the various treatments were applied.

The solution was divided into 35–50-ml portions and held 4–14 hr at −20°F in a blast freezer. The frozen samples were then removed from their containers, placed in Dow Saran freezer bags, and sealed under vacuum. The extracts were stored either in a walk-in freezer under approx. 25 ft-c of fluorescent light or in a cycling 0°F frozen meat display case under 50–60 ft-c of fluorescent illumination.

The effects of light on the solutions were observed by comparison of frozen extracts stored under 50 ft-c with extracts retained in darkness. The effect of dialysis was studied by dialyzing the extracts in cellulose casings against 4 L of distilled deionized water for three days with two changes of water per day. In some instances, the chloride salts of magnesium, manganese, iron, calcium, and copper were added to the dialyzed solution in the concentrations reported by Mittledorf and Landon (1952). In another trial, the dialysate was boiled down and added back to the residue solution.

The treated samples and controls were removed after varying storage periods and thawed at 98°F. The extracts were again centrifuged at 2500 rpm at 32°F, and filtered as before. The percent metmyoglobin was determined spectrophometrically according to the method described by Mangel (1951) and Townsend (1958).

RESULTS AND DISCUSSION

Effect of light on aqueous extracts. Utilizing steaks to determine color changes is expensive and not easily adaptable to quantitative measurement. An aqueous meat extract provides homogeneneity that is more suitable for spectrophotometric analysis. Fig. 1 shows the behavior of frozen extracts when stored under light in the same 0°F case as compared with frozen steak (Townsend, 1958). The increases in metmyoglobin formation are comparable, and the resultant curves do not fluctuate as greatly as when steaks are used.

Effects of dialysis and metal ions. The removal of all dialyzable material from the solution markedly retarded the rate of metmyoglobin formation (Fig. 2), but not completely. It may be that some substance, or combination of substances, removed by dialysis causes a supplementary effect on

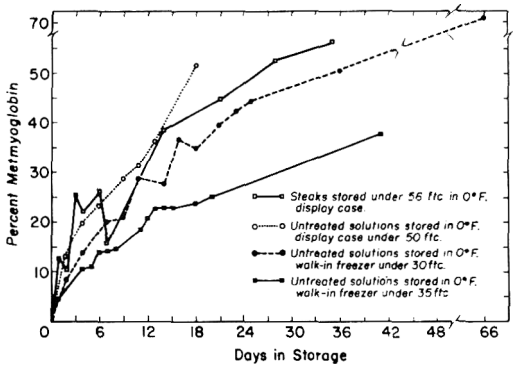

Fig. 1. Percent metmyoglobin of frozen steaks and meat solutions stored at 0°F under fluorescent light (from Townsend, 1958).

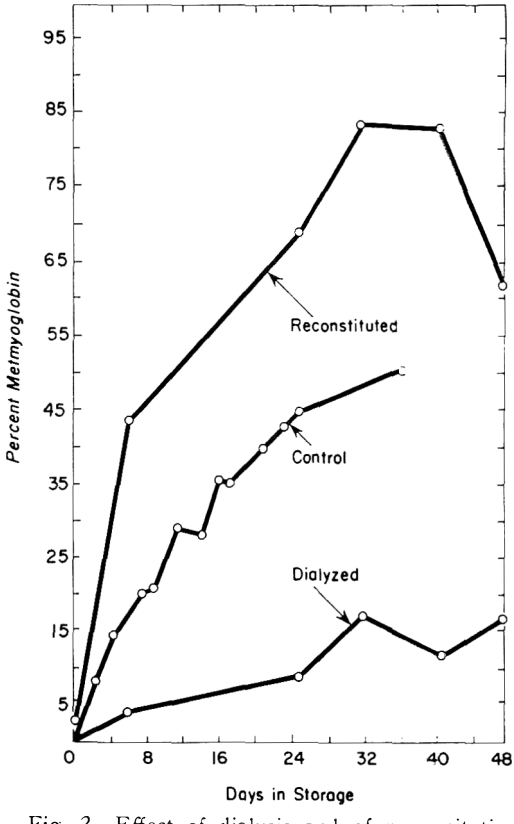

Fig. 2. Effect of dialysis and of reconstitution of a dialyzed solution on metmyoglobin formation of frozen extracts stored at 0°F in a walk-in freezer under 30 ft-c.

the light-catalyzed formation of metmyoglobin over and above the direct effect on the myoglobin molecule itself. Reconstituting the dialyzed aqueous extract, freezing, and storing under light indicated that there is some constituent, or combination, actually removed during dialysis that contributes to the increase in the rate of metmyoglobin formation in frozen extracts stored under light. Although the concentrated dialysate was added to the residue solution at a rate calculated to approximate the original concentration, the high percentage of metmyoglobin may have been due to differences in dialysate concentration in the reconstituted extract. The control curve shown is typical of those obtained when untreated frozen extracts were stored under fluorescent light. The presence of iron, bromine, phosphorus, magnesium, manganese, calcium, and copper was found by partial spectroscopic analysis of the concentrated and ashed dialysate.

The metals selected for study were magnesium, manganese, iron, copper, and calcium. Fig. 3 presents the effects of these ions on the rate of metmyoglobin formation in extracts stored in a 0°F display case under 60 ft-c. The undialyzed control had the highest average percent metmyoglobin (35.2), and the solution with the added magnesium ions had the next highest (21.7 %.) Both of these groups had statistically greater ($P < .05$) amounts of metmyoglobin than the dialyzed control, which had the lowest average percent (10.5). The iron group had the third-highest average percent metmyoglobin (16.1), but was not statistically higher than the dialyzed control. Calcium (10.7%), manganese (11.1 %), and copper (11.8%) were not significantly higher in percent metmyoglobin than the dialyzed control. Another trial in which ions of manganese, magnesium, and iron in the ferrous and ferric form were added to the residue solution showed the same results with no difference due to the form of iron used. It is possible that a combination of ions, or others than those studied, may be responsible for the increased metmyoglobin formation in frozen meat extracts stored under light.

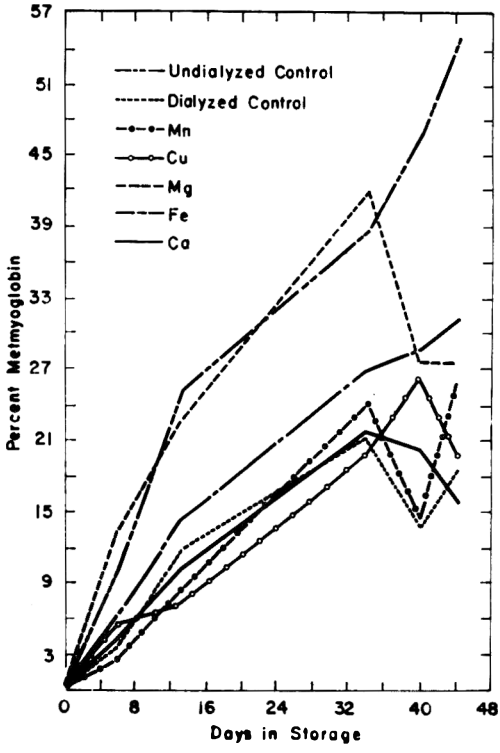

Fig. 3. Effect of metal ions on rate of metmyoglobin formation of dialyzed frozen meat extracts stored under 60 ft-c.

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A Comparison of Two Colorimetric Methods for Determining Reduced Ascorbic Acid in Frozen Peas [a,b]

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SUMMARY

The sodium 2,6-dichlorobenzenoneindophenol method (Method A) and the diazotized 4-methoxy-2-nitroaniline method (Method B) for measuring reduced ascorbic acid in frozen peas were compared. A randomized incomplete block design with 12 replications for each of the two methods of ascorbic acid analysis was used. Very highly significant differences in ascorbic acid values were obtained between Methods A and B, with Method A always the higher.

The retention of reduced ascorbic acid in fruits and vegetables has been one criterion of quality. A number of methods have been used to measure reduced ascorbic acid. Since no one method appeared to be entirely satisfactory for measuring reduced ascorbic acid, it seemed desirable to compare two methods considered applicable to a large number of routine laboratory analyses.

The sodium 2,6-dichlorobenzenoneindophenol method (Loeffler and Ponting, 1942) depends directly on an oxidation-reduction reaction, whereas Schmall *et al.* (1953) suggested that the diazotized 4-methoxy-2-nitroaniline method is highly specific for ascorbic acid, and depends somewhat on the enediol character of ascorbic acid rather than directly on its reducing power. An apparent advantage of the diazotized 4-methoxy-2-nitroaniline method was the longer time allowance of 10 min for reading the Klett-Summerson photoelectric colorimeter after all reagents had been combined. With the sodium 2,6-dichlorobenzenoneindophenol method, readings must be made within 15 sec after the dye and acid are combined.

The purpose of the present investigation was: 1) to compare values for reduced ascorbic acid in individually frozen peas as determined by the sodium 2,6-dichlorobenzenoneindophenol and the diazotized 4-methoxy-2-nitroaniline methods, and 2) to determine any variations that exist in the quantity of reduced ascorbic acid within one lot of grade A frozen peas.

EXPERIMENTAL PROCEDURE

A randomized incomplete block design, with 12 replications for each method of ascorbic acid analysis was used. Data were analyzed by analysis of variance; Fisher's test (Fisher, 1949) for least significant difference, when appropriate; the t-test; and the Bartlett test (Snedecor, 1956).

Six 2-lb packages from one lot of U. S. Grade A fancy individually frozen peas were purchased from a local market in midsummer. Each two-pound package was gently tapped to separate the peas, and the contents emptied into a plastic bowl for thorough mixing. From the bowl, 100-g samples of peas were weighed into polyethylene bags marked to indicate the number of the 2-lb package, number of the sample, method of analysis, date of analysis, and time of analysis (morning or afternoon). Weighing was done rapidly to avoid thawing. All samples from each 2-lb package were closed, tied with "Twist-em" covered wire, and stored on one shelf of a home freezer.

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A thermostatically controlled gas burner was used to cook the frozen peas. Each sample was dropped into 80 ml of boiling distilled water in a 1-qt aluminum saucepan with a vented lid. When the water returned to a boil, the peas were cooked for 1 min with the temperature control at the highest point. Vigorous boiling was reduced by adjusting the temperature control to between 200 and 225°F, and the cooking continued for 6 min. Immediately after cooking, the peas and remaining cooking liquid were analyzed for reduced ascorbic acid by two methods. Method A was a modification of the sodium 2,6-dichlorobenzenoneindophenol method of Loeffler and Ponting (1942), and Method B was the diazotized 4-methoxy-2-nitroaniline method reported by Schmall *et al.* (1953).

The modifications of the method of Loeffler and Ponting (1942) were: 1) an increase in the size of the sample from 25 to 50 g to 100 g; 2) the addition of 5 ml of diluted filtrate to 5 ml of dye instead of adding 9 ml of dye to 1 ml of filtrate; 3) the substitution of a Klett-Summerson photoelectric colorimeter for the Evelyn photoelectric colorimeter; and 4) the use of the following formula for calculating the ascorbic acid factor.

$$\text{ascorbic acid factor} = \frac{\text{concentration of ascorbic acid in dilution}}{\text{blank reading} - \text{ascorbic acid reading}}$$

The factor, then, was used in calculating the reduced ascorbic acid concentration of the unknown solution as follows:

$$\frac{\text{ascorbic acid factor} \times \text{corrected unknown reading (blank reading} - \text{unknown reading)}}{\text{aliquot portion}} \times \text{dilution} = \text{mg reduced ascorbic acid/100 g sample}$$

RESULTS AND DISCUSSION

The investigation was completed within 7 weeks after the peas were purchased and stored. The ascorbic acid values of the peas were subjected to analysis of variance (Table 1). When appropriate, least significant difference (Fisher, 1949) was calculated.

Differences among packages were nonsignificant (Table 1), whereas differences attributable to method of analysis and the interaction of packages × methods were respectively significant at the 0.1% and 5.0% levels. The significant differences attributable to the interaction of packages × methods probably occurred in package V (Table 2). The mean value for package V analyzed by Method B was significantly higher than that for any other package analyzed by Method B; whereas the mean value for package V analyzed by Method A was significantly lower than for either package III or IV. The lowest mean value for all packages analyzed by Method A was obtained from package V.

Table 1. Analysis of variance of reduced ascorbic acid in peas as determined by two methods.

Source of variation	D/F	Ms and significance
Packages	5	0.2704
Methods	1	221.2**
Packages × methods	36	0.9921*
Remainder	36	0.1886
Total	47	

All packages contained peas that varied widely in size from large mature to small, less mature. Ascorbic acid values that occurred within and among packages, analyzed by a given method, are illustrated in Figs. 1 and 2. According to the Bartlett test described by Snedecor (1956), the sampling variances within the same treatment and package were homogeneous. For individual samples analyzed by the sodium 2,6-dichlorobenzenoneindophenol method (Method A), the ascorbic acid values ranged from 15.77 mg/100 g peas to 17.35 mg/100 g. A range of 11.25 mg/100 g to 13.75 mg/100 g was found with the diazotized 4-methoxy-2-

Table 2. Mean ascorbic acid values of methods [a] with the interaction of packages × methods as the source of variation.

Packages	Methods A (mg/100 g peas)	Methods B (mg/100 g peas)
I	16.49	11.93
II	16.76	12.34
III	16.96	12.11
IV	17.09	12.21
V	16.17	13.18
VI	16.36	12.29
	lsd* = 0.62	

[a] A = sodium 2,6-dichlorobenzenoneindophenol method; B = diazotized 4-methoxy-2-nitroaniline method; lsd* = least significant difference at the 5.0% level.

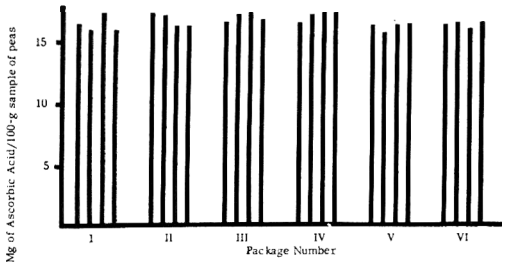

Fig. 1. Ascorbic acid values of 100-g pea samples within a package analyzed by Method A (sodium 2,6-dichlorobenzenoneindophenol method).

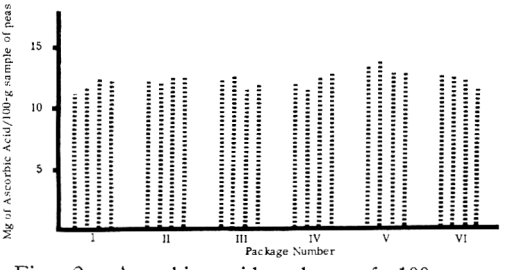

Fig. 2. Ascorbic acid values of 100-g pea samples within a package analyzed by Method B (diazotized 4-methoxy-2-nitroaniline method).

nitroaniline method (Method B). The values found with Method A were closer than the values found with Method B to the average values reported by Burger *et al.* (1956) for frozen green sweet peas (17.1 mg/100 g) and by Watt *et al.* (1950) and Bowes and Church (1956) for cooked peas (15 mg/100 g) and frozen peas (18 mg/100 g). Therefore, it appeared that Method A most nearly reflected the reduced ascorbic acid concentration of the peas analyzed and would be the method recommended for subsequent analyses. Table 2 shows variations in mean ascorbic acid values for packages of one lot of U. S. Grade A Fancy peas analyzed by each method.

Although the differences in reduced ascorbic acid among individual samples of peas seemed small (Figs. 1 and 2), some of the mean values (Table 2) were significantly ($P < .05$) different from each other. With Method A the mean ascorbic acid value was significantly greater for package IV than for packages V and VI. Package III also had significantly more ascorbic acid than package V. All other differences among packages analyzed by Method A were nonsignificant. The Method B analysis indicated that package V contained significantly more ascorbic acid than any other package and that differences among the other packages were nonsignificant.

The interaction of packages $\times$ methods was significant; packages and methods were confounded with the time of analysis. Therefore, t-tests were made to determine if there were significant variations in ascorbic acid values. Consideration of each method separately resulted in no difference attributable to the time of analysis. When Method A was compared with Method B, and when the analyses done in the morning and the afternoon were considered separately, Method A gave decidedly higher numerical values than Method B at each time of day.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. H. C. Fryer, Department of Statistics, for assistance in planning the experiment and analyzing the data.

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Effect of Various Sugars on Browning [a]

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(Manuscript received December 19, 1961)

SUMMARY

The rate of browning of 20 sugars or sugar derivatives was followed by reflectance measurements of cookies and spectrophotometric measurements of browning of dilute buffered solutions of the sugars and glycine or lysine, heated at 114°C in an autoclave. Both in cookies and in solution, the order of decreasing reactivity for the pentoses was: ribose, xylose, and arabinose. Among the hexoses, galactose was the most reactive and rhamnose the least. Because the conditions of pH and presence of amino acids were varied, no over-all order of reactivity could be established for the other hexoses. Disaccharides with reducing groups, except melibiose, showed little reactivity. Sugars without reducing groups failed to exhibit browning.

The browning of food products during processing is of considerable interest from both the theoretical and practical viewpoints. A number of excellent reviews are available (Stadtman, 1948; Coulter *et al.*, 1951; Danehy and Pigman, 1951; Hodge, 1953; Ellis, 1959; Heyns and Paulsen, 1960). The storage of cereal grains and the acceptability of processed cereals are closely related to browning. The browning of baked products has received relatively little attention, although such browning is generally understood to be predominantly of the Maillard type (Johnson and Miller, 1961). Most of the work reported on the browning of baked products has been concerned with glucose, sucrose, or maltose. There is accordingly a dearth of information on the effect of various other sugars on browning. Heyns and Paulsen (1960) have indicated that, in model systems, sugars react in the following order of decreasing activity: xylose, arabinose, galactose, mannose, glucose, lactose, and maltose; sucrose shows no tendency to brown. Several of the above sugars, including pentoses, occur naturally in cereals (Montgomery and Smith, 1956), and may play an important role in browning.

This study was therefore undertaken to establish the reactivity of various sugars in the browning of baked products. To clarify the mechanism of browning in cookies, experiments were also conducted on model systems employing various sugars in dilute solutions, in both the presence and absence of amino acids, and under various pH conditions.

MATERIALS AND METHODS

The flour used was a commercially milled pastry flour having a moisture content of 12.4% and a protein content of 8.9%. The sugars used were analytically pure. The cookies were prepared according to the following basic formula: 100 g flour, 60.0 g sucrose, 30.0 g shortening, 1.75 g sodium bicarbonate, 1.0 g sodium chloride, and 40 g water.

The test sugars were added, where applicable, at either 3.0- or 5.0-g levels, and the amount of sucrose used was reduced by a corresponding amount. The flour, sugar, shortening, sodium bicarbonate, and salt were creamed at medium speed with a Hobart N-50 mixer; then water was added and the dough mixed until homogeneous. The dough was rolled 6 mm thick and cut into pieces 59 mm in diameter. The cookies were baked 12 min at 425°F. After the cookies were cool, external color was determined with a Photovolt Reflectometer, Model 610, equipped with a green tristimulus filter. Three determinations of percent reflectance were made on each of the 6 cookies produced from each batch.

Also studied was the effect of various sugars on rate of browning in model systems in solutions with glycine and lysine. These two amino acids

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were chosen since glycine has been shown (Ellis, 1959) to have a low reactivity and lysine a high reactivity in browning. Since the pH of various baked products ranges from 4.5 to 9.0 (Pyler, 1952), a series of mixtures of the respective sugars and amino acids was prepared at 5 pH levels: 4.6, 5.7, 6.6, 7.2, and 8.9.

Browning observations were made on solutions that were 0.05*M* in both carbohydrates and amino acids, and 0.1*M* in phosphate buffer. Five-ml lots were pipetted into test tubes, covered with metallic caps, and heated 20 min at 114°C in an autoclave. Used as a measure of brown color formation was absorbance at 500 mμ in a Beckman Model DU spectrophotometer. Also followed with this instrument was absorbance in the uv (278 mμ) as a measure of carbonyl compound development. The ultraviolet and browning data have been corrected for absorbance of the amino acids alone in the appropriate phosphate buffers, at the respective wavelengths.

RESULTS AND DISCUSSION

Table 1 shows the effects on cookie color of substituting various sugars for sucrose.

Table 1. Effect on color of cookies of substituting various sugars for sucrose.

Test sugar	Level added (g)	Reflectance (%)
Control	.....	57.5
D(−)arabinose	3	38.9
D(−)ribose	3	38.4
D(+)xylose	3	38.6
D(+)xylose	5	37.7
D(+)galactose	3	41.5
D(+)galactose	5	41.6
D(+)glucose	3	46.1
D(+)glucose	5	44.1
D(−)levulose	3	44.7
D(+)mannose	3	45.0
L(−)sorbose	3	46.1
L(−)sorbose	5	38.9
L(+)rhamnose	3	55.6
sorbitol	3	57.1
α-D-glucose pentaacetate	5	36.5
D-glucose amine hydrochloride	5	33.9
cellobiose	3	55.2
lactose	3	54.0
lactose	5	52.3
maltose	3	53.0
maltose	5	51.8
melibiose	3	44.3
melibiose	5	40.3
melezitose	3	56.9
raffinose	3	57.1
trehalose	3	56.1

The pentoses exhibited the highest reactivity among the sugars employed. Under the conditions of the experiment, the extent of browning caused by pentoses decreased slightly in the following order: ribose, xylose, and arabinose. Among the hexoses tested, galactose was most reactive followed by levulose, mannose, sorbose, and glucose, all four essentially alike. The practically nil reactivity of sorbitol, the hexahydric straight-chain alcohol, is to be expected since the reduction of the keto-group of sorbose would prevent the Maillard reactions. The very low reactivity of rhamnose (6-deoxy-L-mannose) may be attributed to the change in the 6 position of L-mannose. Whereas this change probably would not affect the initial interaction between the amino acids and sugars, the data indicate that the entire sugar molecule is involved in formation of the brown condensation products. With α-D-glucose-pentaacetate, no browning occurred. The low reflectance recorded in Table 1 was due to a decided graying of the cookies. The pronounced browning of cookies containing D-glucose-amine-hydrochloride suggests that this substitution at the 2 position may have resulted in a compound of reactive properties similar to the one formed in condensation of an amino acid and sugar. No browning was obtained with the nonreducing disaccharides, namely, sucrose and trehalose. Adding cellobiose, lactose, and maltose resulted in some slight browning. The low reactivity of 5% maltose, as compared to 3% glucose, points to the fact that the rate of reaction is controlled not only by the reducing group but by the entire sugar molecule. The effect of using melibiose with a 1,6-linkage seemed to demonstrate that under the conditions of the test, two reactive hexoses are produced. One of the reactive hexoses is likely galactose. This could account for the higher reactivity of melibiose than of other disaccharides with a 1,4-linkage. The low reactivity of melezitose, raffinose, and tre-

Table 2. Absorbance at 500 mμ of sugar–amino acid solutions.

Sugar	pH levels without amino acid					pH levels with lysine added					pH levels with glycine added				
	4.6	5.7	6.6	7.2	8.9	4.6	5.7	6.6	7.2	8.9	4.6	5.7	6.6	7.2	8.9
D(−)arabinose	0	0	0	.010	.118	.035	.345	.760	1.000	1.080	.005	.070	.250	.325	.530
L(+)arabinose	0	.005	.008	.008	.110	.050	.350	.750	.890	.990	.005	.075	.232	.355	.510
D(−)ribose	0	.010	.015	.020	.160	.195	1.050	1.430	1.370	1.370	.015	.271	.590	.710	.900
D(+)xylose	0	.005	.005	.012	.202	.125	.710	1.080	1.210	1.180	.008	.142	.368	.553	.745
D(+)galactose	0	.005	0	.005	.087	0	.118	.645	1.000	1.180	0	.018	.108	.183	.287
D(+)glucose	0	0	0	.005	.095	0	.055	.308	.520	.900	0	.008	.050	.085	.150
D(−)levulose	0	.015	.010	.025	.215	.005	.032	.065	.188	.418	.015	.023	.062	.160	.355
D(+)mannose	0	.010	.055	.070	.090	.010	.072	.322	.650	.820	0	.015	.065	.107	.165
L(−)sorbose	.005	.018	.025	.052	.400	.035	.115	.190	.545	.780	.022	.055	.142	.315	.620
L(+)rhamnose	0	0	.010	.005	.050	.005	.033	.107	.185	.280	0	.005	.035	.055	.087
D-glucose amine hydrochloride	.025	.172	.575	.780	.900	.055	.432	1.030	1.120	1.210	.035	.300	1.100	1.330	1.650
cellobiose	0	.005	.005	.028	.157	0	.020	.137	.275	.460	0	.008	.040	.063	.167
lactose	0	0	.062	.010	.168	0	.020	.093	.183	.365	0	.008	.022	.055	.140
maltose	0	.005	.008	.025	.215	.005	.022	.087	.177	.345	0	.015	.040	.067	.175
melibiose	0	.005	.005	.005	.255	0	.110	.651	1.250	1.500	0	.017	.103	.192	.330
melezitose	0	0	0	0	.005	0	0	0	0	.005	0	0	0	0	0
raffinose	0	.005	0	0	.005	0	0	0	.010	.005	0	0	0	0	0
sucrose	0	0	0	0	.008	0	0	0	0	0	0	0	0	.005	0

Table 3. Absorbance at 278 mμ of sugar–amino acid solutions.

Sugar	pH levels without amino acid					pH levels with lysine added					pH levels with glycine added				
	4.6	5.7	6.6	7.2	8.9	4.6	5.7	6.6	7.2	8.9	4.6	5.7	6.6	7.2	8.9
D(−)arabinose	.057	.285	.510	.500	.83*	.79*	.83*	.83*	.83*	.84*	.458	.80*	.82*	.80*	.80*
L(+)arabinose	.060	.243	.350	.403	.85*	.82*	.83*	.83*	.85*	.80*	.450	.80*	.80*	.81*	.80*
D(−)ribose	.305	.68	1.53	.79*	.83*	.82*	.82*	.83*	.83*	.80*	1.60	.80*	.81*	.81*	.79*
D(+)xylose	.060	.240	.375	.655	.85*	.83*	.82*	.83*	.84*	.78*	.690	.80*	.80*	.81*	.80*
D(+)galactose	.018	.225	.275	.215	.79*	.200	.82*	.82*	.83*	.84*	.133	1.35	.81*	.79*	.79*
D(+)glucose	.005	.120	.150	.145	.83*	.055	.83*	.84*	.84*	.84*	.032	.66	.79*	.79*	.79*
D(−)levulose	.550	.780	.760	.930	.85*	.380	1.18	.83*	.84*	.81*	.780	1.08	.78*	.79*	.79*
D(+)mannose	.100	.232	.215	.270	.82*	.217	.82*	.83*	.83*	.81*	.165	.94	.80*	.79*	.79*
L(−)sorbose	1.05	1.03	1.10	.80*	.84*	1.05	.82*	.84*	.84*	.81*	1.55	.77*	.79*	.81*	.81*
L(+)rhamnose	.010	.163	.170	.160	.82*	.195	.83*	.84*	.84*	.81*	.060	1.23	.81*	.81*	.79*
D-glucose amine hydrochloride	.84*	.83*	.83*	.81*	.82*	.82*	.82*	.83*	.83*	.84*	.81*	.80*	.80*	.79*	.79*
cellobiose		.110	.200	.278	.81*	.090	.74*	.82*	.83*	.84*	.040	.425	1.45	.76*	.79*
lactose	.018	.150	.225	.322	.81*	.073	.68*	.83*	.82*	.84*	.042	.425	1.15	.74*	.79*
maltose	.065	.245	.390	.530	.83*	.168	.82*	.83*	.83*	.81*	.115	.78	.80*	.79*	.79*
melibiose	.025	.255	.350	.445	.84*	.230	.83*	.83*	.84*	.80*	.120	1.28	.81*	.82*	.79*
melezitose	0	0	.020	.030	.040	0	.037	.140	.245	.200	0	.035	.035	.030	.032
raffinose	0	0	.025	.057	.035	.011	.115	.320	.470	.400	.010	.050	.115	.090	.025
sucrose	0	0	.025	.070	.075	.008	.053	.210	.375	.410	.010	.050	.070	.090	.035

*Read at selector switch of 0.1.

halose was expected from their structure.

The amount of color developed in the various synthetic reactions is recorded in Table 2 as absorbance at 500 mμ, and in Table 3 as absorbance at 278 mμ. These results confirm those found in the "dry state" as exemplified by experiments with cookies. The pentoses were the most chromogenic, and the absence of sugars with free reducing groups eliminated browning completely or greatly reduced it. Among the pentoses, ribose was most active, followed by xylose and arabinose. Of the hexoses, galactose was the most reactive and rhamnose the least. The order of reactivity of the other sugars varied with pH and the amino acid present. In the absence of amino acids, the keto sugars (sorbose and levulose) were more reactive than the sugars with an aldehyde group. In the presence of amino acids, no consistent overall trend was obtained. Here again, there was a very high absorbance of D-glucose amine hydrochloride. The reactivity of oligosaccharides paralleled the order observed with the cookies. The high reactivity of melibiose was confirmed under all the tested conditions.

Raising the pH into the alkaline range enhanced browning with all the sugars tested. Lysine was more active in inducing browning than was glycine. Willits *et al.* (1958) and Underwood *et al.* (1959) have shown that in solutions that are 0.125*M* or 0.1*M* with respect to amino acids and 0.025*M* or 0.1*M* in glucose, browning did not occur below pH 6, but above this point browning developed in increasing amounts as the alkalinity of the solution was raised. In this study employing solutions of 0.05*M* in both carbohydrates and amino acids, the use of sugars more reactive than glucose resulted in measurable browning at pH 4.6 with lysine, and at pH 5.7 with glycine.

The data from this study show that under the conditions of the test, browning products are produced from the highly reactive reducing sugars, at all pH levels, independent of the presence of amino acids. Even though the highly reactive sugars browned readily in the absence of amino acids, the rate of browning was enhanced by the presence of amino acids. The presence of highly reactive pentoses in cereals, especially those containing high levels of wheat embryos or rye flours, might be responsible for their pronounced browning despite the low pH of such foods. With some products having a high pH, such as certain cakes and cookies, browning may take place in the absence of highly reactive sugars.

ACKNOWLEDGMENT

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The Flavor Spectrum of Apple-Wine Volatiles [a]

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SUMMARY

Sixteen compounds in the volatile fraction of apple wine were isolated and identified. Ethyl chloride was preferred over pentane or iso-pentane in extraction of the volatiles because it gave higher yields and its lower boiling point allowed almost solvent-free extracts in a very short time. Gas-liquid chromatography was used in separation and purification of the volatiles. The compounds were identified by comparing retention times, infrared spectra, and melting point of derivatives with those of known compounds.

The chemical composition of the volatile components of various food products has recently become the subject of many research projects. Interest in this matter has always been high, but many workers were discouraged by the arduous methods of separation that were necessary before the development of gas chromatography. The chief concern in the writers' laboratory has been to define and differentiate flavors and off-flavors in apple wine. As the first step in elucidating this problem by chemical means, identification of the volatile organic materials in apple wine has been attempted. There is considerable precedent for this work since the volatile compounds of beer (Jenard, 1959; Van der Kloot *et al.*, 1958), brandy (Bouthilet and Lowrey, 1959), grain spirits (Austin and Boruff, 1960), whiskey (Caroll and O'Brien, 1959), and other fermentation products (Baraud and Genevois, 1958) have been examined. Several compounds in apple juice have been identified by chemical methods (Williaman, 1950); because of varietal and cultural differences as well as changes taking place during fermentation, however, it would not be of any significance to compare the results of Willaman with those obtained in the present study.

EXPERIMENTAL

Preparation of volatile material from apple wine. Duchess apples (1½ tons) were ground in a Fitzmill and pressed, and the juice filtered through cheesecloth. Sugar was added to give a refractometer reading of 15% total soluble solids. A 2% SO_2 solution was added to the juice during pressing to give 100 ppm SO_2 concentration as determined by a modification of the Monier-Williams method (Ruck, 1956).

Apple wine was made by the rapid method of Atkinson *et al.* (1959). The wine volatiles were concentrated approximately fivefold by stripping in a rising film evaporator and condensing in two heat exchangers arranged in series. The first stage was cooled with tap water, and the second with ice water. The wine residue, after stripping, had almost no odor. The condensate was held at 0°C until required for extraction.

Preparation of extract. Pentane, iso-pentane, and ethyl chloride were examined for their suitability as extracting solvents. The evaporation of both pentane and iso-pentane from the extract required slight heating, with concomitant loss of flavor components. Ethyl chloride was chosen as the extracting solvent because of the ease with which it could be removed from the extract at room temperature, and the higher yields of low-boiling compounds obtained.

The method of extraction was as follows: To 1000 ml of apple-wine condensate enough NaCl was added to saturate the solution. Samples of 150 ml were extracted with 15 ml of ethyl chloride in a separatory funnel, which was gently manipulated for 1 min every 5 min. After ½ hr,

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the lower aqueous layer was discarded and the upper layer containing the ethyl chloride and volatile extract was placed in a small beaker. The extract in the beaker was decanted from the small remaining portion of the aqueous layer into a 50-ml Erlenmeyer flask, which was then immersed in water at room temperature. After immersion the flask was immediately fitted with a glass tube leading to a second 50-ml Erlenmeyer flask in a dry-ice-and-alcohol bath. Most of the ethyl chloride was evaporated from the extract and condensed in the cold trap. The condensate containing ethyl chloride and a small amount of wine volatiles was reused in subsequent extractions.

Separation and identification of volatiles. The volatiles were separated into three main fractions with the aid of a Beckman GC-2 gas chromatograph equipped with a 10-ft × 5/8-in. copper preparative column and a fraction collector. Many types of solid and stationary-phase materials were tested before it was found that 30% by weight Carbowax 400 (polyethylene glycol; Union Carbide Chemical Co.) on acid-washed 40–60-mesh firebrick C-22 (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) effected an efficient separation of the extract components.

After rough separation into three fractions using the 10-ft preparative column (Fig. 1) finer separations were made using a 6-ft × 1/4-in. stainless-steel column. When enough material from each of the three major fractions had been collected, the components of each fraction were collected (Fig. 2, 3, 4). Each component was re-run and collected until 0.005 ml produced only one well-defined peak.

From 0.003 to 0.02 ml of the various components were recovered in what was thought to be the pure state.

The isolated components were identified by preparing derivatives, infrared analysis, and measurement of the retention times in a gas chromatograph column.

Acetaldehyde and acetone were identified as derivatives of 2,4-dinitrophenyl hydrazone (Shriner and Fuson, 1946) by measuring the melting point of an admixture of the derivatives and authentic compounds. Further confirmation was obtained by subjecting the derivatives and authentic compounds to ascending paper chromatography according to a modified method of Meigh (1956) utilizing petroleum ether and methanol 80:20 on Whatman paper No. 1.

Two peaks with retention times of 8 and 14 min (Fig. 3) could not be identified by infrared analysis, and therefore might be a mixture of two or more compounds. By using a Perkin Elmer model 154 vapor fractometer equipped with a flame ionization detector and a 1/4-in. × 1-meter column, it was confirmed that the peak representing ethanol and iso-propanol was, in fact, a mixture. By comparing the retention times of the mixtures, using the flame ionization detector, with those of known compounds (Fig. 5), identification of the mixed components was made and confirmed by comparing the infrared spectra of the sample mixture and mixtures of known compounds. The 14-min peak in Fig. 3 could be separated into two distinct components (*n*-butyl acetate and iso-butyl propionate) by using Ucon HB 2000 or DEGS (10% on 60–80-mesh C-22) (Table 3). A slightly differentiated peak at 18 min (Fig. 3) separated clearly on a Ucon LB 550X (10% on 60–80-mesh C-22) column. Comparison of retention times with those of known compounds (Fig. 5) and infrared spectra showed the mixture to be iso-amyl acetate and iso-butanol.

Infrared spectroscopy. Infrared spectra were determined on a Perkin Elmer model 21 spectrometer. When material was sufficient, the thin-film technique was used with the material layered between two NaCl discs. When sample size was seriously restricted, a micro-cavity cell was used to determine the spectra in spectro-grade CCl_4.

The following unsuccessful preliminary experiments were undertaken to determine the best method of separating the flavor components from the wine strippings:

1) Nitrogen was bubbled through calcium-chloride-saturated wine strippings, and the vapors were trapped by a dry-ice cold trap containing purified ethyl benzene. Recovery was poor, and the ethyl benzene broke down rapidly when exposed to light.

2) Instead of trapping the vapors in cold ethyl benzene, they were trapped in a series of cold traps submerged in dry ice and alcohol. A final trap set in liquid oxygen was used. A large percentage of the condensate from each trap consisted of water. Also, aerosol formation allowed a portion of the flavor components to pass through all the traps.

RESULTS AND DISCUSSION

Table 1 shows the 16 compounds isolated and positively identified. They are arranged in order of their elution from a Carbowax 400 column as shown in Fig. 1. In three cases, the quantities of material recovered were too small to permit identification of compounds by other than retention times. Quantitative estimates of the components are rather difficult because the extraction solvent was chosen to eliminate ethanol and, by necessity, methanol. Aside from these two compounds, iso-pentanol, acetaldehyde,

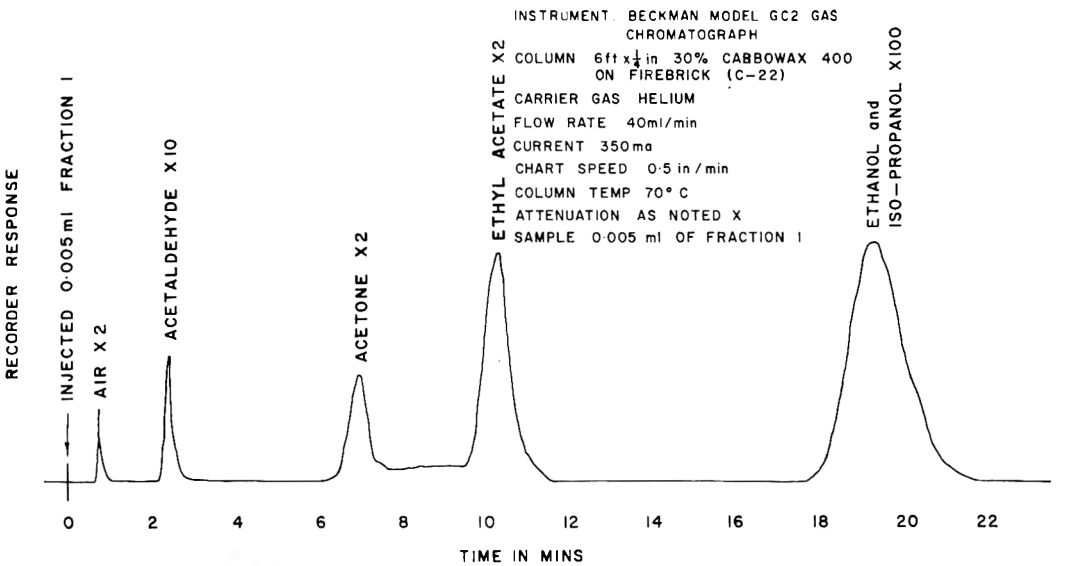

Fig. 2. Chromatogram of fraction 1 of apple-wine extract.

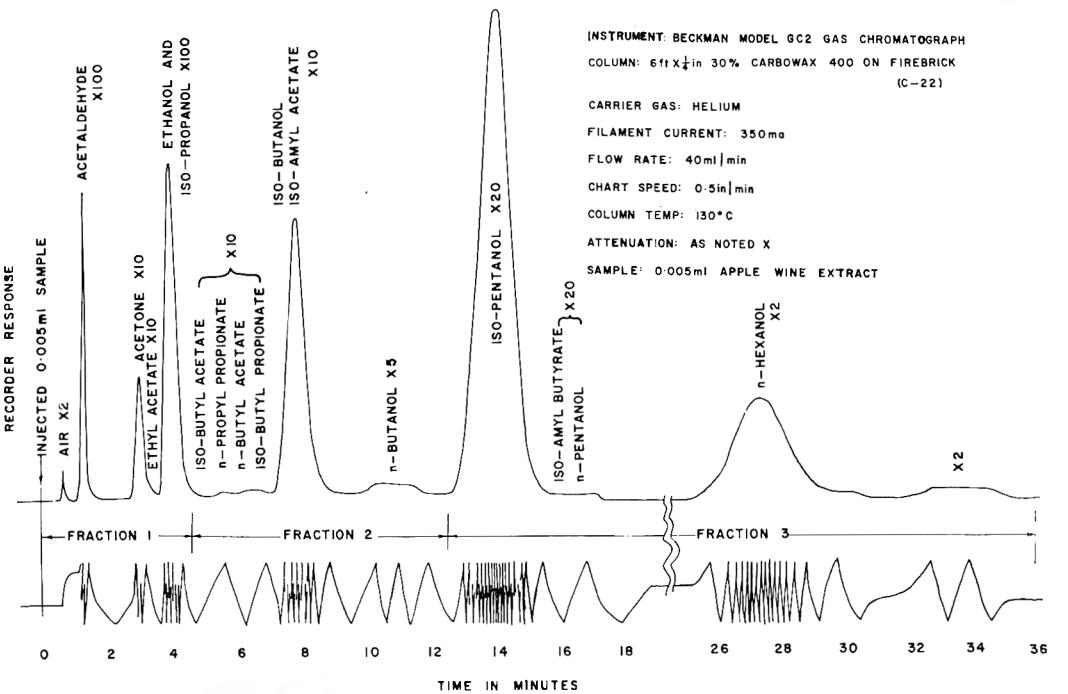

Fig. 1. Chromatogram of apple-wine extract.

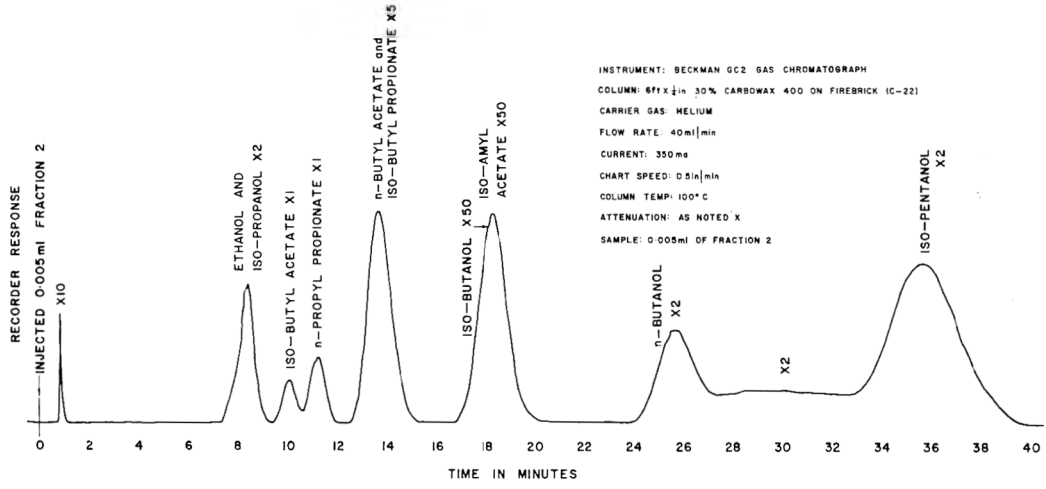

Fig. 3. Chromatogram of fraction 2 of apple-wine extract.

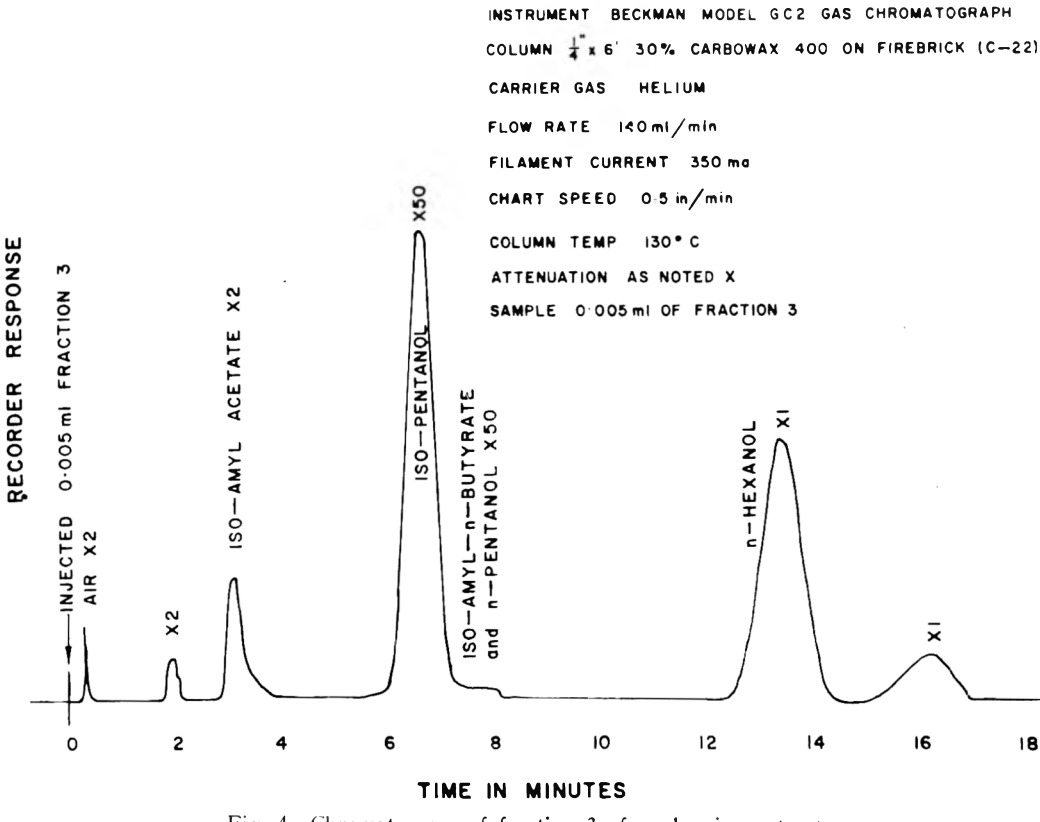

Fig. 4. Chromatogram of fraction 3 of apple-wine extract.

and possibly iso-propanol are the major fractions. All apparent peaks in Fig. 1, except the one after *n*-hexanol, have been identified.

Fraction 1, run at 70°C, is shown in Fig. 2. Acetone and ethyl acetate are separated, but ethanol and iso-propanol are not. This fraction had an acrid, unpleasant odor, with no suggestion of fruitiness. It is possible that components of the fraction (discounting ethanol) form the major volatile organics in apple wine, and that they were partially eliminated in the solvent extraction process.

Fig. 3 illustrates the improved resolution of fraction 2 attained by increasing the relative concentration of the components and decreasing column temperature. There is some carryover of ethanol and iso-propanol from fraction 1. No explanation was found for the long plateau between *n*-butanol and iso-pentanol. Fractions 2 and 3 had an odor characteristic of apple wine.

Table 1. Volatile components found in apple wine.

Component	Method of identification
Acetaldehyde	Derivatives, retention times
Acetone	Derivatives, retention times
Ethyl acetate	Infrared, retention times
Ethanol	Infrared, retention times
iso-Propanol	Infrared, retention times
iso-Butyl acetate	Infrared, retention times
n-Propyl propionate	Retention times
n-Butyl acetate	Infrared, retention times
iso-Butyl propionate	Infrared, retention times
iso-Butanol	Infrared, retention times
iso-Amyl acetate	Infrared, retention times
n-Butanol	Infrared, retention times
iso-Pentanol	Infrared, retention times
iso-Amyl-n-butyrate	Retention times
n-Pentanol	Retention times
n-Hexanol	Infrared, retention times

Some iso-amyl acetate carries over from fraction 2 to fraction 3 (Fig. 4). On the

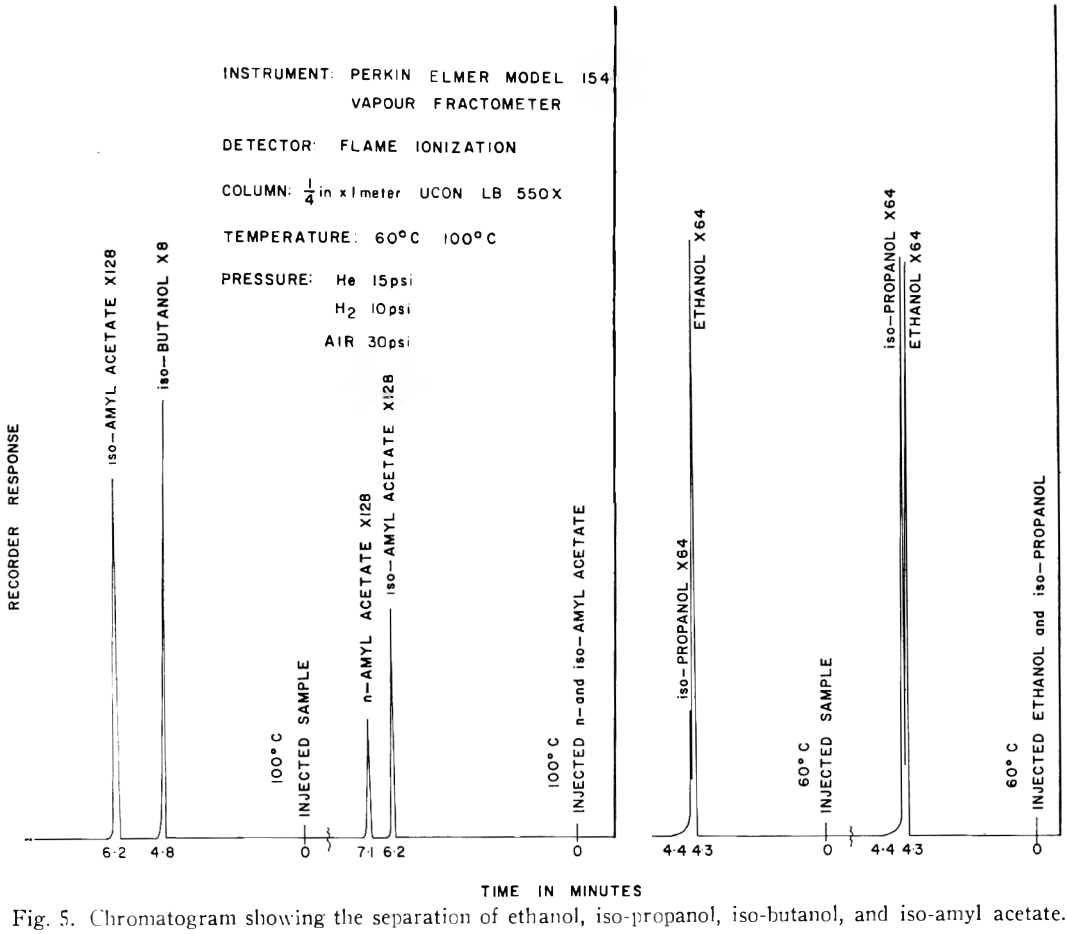

Fig. 5. Chromatogram showing the separation of ethanol, iso-propanol, iso-butanol, and iso-amyl acetate.

Table 2. Retention times of apple-wine components and of authentic compounds.

Sample	Chromatograph sample	Retention times in minutes relative to injection time									
		Carbowax 400[a]		Ucon HB 2000[a]		Ucon LB 55OX[b]		Tergitol NP 27[b]		DEGS[b]	
		Std.	Sample	Std.	Sample	Std.	Sample	Std.	Sample	Std.	Sample
70°C											
1	Acetaldehyde	2.3	2.3	2.5	2.5						
2	Acetone	6.7	6.7	6.0	6.4						
3	Ethylacetate	10.2	10.2	11.0	11.1			3.7	3.7 50°C		
4	Ethanol	18.0	18.5	11.2	11.1	4.3	4.3 } 60°C				
5	iso-Propanol	19.0	18.5	12.1	11.1	4.4	4.4 }				
100°C											
6	iso-Butyl acetate	9.5	10.0	5.6	5.6			4.2	4.2 } 75°C		
7	*n*-Propyl propionate	11.1	11.2	5.6	5.6			4.6	4.6 }		
8	*n*-Butyl acetate	13.2	13.6	7.3	7.3					1.7	1.7 } 100°C
9	iso-Butyl propionate	13.4	13.6	8.3	8.0					2.0	2.0 }
10	iso-Butanol	17.5	18.2	5.6	5.6						
11	iso-Amyl acetate	18.0	18.2	10.3	10.3	6.2	6.2 100°C				
12	*n*-Butanol	24.5	24.2	7.2	7.3					2.7	2.7 100°C
130°C											
13	iso-Pentanol	14.0	14.0	11.1	11.1						
14	iso-Amyl *n*-butyrate	16.2	16.5	24.0	24.5					4.5	4.5 } 100°C
15	*n*-Pentanol	17.0	16.5	13.0	12.5					3.7	3.7 }
16	*n*-Hexanol	28.3	28.1	22.2	23.0					6.1	6.1 }

[a] 6-ft × ¼-in. column, thermal conductivity detector.
[b] ¼-in. × 1-meter column, flame ionization detector.

other hand, the bulk of the iso-pentanol is found in fraction 3, with a smaller amount in fraction 2. *n*-Butanol, although between iso-amyl acetate and iso-pentanol in fraction 2, does not appear in fraction 3. Two unknown peaks at 2 and 16 min appear in Fig. 4. It is probable that many other minor flavor and odor compounds may exist beyond fraction 3 that are not volatile enough to be recovered in significant amounts by vapor stripping.

A finer separation of compounds with similar retention times is illustrated in Fig. 5, using a flame ionization detector. Iso-propanol and ethanol are still very close. Comparison of retention times shows that the 18-min peak of Fig. 3 contains iso-amyl acetate rather than *n*-amyl acetate.

Table 3 tabulates the alcohols and esters, with their acidic components found in this investigation. Iso-propanol, *n*-pentanol, and *n*-hexanol were not esterified in detectable amounts. The number of esters formed from the three acidic components was in the order acetic > propionic > butyric. The major ester peak is iso-amyl acetate, and its alcoholic component is the major free alcohol. Whether iso-pentanol is formed from the hydrolysis of iso-amyl acetate or vice versa, remains to be seen. *n*-Propyl propionate is in the unique position of not having detectable amounts of its parent alcohol present.

Preliminary studies indicate the presence of several other volatile fatty acids not all of which form detectable esters. These acids are being further investigated. The interesting possibility will also be pursued of blending and comparing organoleptically the synthetic homologs of the compounds identified here.

Table 3. Tabulation of alcohols, esters, and acidic ester components found in apple wine.

		Acid ester component		
	Alcohol	Acetic	Propionic	Butyric
Ethanol	+	+		
iso-Propanol	+			
n-Propanol			+	
iso-Butanol	+	+	+	
n-Butanol	+	+		
iso-Pentanol	+	+		+
n-Pentanol	+			
n-Hexanol	+			

ADDENDUM

Since completion of this manuscript the 16-min peak of Fig. 4 has been identified, by infrared spectra and comparison of retention times, to be a mixture of ethyl caprylate and *n*-butyl caproate. Retention times on a 10% Tergitol NP27 on firebrick, ¼-in. × 1-meter column at 100°C with helium as carrier gas and a flame ionization detector, are (in min) as follows:

Major peak	34.0
Ethyl caprylate	34.2
Minor peak	31.4
n-Butyl caproate	31.6

This brings to 18 the number of compounds identified.

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Reaction of Cod Actomyosin with Linoleic and Linolenic Acids

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SUMMARY

Small concentrations of linoleic and linolenic acids reduced the solubility of cod actomyosin rapidly. The extent of insolubilization depended on the structure of the fatty acid, on its concentration, and on the duration of storage of the fatty-acid-treated actomyosin solutions. The results support the hypothesis that the accumulation of free fatty acids in frozen fish muscle causes the actomyosin of the muscle to become inextractable.

INTRODUCTION

The development of toughness is an important factor in the storage life of frozen cod. Several laboratories have correlated sensory evaluation of toughness in frozen cod muscle with measurement of actomyosin extractability (Dyer *et al.*, 1957; Love, 1960; Luijpen, 1957). Similar correlations have been found in other species of fish during frozen storage (Dyer, 1951; Dyer *et al.*, 1956; Dyer and Morton, 1956; Heen, 1954; Nikkilä and Linko, 1954).

Although actomyosin denaturation may be measured by several methods (Connell, 1960a; Partmann, 1957; Sawant and Magar, 1961), the criterion of denaturation that is best correlated with textural changes in fish muscle is loss of extractability. Meat, in contrast with fish, is less likely to toughen during commercial conditions of frozen storage. This difference in storage stability may be related to Connell's (1961) observation that myosin extracted from cold-blooded animals aggregates spontaneously at a faster rate than myosin from warm-blooded animals.

The mechanism by which actomyosin aggregates, loses much of its bound water (forming "drip"), and becomes insoluble is a current subject of investigation in several laboratories. Connell (1958 and 1960b) has postulated that only a small part of the molecular surface of cod myosin is responsible for its relative instability since the other properties of cod and rabbit actomyosin are very similar. Other workers have indicated the possibility of a relationship between lipid hydrolysis and actomyosin denaturation in frozen muscle. Dyer and Fraser (1959) and Olley and Lovern (1960) reported a correlation between increase in free fatty acid content and decrease in actomyosin extractability in frozen cod muscle. Dyer and Fraser (1959) postulated that either the stabilizing effect of intact lipids on actomyosin is destroyed by lipid hydrolysis or that fatty acids formed from lipid hydrolysis cause actomyosin inextractability.

The present investigation is a study of the effect of added fatty acids on the solubility of extracted cod actomyosin.

EXPERIMENTAL METHODS

Materials. Gutted cod [*Gadus morhua;* also *Gadus callarias* (Cohen, 1959)] were obtained commercially. They were kept on ice until they had passed through rigor. After filleting, the midsection of the anterior portion of their skeletal muscle was used.

Linoleic and linolenic acids were obtained from Nutritional Biochemicals Corp. (no endorsement implied) and used immediately after breaking the vial. All inorganic reagents were analytical reagent grade.

Buffer solutions having an ionic strength of 0.05 and a pH of 7.5 were made from 0.00338*M* KH_2PO_4 plus 0.0155*M* Na_2HPO_4.

Buffer solutions were made of 0.5 μ by adding KCl to the 0.05 phosphate buffer. In this paper, μ denotes ionic strength; $\mu = 0.5 \Sigma mz^2$ where m is the molality of each ion and z is its valence (Koch and Hanke, 1953). Final pH was 7.2. It was not changed after adding cod muscle, linoleic, and linolenic acids.

All stock solutions were stored in a 4°C refrigerated room.

Preparation of actomyosin solutions. This method removed sarcoplasmic proteins by washing the sample of muscle tissue before dissolving its actomyosin (Connell, 1960b) instead of precipitating actomyosin from a total protein extract. All of the preparative operations were carried out in a 4°C refrigerated room. An 80-g sample of cod muscle tissue blended with 800 ml of 0.05-μ buffer in a Waring blender whose jar had a Polytron model BEW rotor-stator assembly. A baffle technique (Dyer *et al.*, 1950) was used to reduce foaming. The blender was operated at reduced speed about 7,000 rpm for 90 sec in nine 10-sec periods separated by 5-sec intervals to allow settling of undissolved material. The suspension was centrifuged 20 min at 10,000 × G in a Spinco model L preparative ultracentrifuge using a type-21 rotor. The sedimented material was rewashed and recentrifuged two more times. Then the sedimented material was taken up in 800 ml of 0.5-μ buffer and left overnight. It was then centrifuged 20 min at 10,000 × G to remove muscle debris and other insoluble material. The concentrated actomyosin supernatant was placed in a 4-L aspirator bottle and mixed slowly by a magnetic stirrer while it was diluted with 0.5-μ buffer to a final concentration of about 0.45 mg total soluble protein nitrogen (TSPN) per ml. Stirring was continued for 30 min and was maintained while 350-ml samples of the solution were withdrawn through the bottom outlet of the bottle. These samples were used in the experiments only if they were uniform in protein content (biuret test of Layne, 1957).

Addition of linoleic and linolenic acids to actomyosin solutions. In each experiment, 3–4 different concentrations of linoleic and linolenic acids were dispersed into separate aliquots of soluble actomyosin. The fatty acid was dispersed by blending in the modified Waring blender assembly (described above) for 30 sec in three 10-sec periods separated by 5-sec intervals. During the 5-sec intervals, the preparation was mixed gently with the baffle. In each experiment, control aliquots were treated identically except for the addition of fatty acid.

Although the concentrations of added linoleic and linolenic acids were measured volumetrically (usually 0.025, 0.05, 0.10, and 0.20 ml), they are expressed as weight ratios to the weight of TSPN in the controls after centrifugation, as recommended by Putnam (1948).

Determination of soluble actomyosin. Soluble protein determinations were made on the control and fatty-acid-treated actomyosin samples immediately after preparation or after storage for 1–4 days at 4°C. The supernatants of these samples, after centrifugation for 20 min at 10,000 × G, were analyzed for their TSPN content by a biuret test (Layne, 1957).

RESULTS AND DISCUSSION

Five sets of actomyosin aliquots from five different cod were used. The results are combined in the figures. In the two experiments illustrated in Fig. 2, the TSPN content of the controls was essentially unchanged during storage at 4°C (std. dev. = 0.008 mg TSPN per ml).

The presence of linoleic and linolenic acids, in low concentrations, reduced the solubility of cod actomyosin rapidly (Fig. 1). Further insolubilization took place if the fatty-acid-treated actomyosin solutions were stored 1–4 days at 4°C (Fig. 2). Although some of the details of the reaction between fatty acids and some water-soluble proteins are known (reviewed by Putnam, 1948), the mechanism of the reaction between fatty acids and actomyosin is speculative. However, the end result of adding linoleic and linolenic acids to soluble actomyosin was insolubilization of this protein at rates that depended on the structure of the fatty acid as well as its concentration and the time of storage.

These results give experimental support to Dyer and Fraser's (1959) hypothesis that free fatty acids cause inextractability of actomyosin in frozen cod muscle. The evidence that linoleic and linolenic acids insolubilized extracted actomyosin does not prove that the same reaction occurs in intact frozen cod muscle. However, the concentrations of fatty acid that insolubilized actomyosin in our extracts are similar to the free fatty acid content of frozen stored cod muscle in which actomyosin has become totally inextractable. For example, Dyer and Fraser (1959), in a study of changes in frozen cod muscle stored at −12°C, found that the actomyosin had become totally inextractable after 30 weeks of storage. They also found that the free fatty acid content increased in 30 weeks, from about 0.01 mg per mg TSPN in their fresh cod, to about 0.1 mg (expressed as oleic) per mg of original TSPN. It is apparent from Fig. 2 that 0.1 mg linoleic

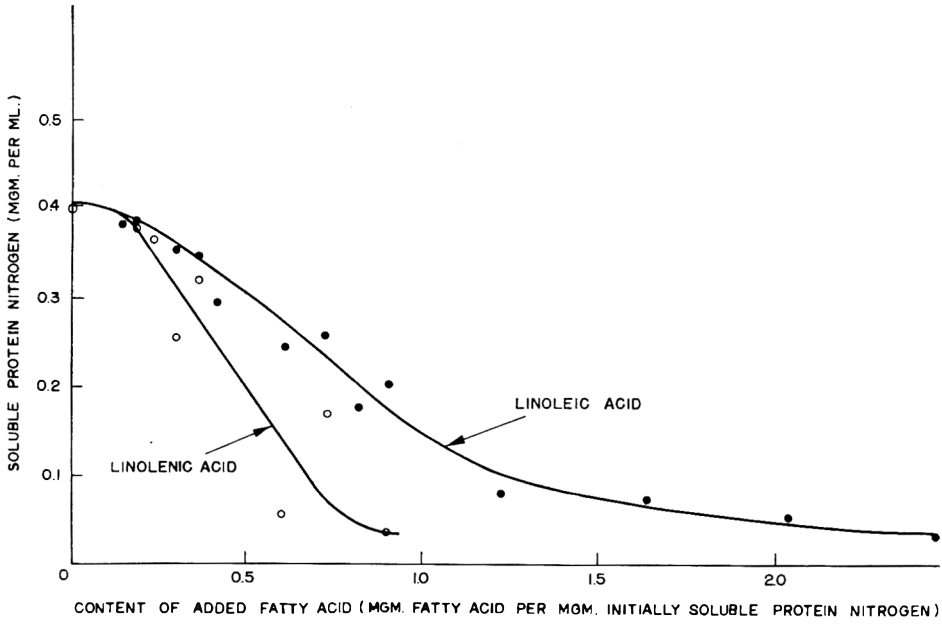

Fig. 1. Soluble protein of actomyosin solutions immediately after adding linoleic (●) or linolenic (○) acid. The results of three experiments have been combined.

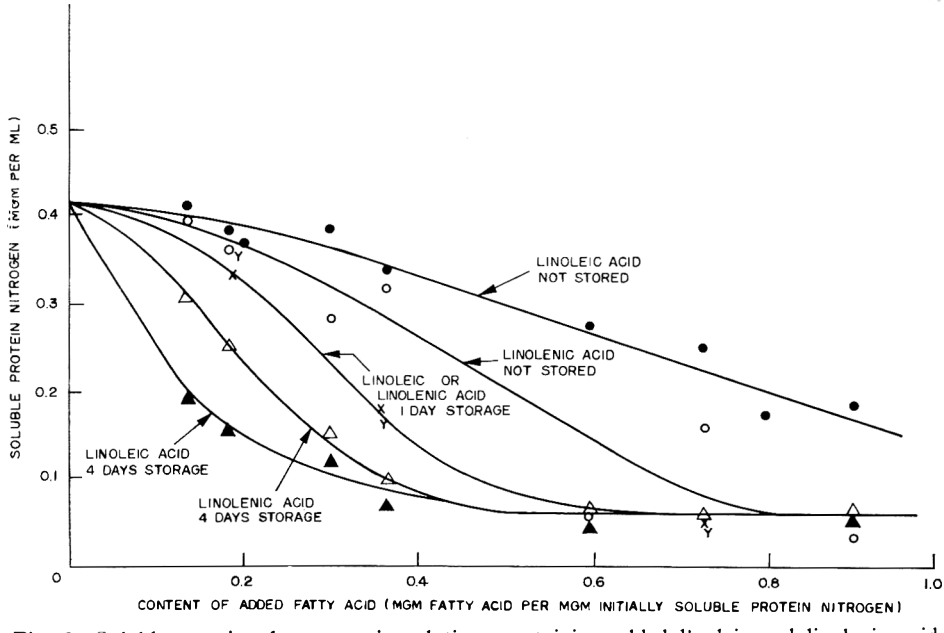

Fig. 2. Soluble protein of actomyosin solutions containing added linoleic and linolenic acids after storage at 4°C. The results of two experiments (not shown in Fig. 1) are combined. Key to symbols: Linoleic acid: not stored (●); 1-day storage (x); 4-day storage (▲). Linolenic acid: not stored (○); 1-day storage (y); 4-day storage (△).

acid per mg TSPN causes insolubilization of about one third of extracted fresh cod actomyosin after 4 days at 4°C.

Since linoleic and linolenic acids insolubilized actomyosin at different rates, the possibility is now being studied that species difference in actomyosin solubility or extractability may be related to species difference in fatty acid composition of muscle lipids.

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Methionine Content of Some South Indian Foods [a]

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(Manuscript received March 7, 1962)

SUMMARY

Methionine content of 24 of the more common foodstuffs used in South India is reported. Two independent methods were used, one based on the McCarthy and Sullivan color reaction and the other on filter-paper disc chromatography.

INTRODUCTION

Best *et al.* (1936) discovered that the livers of animals on a choline-deficient diet containing sucrose accumulated less fat if a liberal ration of protein was included in the diet. Casein in adequate amounts could be substituted for choline as a lipotropic agent (Beeston *et al.*, 1936). Investigations on the active constituents of casein revealed that cysteine exaggerated whereas both D- and L-methionine prevented the deposition of lipid (Tucker and Eckstein, 1937). It was subsequently established that the lipotropic activity of a large number of proteins was directly proportional to the amount of methionine they contained (Singal and Eckstein, 1939). The lipotropic action of methionine depends on its ability to provide methyl groups for the formation of choline from ethanolamine (du Vigneaud *et al.*, 1941). The biological lability of the methyl group, its reattachment to various methyl acceptors, and the biological reversibility of several of these transfer reactions form the essence of transmethylation.

Methionine is an indispensable amino acid for all animals investigated to date, although a large part of the dietary requirement can be met by cysteine (Rose and Wixon, 1955; Rose *et al.*, 1954). It is recognized that animals as well as microorganisms and plants can synthesize the methyl group of methionine from simple one-carbon compounds. But this represents only a minor pathway of methionine formation in animals, which normally ingest adequate amounts of this amino acid.

The choline and methionine in several foodstuffs commonly used in South India were investigated to establish the intake of lipotropic factors by different groups of population. The choline content of several foodstuffs has been reported (Dakshinamurti, 1955). Information on the methionine content of foodstuffs common in Indian diets is scanty, hence the necessity for estimation of methionine in foodstuffs widely used in South India.

EXPERIMENTAL

Samples of food materials were collected by appropriate sampling procedures from the Vellore market at different times of the year. The powdered food material was dried free of moisture and stored until used. Moisture determinations were done on fresh samples.

The material to be analyzed was subjected to acid hydrolysis by refluxing 5 g with 25 ml 20% hydrochloric acid over an oil bath maintained at 125°C for 6-8 hr. The hydrolysate was made up to 100 ml and filtered. Nitrogen determinations in the hydrolysate were done by the micro-Kjeldahl procedure.

Methionine estimation was done by two independent methods: 1) Chitre and Kini's (1946) modification of the McCarthy and Sullivan (1941) color reaction; and 2) a filter-paper-disc chromatographic procedure (Rao and Wadhwani, 1954) using normal butanol–water–acetic acid mixture (40:14:5) as the developing solvent. Recovery experiments were conducted by adding a known quantity of methionine to a sample and

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Table 1. Methionine content (%) of some South Indian foods.

	Moisture	Nitrogen	Methionine		
			McCarthy-Sullivan reaction	Filter-paper chroma-tography	Literature values
Cambu (*Pennisetum typhoideum*)	12.0	1.80	0.22 ± 0.06	0.21 ± 0.04	
Cholam (*Sorghum vulgare*)	10.8	1.20	0.14 ± 0.05	0.13 ± 0.06	
Maize (*Zea mays*)	14.2	1.74	0.18 ± 0.05	0.17 ± 0.03	
Ragi (*Eleusine coracana*)	13.8	1.10	0.18 ± 0.04	0.18 ± 0.04	0.28 [a]
Rice (*Oryza sativa*)	13.3	1.10	0.17 ± 0.07	0.16 ± 0.05	0.168 [b]
Wheat (*Triticum vulgare*)	12.6	1.74	0.13 ± 0.03	0.13 ± 0.05	0.16 [a] 0.138 [b]
Red gram (*Cajanus indicus*)	15.8	3.48	0.28 ± 0.04	0.27 ± 0.05	
Bengal gram (*Cicer arietinum*)	11.5	3.50	0.19 ± 0.03	0.18 ± 0.02	0.13 [a]
Green gram (*Phaseolus radiatus*)	10.2	3.73	0.17 ± 0.05	0.16 ± 0.04	0.14 [a]
Black gram (*Phaseolus mungo*)	10.4	3.64	0.26 ± 0.08	0.22 ± 0.06	0.32 [a]
Peas (*Pisum sativum*)	14.3	3.00	0.22 ± 0.05	0.20 ± 0.03	
Beans (*Dolichos lab lab*)	9.0	3.80	0.29 ± 0.04	0.27 ± 0.05	0.31 [a]
Cow gram (*Vigna catiang*)	11.6	3.87	0.42 ± 0.09	0.41 ± 0.06	
Horse gram (*Dolichos biflorus*)	12.4	3.40	0.43 ± 0.12	0.42 ± 0.09	
Amaranth (*Amaranthus gangeticus*)	86.4	0.74	0.048 ± 0.015	0.042 ± 0.020	
Egg plant (*Solanum melogena*)	91.8	0.20	0.017 ± 0.008	0.016 ± 0.005	
Potato (*Solanum tuberosum*)	75.6	0.22	0.023 ± 0.010	0.021 ± 0.007	
Sweet potato (*Ipomoea batatas*)	68.0	0.20	0.013 ± 0.003	0.013 ± 0.005	
Colacasia (*Colacasia antiquorum*)	74.0	0.45	0.028 ± 0.005	0.022 ± 0.005	
Tomato (*Lycopersicum esculentum*)	93.2	0.30	0.021 ± 0.002	0.019 ± 0.005	
Yam (*Typhonium trilobatum*)	70.2	0.20	0.014 ± 0.005	0.012 ± 0.004	
Banana (*Musa paradisciaca*)	84.0	0.20	0.014 ± 0.002	0.012 ± 0.004	
Spinach (*Spinacia oleracea*)	92.0	0.30	0.058 ± 0.007	0.050 ± 0.006	
Onion (*Allium cepa*)	85.2	0.20	0.014 ± 0.002	0.011 ± 0.005	

[a] Chitre and Kini, 1946.

[b] Balasubramaniam *et al.*, 1952.

estimating the total methionine. Recoveries were 95–103% by both methods. Twenty estimations were made for each foodstuff.

RESULTS

The methionine content of foodstuffs common in South India are given in Table 1. The chemical method generally gave values slightly higher than those obtained with the chromatographic method. Vegetables are much inferior to cereals as source of methionine. Of the plant foods, the pulses are the richest in methionine.

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Colorimetric Assay for Potassium Sorbate in Dried Fruits[a]

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(Manuscript received March 30, 1962)

SUMMARY

A simple, direct colorimetric method requiring no distillation is described for the determination of potassium sorbate in dried fruits. The method makes use of the oxidation of sorbic acid to malonaldehyde, which reacts with thiobarbituric acid to form a red pigment that is measured photometrically.

Increased demands for high-moisture (28% and over) dried fruits, primarily prunes and figs, have resulted in a need for a safe, effective antimycotic agent for treatment of thousands of tons of these products. The effect of various antimycotic agents on high-moisture dried fruits has been reported by Nury *et al.* (1960). Potassium sorbate was the most effective agent studied; under the conditions described, it protected high-moisture prunes from molds and yeasts *(Aspergillus glaucus* and *Saccharomyces rouxii)* commonly associated with prune spoilage. Another advantage of this additive was its residual effect, which protected open packages of high-moisture prunes, thus making it of particular value for the bulk high-moisture pack. Potassium sorbate is now being used by many prune processors and at least one fig processor.

An adequate and simple method is needed for determination of potassium sorbate in dried fruits, both for process control and for tests to assure that the sorbate content in fruit is not above its taste threshold (determined in this laboratory to be above 600 ppm). An analytical method would be adequate if measurements could be made in the range of 0–800 ppm sorbate in the fruit. Antimycotic effect is obtained by 200–600 ppm (as determined in this laboratory). The concentration required depends on the moisture content of the dried fruits.

Methods for determination of sorbic acid in food products include distillation and subsequent determination by ultraviolet absorption and titration techniques (Alderton and Lewis, 1958; Harrison, 1961; Spanyár and Sándor, 1958). Distillation is time-consuming, and the ultraviolet absorption method is not adaptable for use by small processors, because they lack technical personnel and equipment costs are high. The procedure reported here for dried fruits is a modification of the Schmidt (1960) method. It is based on the color reaction of malonaldehyde and thiobarbituric acid, which has been reported by several authors (Bernheim *et al.*, 1947; Biggs and Bryant, 1953; Dox and Plaisance, 1916; Jennings *et al.*, 1955; Sinnhuber *et al.*, 1958) in studies on the rancidity of lipids. No distillation is required, and a single analysis can be completed in 25 min or less.

MATERIALS AND METHODS

Thiobarbituric acid. Dissolve 0.5 g of 2-thiobarbituric acid (TBA) in 20 ml water and 10 ml of 1*N* NaOH in 100-ml volumetric flask. After dissolving, add 11 ml of 1*N* HCl and make to volume. The 2-thiobarbituric acid must be made fresh daily. The reagent remains relatively unchanged for a 6-hr period, but after one day absorbance values are lower, resulting in erroneous analytical results. The amount of loss is about

[a] Presented at the 14th Annual Meeting of the Institute of Food Technologists, Miami, Florida, June 10–14, 1962.

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40% after 14 days and 100% after 30 days at ambient temperature.

Potassium dichromate-sulfuric acid. Mix 0.01*N* $K_2Cr_2O_7$ and 0.3*N* H_2SO_4 in 1:1 proportions.

Potassium sorbate. Dissolve 250 mg of potassium sorbate in water in a 250-ml volumetric flask and dilute to mark.

Standard curve. For determining the standard curve, use dried fruit that has not been treated with potassium sorbate. Blend 10 g of ground fruit for 2 min in 90 ml water in a blender. Add 10 g of slurry to each of five 250-ml volumetric flasks. Add 0, 0.25, 0.5, 0.75 and 1.0 ml of the standard sorbate solution (0.1% potassium sorbate solution) and make up to volume. In each case, filter and add 2 ml of the filtrate to a test tube containing 2 ml of potassium dichromate-sulfuric acid solution. Heat 5 min at 100°C in an oil bath. Add 2 ml of thiobarbituric acid to the tube and allow it to remain in the bath for an additional 10 min. Remove and cool quickly in running tap water. Measure the absorbance at 530 mμ in a spectrophotometer; use water as a blank (for 100% transmission). Plot absorbance vs. μg potassium sorbate. The colored solution, after cooling, is stable for at least one-half hour.

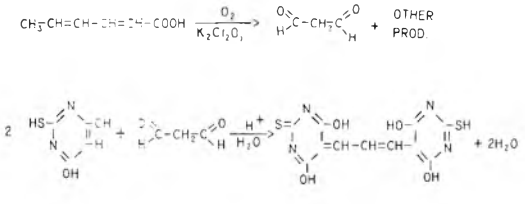
Sample preparation. Prepare samples to be anlayzed for potassium sorbate exactly as described above except that no potassium sorbate solution is added to the volumetric flasks.

Calculation. Refer optical absorbance to the standard curve for conversion to μg potassium sorbate.

μg potassium sorbate × 125 = ppm potassium sorbate in the fruit.

RESULTS AND DISCUSSION

The method is based on conversion of a sorbate to sorbic acid and its subsequent oxidation to malonaldehyde, which reacts with 2-thiobarbituric acid to form a colored compound (Sinnhuber, 1958) as follows:



The absorption spectrum for potassium sorbate in figs and prunes is shown in Fig. 1. The recovery of potassium sorbate, by the colorimetric method, from samples is shown in Table 1. Table 2 shows com-

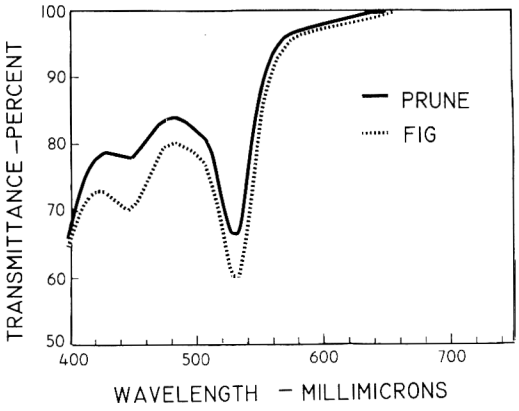


Fig. 1. Absorption spectra of the thiobarbituric acid–malonaldehyde pigment.

Table 1. Recovery of potassium sorbate that had been added to dried fruit samples.

Fruit	Potassium sorbate added (mg)	Recovered (mg)	Recovered (%)
Figs	.25	.255	100
	.25	.258	104
	.50	.512	102
	.50	.518	104
	1.00	1.00	100
	1.00	1.00	100
Prunes	.25	.243	97
	.25	.250	100
	.50	.518	103
	.50	.516	101
	1.00	.906	91
	1.00	.962	96

Table 2. Comparison of colorimetric method with ultraviolet absorption method for assay of potassium sorbate.

Fruit	Potassium sorbate added (mg)	Colorimetric method (ppm)	U. V. method (ppm)
Figs	.25	255	200
	.25	258	190
	.50	512	500
	.50	518	470
	1.00	1000	770
	1.00	1000	750
Prunes	.50	518	.....
	.50	516	450
	1.00	906	830
	1.00	962	760

parative analyses by ultraviolet absorption method (Alderton and Lewis, 1958) and the present technique.

Fig. 2 shows typical standard curves for prunes and figs. Variation in sugar contents of dried fruits did not affect the result of the analyses. This was determined by adding fructose and glucose, principal components of figs and prunes, to samples. They did not in any way affect the results.

To test reproducibility, 20 analyses were run on a single prune sample. The coefficient of variation was 3.14%.

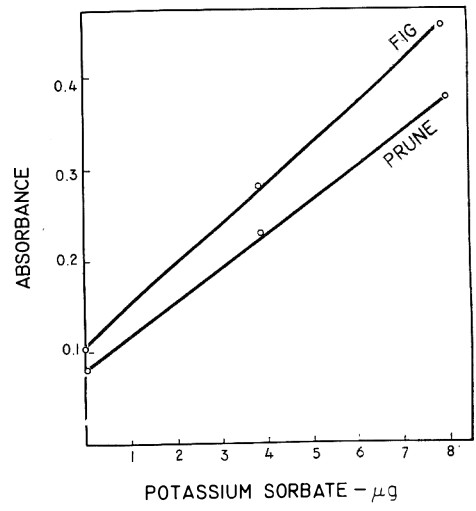


Fig. 2. Typical standard curve for figs and prunes.

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Variety and Location Effects on Ascorbic Acid in Potatoes[a]

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(Manuscript received January 26, 1962)

SUMMARY

The data from analyses of six potato varieties grown at nine widely different locations over two years showed that varietal comparisons for ascorbic acid content are relatively unaffected by location. Thus, in ascertaining the potential value of potato varieties as a source of ascorbic acid, widespread testing appears to offer no advantage over the determination of varietal differences for this factor in a more confined area.

Potatoes are known to be an important dietary source of ascorbic acid. The antiscorbutic activity of potatoes is due to the content of reduced ascorbic acid and its conversion product, dehydroascorbic acid (Leichsenring *et al.*, 1957). Some attempts have been made to determine whether the ascorbic acid level in potatoes is related to variety, location, and storage conditions. It has been well established that the ascorbic acid content of potatoes decreases during storage (Allison and Driver, 1952; Barker, 1950; Karrika *et al.*, 1944; Leichsenring *et al.*, 1957; Panalaks and Pelletier, 1960), the rate varying with storage temperature. Allison and Driver (1953) reported that variety, location, and storage all had highly significant effects on the ascorbic acid level in potatoes. Karrika *et al.* (1944) found that the varieties Katahdin and Houma were consistently high in ascorbic acid; Irish Cobbler, Warba, and Sebago were intermediate; Chippewa was low; and Earlaine and Green Mountain were variable. In a recent literature review, Talburt and Smith (1959) reported that the effect of variety on ascorbic acid is still unsettled.

The availability of potato varieties grown at widely different locations in eight provinces of Canada, over a two-year period, made possible an intensive study of variety and location effects on the ascorbic acid content of potatoes after a short period of storage. Only the reduced ascorbic acid content was determined, since it is the important source of antiscorbutic activity in the potato; the dehydroascorbic fraction is practically eliminated by cooking (Leichsenring *et al.* 1957).

MATERIALS AND METHODS

Six potato varieties were grown at nine different locations in eight provinces of Canada during 1958 and 1959 as part of the national potato trials. The varieties were: F4834, F4932, F5025, F5080, F5216, and Norgleam. The numbered varieties were developed at the Canada Department of Agriculture Research Station at Fredericton, New Brunswick, a center for potato breeding in Canada. For comparisons, the standard varieties Green Mountain, Irish Cobbler, Katahdin, and Warba were grown at several of the nine locations and at the same locations for each variety in both years.

Fifteen-pound samples of the freshly harvested potato varieties were shipped to the Morden laboratory, where they were stored 1 month at 40°F. The tubers were conditioned for 7 days at 65°F before testing.

A random six-tuber sample of each lot of potatoes was peeled, and transverse sections were cut and halved. Duplicate samples were prepared in oxalic acid from the half sections according to the method described by Ruck (1956). Analyses for reduced ascorbic acid were performed with the 2,6-dichlorophenolindophenol titration technique (Association of Vitamin Chemists, 1951).

RESULTS

The data in Table 1 give the ascorbic acid contents of six potato varieties grown at nine locations in 1958 and 1959. These data are arranged in descending order according to the means for

[a] Contribution No. 27 from the Experimental Farm, Research Branch, Canada Department of Agriculture, Morden, Manitoba.

each variety and each location in 1958. Analysis of variance of the data showed highly significant differences in ascorbic acid content between varieties and between locations in both years. Also, there was a highly significant variety-location interaction for this factor. When the varietal means for all locations for each year were compared, a highly significant correlation coefficient ($r = +.979$) was obtained.

Applying Duncan's multiple-range test (1955) to the data for the six varieties at each individual location (Table 1) indicated that the varieties could be separated into two groups of three by ascorbic acid level. Norgleam, F5216, and F4932 were higher, and F4834, F5025, and F5080 lower at eight of nine locations in 1958, and at seven of nine in 1959.

The mean two-year data for ascorbic acid in the standard varieties were: Katahdin, 22.8; Irish Cobbler, 20.4; Green Mountain, 18.4; and Warba, 13.0 mg/100g.

DISCUSSION

Since a significant variety-location interaction occurred in the analysis of variance for both 1958 and 1959 data, it was necessary to examine results from each location separately. The error mean squares at each location were found to differ significantly[a]; therefore it was concluded that the pooled data were not adequately tested, because of the error heterogeneity. Thus, the application of Duncan's multiple-range test to the data from each individual location appeared to be the best method of analyzing the data. This test showed that distribution of the six test varieties into two distinct groups by ascorbic acid content occurred at 15 of 18 widely different locations over a two-year period. Also, at nine locations out of the 15 at which this grouping occurred there was a significant difference between the lowest-ranking variety of the high group and the highest-ranking variety of the low group. Thus, division between the two groups was reasonably clear, and the average performance of each was affected by location in a similar manner. The location effects were undoubtedly influenced by a combination of climatic and cultural conditions as well as storage temperatures prior to and during shipment to the central location. For example, at one location (Lethbridge, Alta.) in 1959, ascorbic acid content was very low (Table 1). In data not shown, the reducing sugar content of the potatoes from the same location was high.

Table 1. Ascorbic acid contents of six varieties of potatoes grown at nine locations in each of two years.

Variety	St. Anne Que.	Smithfield Ont.	Melfort Sask.	Fredericton N. B.	Lethbridge Alta.	Guelph Ont.	Morden Man.	Agassiz B. C.	Kentville N. S.	Varietal means
1958										
Norgleam	29.2 [a] a [b]	23.6 b	30.3 a	23.2 b	30.3 a	30.3 a	29.1 a	25.1 a	17.5 a	26.5
F5216	25.4 b	24.6 a	24.2 b	26.2 a	26.9 b	22.0 b	25.9 b	21.2 b	18.5 a	23.9
F4932	25.7 b	24.2 ab	19.6 c	22.5 b	21.6 c	18.4 c	13.1 d	18.0 c	17.0 a	20.0
F4834	17.6 c	22.1 c	16.8 d	18.2 c	14.4 e	17.2 d	13.1 d	16.9 c	13.3 b	16.6
F5025	19.9 c	16.8 e	16.7 d	17.1 d	15.0 de	14.6 f	16.0 c	16.6 c	13.6 b	16.2
F5080	19.8 c	17.8 d	18.4 cd	16.4 d	15.6 d	16.2 e	16.3 c	13.0 d	12.6 b	16.2
Location means	22.9	21.5	21.0	20.6	20.6	19.8	18.9	18.5	15.4	
1959										
Norgleam	27.6 a	25.3 a	19.4 a	29.6 a	12.4 b	28.6 a	21.8 a	28.8 b	22.0 a	23.9
F5216	18.4 d	23.4 a	16.8 b	19.0 b	12.2 b	25.8 b	17.4 b	30.2 a	19.4 b	20.3
F4932	23.6 b	19.8 b	14.2 bc	14.6 d	13.0 a	19.8 c	15.8 c	17.0 c	18.6 b	17.4
F4834	21.4 c	18.7 bc	13.2 c	14.9 d	10.6 c	18.4 cd	13.2 d	16.4 cd	14.2 c	15.7
F5025	17.3 de	16.8 c	10.0 d	12.6 c	9.8 d	17.6 d	14.0 d	15.0 d	15.4 c	14.3
F5080	16.2 f	12.4 d	9.5 e	16.2 c	9.0 e	14.0 e	14.2 d	11.4 e	14.7 c	13.1
Location means	20.8	19.4	13.8	17.8	11.2	20.7	16.1	19.8	17.4	

[a] Each value represents the mean of a duplicate determination expressed as mg per 100 g.
[b] Significant differences between means according to Duncan's multiple-range test ($P = .05$). Means with the same letter are not significantly different.

Since both effects can result from low temperatures (Talburt and Smith, 1959), it is logical to assume that these potatoes were chilled before or after harvest or during shipment. Since all tubers were stored similarly at the laboratory, losses in ascorbic acid which probably occurred at that time did not obscure previous effects.

The results suggest that varietal comparisons of potatoes for ascorbic acid content are relatively unaffected by location. Therefore, widespread testing does not appear to offer any added advantage when establishing the potential value of potato varieties as a dietary source of ascorbic acid. Evaluations of varieties for this factor would be more representative if confined to a limited area with ready access to an analytical laboratory.

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A Rating-Scale Method for Determining Absolute Taste Thresholds

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SUMMARY

Absolute taste thresholds for salt and sweetness were determined by a combination of ascending-series and rating-scale methods: for salt a value only very slightly under previous quoted figures was obtained, for sugar a value of .002 p/p was obtained. The results also permitted a scaling of each taste description on the rating-scales in terms of both j.n.d's above threshold and solution concentrations. Previous workers have doubted whether there is one single absolute threshold for taste: the present results suggest a number of thresholds in an ascending series may be defined operationally.

INTRODUCTION

The absolute taste threshold has been the subject of a number of definitions, and a variety of methods have been used to determine it. These two sources of variation superimposed upon individual differences and errors have resulted in a wide range of quoted values for absolute threshold concentrations of the four primary tastes: salt, sweet, acid and bitter.

Threshold determination is more difficult to achieve in taste than in some other sensory channels, partly because there is still doubt about what the taste receptors do. We may distinguish three sorts of threshold definition—chemical, physiological and psychological—and here we are dealing with the third. A chemical definition of "threshold" demands that the level of explanation be chemical. The precise identification of the events at or on the surface of the receptor organs should be in terms of reaction equations, with the psychophysical relation one between the report of a sensation and the occurrence of a chemical change at the receptors.

By physiological threshold we refer to those definitions that seek to relate a minimum concentration of chemically-defined taste stimulus to neurone activity expressed in terms of changes in electrical potential. Some quoted values for taste threshold seem to aim at this sort of definition in the view that it leads to "pure" values as a baseline from which may be assessed all other values determined with a complete intact organism. It is a common feature of chemical and physiological threshold determinations that they aim at producing deterministic rather than probabilistic values for a threshold, and they both by-pass the idea of sensations by measuring objective correlates of flavor sensations rather than involving sensations directly. The difference thresholds associated with absolute values are supposedly minimal quanta ideally: in chemical terms the change in structure of a single molecule to release energy, in physiological terms the elicitation of a single action potential. By comparison with this type of definition a psychological description of the responses of a sample of intact normal adults to flavors as normally encountered seems imprecise and subject to so many quantitative indeterminate effects (learning, fatigue, set, individual expectations, etc.) as to be elusive to the point of indefinability.

We would expect that threshold values reported by the few psychologists who have worked in this field might be higher than those given by physiologists or chemists, since the usual psychophysical method of defining absolute threshold as being at the mid-point of the cumulative probability distribution of response over the transition

zone near threshold is not always used by chemists, who instead may use some other statistical (and equally arbitrary) definition leading to lower values. And we might expect that methods that attempt to take account of the complex phenomenology of taste near threshold will, though giving a more complete account of known experience, blur the quantitative picture.

This paper submits evidence suggesting that a method in the psychophysical tradition, with comparatively few constraints on the subjects' response choices, can give values that bear favorable comparison with earlier studies. This method attempts to quantify some of the judgments frequently made about weak flavors.

METHOD

The procedure used was a combination of the ascending-series method and a rating-scale method; both are well known but a combination of the two appears to be novel as applied to taste.

In the ascending-series method the subject tastes a series of solutions of increasing concentration, each twice as strong as the preceding one. Two supposedly convenient properties of the series are thus employed:

1) The sum of all stimulus intensities tasted is never greater than that of the next stimulus to be tasted, so that residual tastes, if cumulative, are not expected to completely mask the next stimulus, and

2) the subjective intensity of taste is proportional to the log of the concentration strength, and so the scale is roughly a subjective equal-interval scale above threshold.

The rating-scale method simply consists of having a series of statements corresponding to increasing taste sensations—from "the same as water" to "a strong sweet taste," for example—and the subject reads out the most appropriate statement (for him) after tasting each stimulus.

Traditionally the ascending-series method is one of the constant methods (Guilford, 1954) and usually all that is asked for is one of the two responses "same" or "different." This method gives accurate results and is mathematically well explored, but with taste stimuli (as opposed to weights, sounds or lights) subjects give a large number of "doubtful" answers, and in the case of substances like sodium benzoate may change their responses from "sour" or "sweet" to "bitter" and back again as the solution strength increases. The naive subject also frequently tends to report odd or unpredicted sensations, and to constrain him to two, or at the most three, categories of permitted response is to impose a task that may seem unreal and be resented. The effect of attitude on taste discrimination has been noted (Baryscheva, 1936, and others), and it would seem desirable to permit the subject as much variation in response as is consistent with getting a meaningful determination of the threshold.

For each stimulus tasted, then, the subject selected a description of it from a card of seven descriptions before him, and this was recorded as a Likert-scale value.

The full experimental procedure was as follows:

Solutions in a range of concentrations from well above the reported threshold for the primary taste in question were prepared with tap water: the solutions stood half an hour or more, the subject was asked to rinse his/her mouth with plain tap water and note its taste. A rating card was presented in "negative information" form, the subject then tasted the stimulus series in order and described each as he/she thought appropriate, as follows: same as water; almost certainly the same as water; doubtful if water; very slightly different from water; slightly different from water; different from water; certainly different from water.

This series was used as a trial run so that the stimuli were made familiar to the subject, and all subjects had comparable advance information within the experimental situation, without themselves having practice on the "positive information" rating-scale descriptions (given in Table 1).

The word used in the scale is "sour" with citric acid and "sweet" with sucrose.

A second mouth-rinse with tap water was then made, the "positive information" rating-scale was substituted and the series was tasted again. Because some subjects found higher concentrations exceedingly unpleasant, the more concentrated salt solutions were omitted once the threshold had been clearly passed.

For this experiment the threshold was defined in terms of the location of a jump in the rating-

Table 1. Positive information rating-scale taste descriptions.

Response	Likert (numerical) values (used in analysis but never shown to subjects)
Same as water	1
Doubtful if pure water	2
A very faint taste, can't say what	3
A very faint sour taste	4
A faint sour taste	5
A weak sour taste	6
A clear sour taste	7

scale descriptions, to the last time that the description "a very faint _____ taste" (Likert-value 4) was used by the subject: we specified the last time the jump occurred because some subjects in tasting a long series may anticipatorily cross the threshold, even on a tap-water dummy stimulus interposed in the ascending series. In this way our threshold values should be conservative ones. In theory a separate threshold can be associated with each of the intervals between rating-scale descriptions—as has long been known. The gap between the lowest and highest thresholds thus defined is usually called the "interval of uncertainty." The particular threshold definition we chose was taken to give values nearest to the two-category threshold and hence compare with previously reported figures.

Seventy-nine subjects took part in the salt threshold experiment, not all tasting all stimuli because of their omitting above-threshold concentrations. Seventy-seven subjects, not all the same persons as for salt, took part in the sweetness threshold experiment. All were drawn from a pool of adult factory and office staff.

RESULTS

Salt

Table 2 shows the mean ratings for each stimulus concentration tasted in ascending series, and the cumulative threshold proportions where threshold is defined as at the highest concentration in the series where the Likert-value of the rating scale changes from less than 4 to 4 or greater than 4.

Table 2 is based on truncated stimulus series runs; each subject started tasting about two concentrations below where the trial run suggested threshold might be and stopped tasting as soon as certitude (Likert-value 6 or 7) was attained. This method gives a good threshold estimation but the just-noticeable-difference (j.n.d.) value may be in error because of the error of the variance of the rating-scale values for each concentration.

Plotting cumulative proportions of thresholds on arithmetic probability paper against concentration strength, and locating each threshold proportion midway between the adjacent stimuli, gave a linear plot with absolute threshold at the fiftieth percentile (the usual psychophysical definition) corresponding to a concentration of $2^{-3.05}$% or 0.000647 p/p. This compares with the value of 0.0007 given by previous workers (Ritcher and MacLean, 1939) and usually quoted in relevant texts (Moncrieff, 1944; Stevens, 1951; Morgan and Stellar, 1950). This previous value is based on 53 subjects.

Table 2. Increasing concentrations of sodium chloride in tap water (based on 79 subjects tasting).

Concentration (parts of 1%)	Mean ratings [a] R	Mean ratings [a] R^1	Threshold cumulative proportion of 79	No. of judgments
$1/128 = 2^{-7}$	1.5	8.33		12
			.012	
$1/64 = 2^{-6}$	1.52	8.60		31
			.063	
$1/32 = 2^{-5}$	2.7	19.45		54
			.304	
$1/16 = 2^{-4}$	2.9	31.82		77
			.709	
$1/8 = 2^{-3}$	4.61	60.13		74
			.937	
$1/4 = 2^{-2}$	5.60	76.58		42
			1.000	
$1/2 = 2^{-1}$	6.20	86.67		10

[a] $R^1 = \frac{100(R-1)}{6}$ by definition; for convenience $0 \leqslant R^1 \leqslant 100$.

The associated j.n.d., defined as the semi-interquartile range when concentration is expressed in powers of base two (a log scale), is $2^{-0.75}$%. This gives upper and lower concentration limits to the threshold of $2^{-3.2}$ and $2^{-4.7}$ approximately, or concentrations of .00109 and .00038 p/p. The highest limit to the threshold quoted by previous workers is about .0019 for the drop method (one drop deposited at a point location on the tongue). This appears to be about 3 j.n.d. above threshold, a not unreasonable difference between minimum and maximum information conditions, if that is what the two methods involved exemplify.

Exploiting the fact that cumulative threshold proportions and cumulative mean ratings both gave a linear plot against log concentration for NaCl, scale values were plotted against concentration giving the results of Table 3.

Sugar

The procedure outlined for salt was repeated for sucrose solutions; the differences in the data

Table 3. Rating-scale values, concentrations, and subjective intensity, for salt.

Rating-scale value (R)	Concentration % in powers of 2 (1% = 2^0) of A.R. NaCl	As j.n.d. (d) units about abs. threshold (a).
2	$2^{-5.15}$	a − 1.33d
3	$2^{-4.12}$	a − 0.23d
4	$2^{-3.25}$	a + 0.93d
5	$2^{-2.45}$	a + 2.00d
6	$2^{-1.45}$	a + 3.30d

center on the fact that all subjects tasting the sucrose series tasted all concentrations up to 1% and most on to 2%, well above threshold. Table 4 gives rating-scale values, concentrations, and

Table 4. Rating-scale values, concentrations, and subjective intensity, for sweetness.

Rating-scale value (R)	Concentration % in powers of 2 ($1\% = 2^n$) of sucrose	As j.n.d. (d) units about abs. threshold (a).
2	$2^{-1.0}$	a − 3.70d
3	$2^{-2.0}$	a − 0.64d
4	$2^{-1.85}$	a + 0.86d
5	$2^{-1.0}$	a + 2.07d
6	$2^{-0.25}$	a + 3.14d

j.n.d. units obtained in the same manner as those values in Table 3, q.v. Comparable data to Table 2 are given in Table 5. It will be seen that the j.n.d. values for the three above-threshold

Table 5. Increasing concentrations of sucrose in tap water (based on 77 subjects tasting).

Concentration (parts of 1%)	Mean ratings[a] R	Mean ratings[a] R'	Threshold cumulative proportions of 77	No. of judgments
$1/64 = 2^{-6}$	1.60	9.95		77
			.0	
$1/32 = 2^{-5}$	1.83	13.85		77
			.026	
$1/16 = 2^{-4}$	2.23	20.57		77
			.156	
$1/8 = 2^{-3}$	2.95	32.47		77
			.377	
$1/4 = 2^{-2}$	3.21	36.80		77
			.844	
$1/2 = 2^{-1}$	4.96	66.02		77
			1.000	
$1 = 2^{0}$	6.34	89.03		76
			1.000	
$2 = 2^{1}$	6.83	97.12		75

[a] $R' = \frac{100(R-1)}{6}$ by definition, as in Table 2.

rating-scale values show close agreement between NaCl and sucrose, though actual concentration strengths do not. In other words, the rating scale is a response-centered measure, not a stimulus-centered measure, if we define the stimuli in percentage concentrations. (If we define the stimuli in gram-mols the point is even more obvious.)

The absolute threshold for sucrose, by the same defined procedure as that used for salt, is $2^{-2.15}$% or 0.00183 p/p. (Previous quoted values tend to be higher: 0.005, Howell, 1922.) The associated j.n.d. is $2^{-0.70}$%; this gives upper and lower limits to the threshold of approximately $2^{-1.75}$ and $2^{-3.15}$, or concentrations of 0.00297 and 0.00113 p/p.

The higher absolute threshold for sugar than for salt agrees with previous findings. The slight difference in j.n.d. values is probably error: it represents the difference between 0.00297 and 0.00292 p/p at the highest concentration mentioned so far (a + 1d for sucrose), which is clearly within experimental error. Since the Weber fraction for taste is usually quoted as one constant value for all tastes without respect to the actual substances involved it is probably legitimate that the j.n.d. should be independent of the absolute threshold, but this needs checking experimentally.

DISCUSSION

The results are consistent with previous findings with other methods. In terms of the actual numerical values obtained they are not of great interest, for in any practical situation it is fairly usual to work with substances that are clearly above or below thresholds; either they are intended to be tasted or they are intended not to be tasted. Further, actual determination was by graphical means, and the thresholds, as defined, were assumed to take place in the middle of the stimulus series intervals concerned. Clearly, more precise locations might be obtained by applying second-order corrections and by repeating the whole procedure with new stimulus series that are not powers of 2 of 1% standard but powers of 2 of, say, 1.05% and 0.95% standards.

The points of interest to a psychologist are, we think, that a series of statement-forms representing various subjective intensities or levels of certainty about subjective intensities can be ordered and mapped on to a stimulus series that is chemically definable. The relation between the threshold and j.n.d. units and the scale values must follow by definition if a large sample of data with monotonically increasing probabilities of each scale value for stimulus concentration obtain; in this sense it is mathematically trivial. If we can so scale any series of statements, and the procedure is related, in principle, to the semantic scaling of Osgood *et al.* (1957), and partic-

ularly that of Cliff (1959) on adverbs as multipliers, and we can define the absolute threshold as the jump between one statement-form and the next in their ordered series, then we can consider as rather arbitrary activities the traditional pursuit of how many categories are pertinent to threshold determination, and the more recent attempts by Gridgeman (1959) and others to define not one absolute taste threshold but three (threshold of knowing the substance is not water, threshold of knowing what the substance is, threshold of what intensity the substance is).

Given that a zone of uncertain perception surrounds a change that is capable of precise chemical or physiological definition—in theory, at any rate—then there are, psychologically, as many thresholds or as few thresholds as one is able to conceptualize statement-forms ordered over the zone of uncertain perception. Whether there is a limit to the number of statements one can scale in this way depends on the reliability and precision of language as well as the reliability and precision of taste perception; but it seems reasonable that we can have more than three thresholds, and for some subjects who are practiced in the use of scales, seven values might he obtained. We failed to get separation for the pairs of statements at the ends of the scales; subjects had response preferences, and the extremes of scales are often unscalable for mathematical reasons.

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Objective Characterization of the Mouthfeel of Gum Solutions

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SUMMARY

A correlation was established between the mouthfeel characteristics of gum solutions and their rheological behavior. Gums that are very slimy in the mouth deviate only slightly from Newtonian viscosity. The degree of sliminess decreases with increasing deviations from the Newtonian character, and gums that exhibit a high degree of shear thinning are nonslimy in the mouth. This finding enables selection of proper gums for specific consistency effects by simple reference to the shape of the viscosity vs. the rate-of-shear curve.

INTRODUCTION

Gums are amorphous substances of high molecular weight occurring widely in the vegetable kingdom. They are mostly hydrophilic polysaccharides or their derivatives, and are characterized by dispersibility in cold or hot water, leading to production of viscous dispersions or solutions and, in certain cases, to gel formation (Cook and Peterson, 1958; Hirst and Jones, 1951; Jones and Smith, 1949; Walder, 1948). In recent years, the meaning of the term "gums" has been extended to include materials of similar properties produced by microbial cells and synthetic polymers produced in the laboratory (Anon., 1960).

Gums find important uses in the food industry as stabilizers, thickeners, humectants, protective colloids, coagulants, gel formers, emulsifiers, ice crystal inhibitors, coating agents, and flavor fixatives (Waldt, 1961; Werbin, 1953). Specific gums are selected for specific purposes on the basis of physical and chemical properties. Unfortunately, no method, except sensory evaluation, is available for predicting the mouthfeel imparted to a liquid or semiliquid product by the presence of a gum. This is especially important when gums are used at concentrations sufficient to affect the consistency of a food, as is the case when they are used as thickeners and bodying agents. Experience has indicated that in such instances some gums are slimy in the mouth and difficult to swallow whereas others are hardly noticeable.

An investigation was made to correlate the mouthfeel of gum solutions with their viscosity behavior, thereby establishing an objective method for predicting organoleptic properties of gum solutions that could be useful in selecting specific gums for desired consistency effects in food products.

EXPERIMENTAL

Materials. Thirty major and minor gums were procured from commercial suppliers. All the gums, except four, were natural plant materials. The following synthetic gums were used: carboxypolymethylene (sodium salt of Carbopol 934, made by Goodrich Chemical Company), polyvinyl alcohol (Elvanol 72-60, made by DuPont de Nemours & Company), carboxymethyl cellulose (CMC-grade, 7H XSP, made by Hercules Powder Company), and methyl cellulose (Methocel 400, made by Dow Chemical Company). Two microbial gums (polysaccharide B-1459 and phosphomannan Y-2448) were obtained from the Northern Regional Research Laboratory at Peoria, Illinois, and the third one (dextran B-512) was obtained from a commercial laboratory (Cherokee Laboratory, Tulsa, Oklahoma). The gums were dispersed or dissolved in water by standard published methods for each gum. Cold water was used for cold-water-soluble gums, and the aqueous dispersions were heated to proper temperatures for hot-water-soluble gums. The suspension of cornstarch was brought to a boil to gelatinize starch granules. Concentrations were selected in such a way that the solutions had a starting viscosity of 1200 cps at 0.5 rpm as measured with the Brookfield viscosimeter.

Viscosity measurements. Viscosity was measured at room temperature with a Brookfield vis-

cosimeter, model HAT, equipped with a heliopath attachment. Viscosities were measured at different spindle speeds, and the obtained values were converted to centipoises.

Organoleptic evaluation. With solid materials, mastication consists of shearing and grinding the product with the teeth. The tongue, cheeks, and lips control and direct the food between the teeth. With liquid or semisolid materials, which do not require much effort for disintegration, the tongue plays a more important role than the teeth. The movements of the tongue help to shear the food and dilute it with saliva to the point where its viscosity is low enough for swallowing. During eating, the tongue moves about 30 times per minute.

Sixteen selected gum solutions were subjected to organoleptic evaluation by a trained texture-profiling panel and rated on a 7-point scale for degree of sliminess: 1, nonslimy; 2, very slightly slimy; 3, somewhat slimy; 4, moderately slimy; 5, slimy; 6, very slimy; 7, extremely slimy. Ratings by semitrained tasters gave good agreement with ratings by the trained judges.

Definition of sliminess. For organoleptic evaluation, a slimy material was defined as one that is thick, coats the mouth, and is difficult to swallow.

RESULTS AND DISCUSSION

When the viscosity of the gum solutions was plotted against rate of shear, the curves obtained showed different degrees of deviation from a straight-line characteristic of a Newtonian liquid. Fig. 1 illustrates this point, using typical gums as examples. It

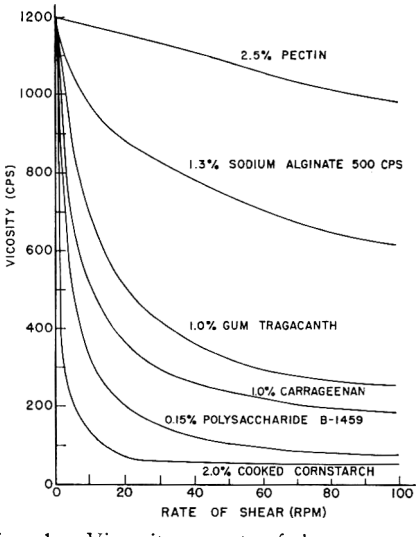

Fig. 1. Viscosity-vs.-rate-of-shear curves of typical gums.

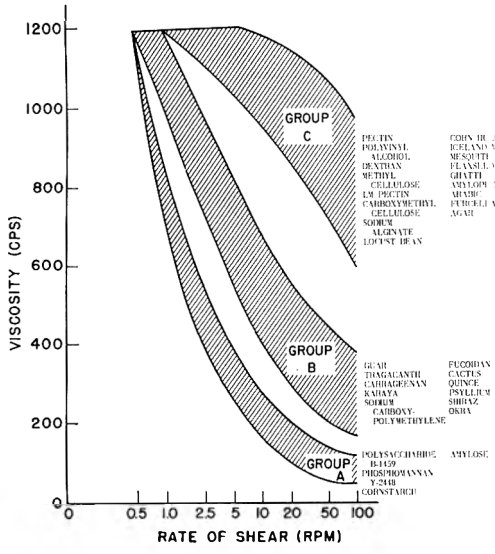

Fig. 2. Effect of shear rate on the viscosity of gum solutions.

will be seen that the cooked cornstarch paste and the solution of polysaccharide B-1459 show a very sharp drop in viscosity with increasing rate of shear. Gum tragacanth and carrageenan exhibit a slower decline of viscosity with increasing rate of shear, and pectin shows relatively little change. When one notices the differences between viscosity at 0.5 rpm, about the environment of the sample when it is first placed in the mouth (obviously, it is not possible to measure viscosity at 0 rpm), and that at 30 rpm, about the condition of the sample during mastication and before it is ready to be swallowed, the contrast between the various gum solutions becomes very evident.

Based on the rate of viscosity drop with increasing rate of shear, the studied gum solutions can be divided into three groups as shown by semilog plot in Fig. 2. Group A comprises gums that exhibit a very sharp viscosity decrease. Group C includes gums that show a small to moderate change with increasing rate of shear. Group B encompasses the intermediate gums. The boundaries for the areas comprising the different groups were defined by the actual curves of the first and last member of the group as listed to the right of the graph. Gums listed in the left-hand column were subjected to organoleptic evaluation, whereas those listed in the right-hand column are classified into

Table 1. Correlation between mouthfeel and viscosity-vs.-rate-of-shear behavior.

Gum	Type	Concentration (%)	Organoleptic evaluation		Viscosity vs. rate of shear
			Rating	How slimy	Group
Starch	Cooked, corn	2.0	1	Non	Group A
Phosphomannan Y-2448	——	0.75	1	Non	Group A
Polysaccharide B-1459	——	0.15	1	Non	Group A
Sodium carboxy-polymethylene	Sodium salt of Carbopol 934	0.3	2	Very slt.	Group B
Carrageenan	Seakem 9	1.0	3	Somewhat	Group B
Gum karaya	——	1.0	3	Somewhat	Group B
Gum tragacanth	——	1.0	2	Very slt.	Group B
Gum guar	——	0.6	2–3	Very slt. to somewhat	Group B
Locust bean gum	——	0.7	3	Somewhat	Group C
Carboxymethyl cellulose	CMC 7H XSP	1.0	4	Moderate	Group C
Sodium alginate	500 cps	1.3	5–6	Slimy to very	Group C
Low-methoxy pectin	Exchange 466	5.0	7	Extremely	Group C
Methyl cellulose	Methocel 400	2.6	6	Very	Group C
Polyvinyl alcohol	Elvanol 72-60	7.0	6	Very	Group C
Dextran B-512	Cherokee Lab.	18.0	7	Extremely	Group C
Pectin	Exchange 681	2.5	7	Extremely	Group C

the respective groups solely on the basis of the shape of the curve for viscosity vs. rate of shear.

Table 1 shows the relation between the classification based on viscosity behavior and organoleptic rating. It will be seen that, with only one exception, the sliminess as perceived in the mouth follows very closely the shape of the curve for viscosity vs. rate of shear. The exception is locust bean gum, which, though rated organoleptically as somewhat slimy, had to be included in Group C, because of its viscosity behavior.

The shape of the curve for viscosity vs. rate of shear changes somewhat with concentration and the molecular size of the gum. This change, however, is not drastic enough to affect the above classification. For example, a 2.6% solution of Methocel 400 decreases only slightly with increasing rate of shear, whereas a 3% solution of Methocel 100 behaves almost like a Newtonian liquid. A similar observation was made for solutions of sodium alginate. The shorter the chain length of the alginate molecule, the more Newtonian the viscosity behavior, whereas the longer the chain, the greater the dependence of viscosity on the rate of shear. However, in no case was the effect of molecular size large enough to move the gum to another group. It may therefore be stated that the slimy mouthfeel is related to a basic character of the gum and is affected only slightly by variations in the size of the gum molecule. The effect of concentration varied depending upon the sharpness of viscosity drop with shear and the position of the gum in the respective group. In the case of pectin or alginate, a decrease in concentration had no effect on the general shape of the curve for viscosity vs. rate of shear. The same was true of cornstarch when its concentration was increased. With borderline cases such as locust bean gum, however, a decrease in concentration caused a sharper viscosity drop with increasing shear, and moved that gum from Group C to Group B. It may be stated that the viscosity pattern of gums that deviate only slightly or very greatly from the Newtonian character is not appreciably affected by concentration, whereas gums that fall close to the borderline of the classification shown in Fig. 2

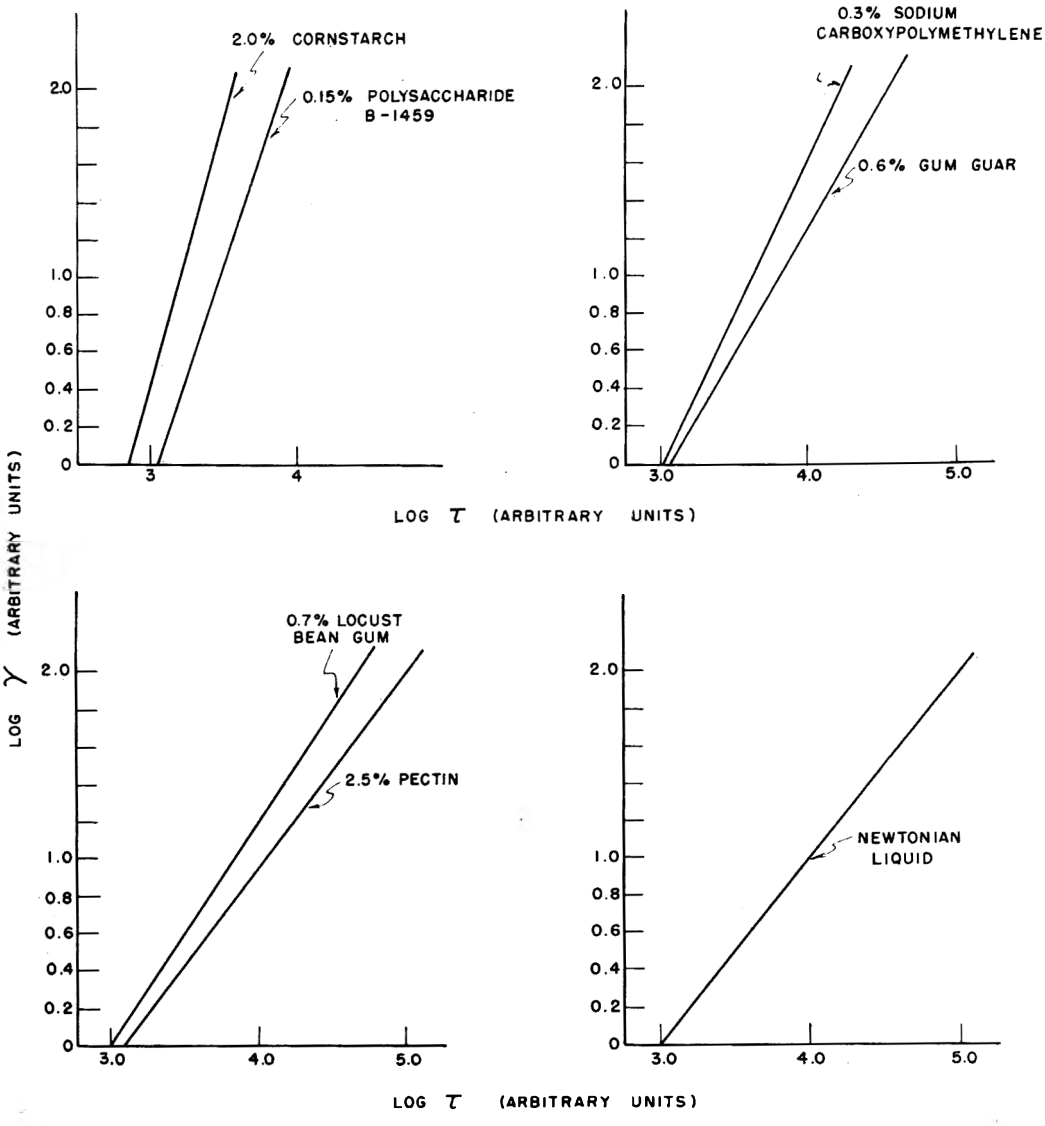

Fig. 3. Stress-shear plots of selected gums as compared to a Newtonian liquid.

may move from group to group with changes in concentration.

Fig. 3 shows a plot of log stress (τ) vs. log shear rate (γ) for six gums which outline the boundaries of the three classification groups. A Newtonian liquid is included for comparison. The slopes of the straight lines obtained are quantitative measures of deviation from the Newtonian behavior. These are listed in Table 2. The slopes of other gums classified in the same group fell in between the given values. Thus, gums which are nonslimy are expected to show a slope greater than 2, those which are slightly slimy a slope in the neighborhood of 1.5 and those that are definitely slimy a slope close to 1.

The viscosity of gums was measured at the sample pH, i.e., the pH was not adjusted to any specific value, and no electrolytes were added to the solutions except those that might have been present in the commercial material. As Masson and Goring (1955) showed with carrageenan, the shear dependence of the electrolyte-sensitive gums varies with the electrolyte concentration. They re-

Table 2. Quantitative deviation of gum solutions from Newtonian viscosity.

Group	Gum	N (slope of lines in Fig. 3)
A	Cornstarch	2.80
	Polysaccharide B-1459	2.40
B	Sodium carboxypolymethylene	1.60
	Gum guar	1.36
C	Locust bean gum	1.20
	Pectin	1.04
—	Newtonian liquid	1.00

ported that the effect of rate of shear on the viscosity of carrageenan decreased with an increase in the concentration of the added electrolyte.

A number of gums (not evaluated organoleptically) whose viscosity behavior places them with the slimy gums (Group C), behave as dilatant materials and show an increased viscosity with increasing rates of shear. These gums are: agar, gum arabic, corn-hull gum, flaxseed gum, Iceland moss, and mesquite gum. All of these gums have very low solution viscosities and are not used as thickening agents in liquid or semiliquid products. Of special interest is furcellaran, which showed the least shear dependence of all the gums studied.

The correlation between organoleptic sliminess and degree of viscosity decrease with increasing rate of shear is logical and follows the definition of sliminess. The faster the solution decreases in viscosity under the revolving motion of the tongue, the faster and easier it can be swallowed. The slower the change in viscosity in the mouth, the more difficult it is to swallow. The reason for the different behavior of different gums undoubtedly lies in the degree of particle dispersion, molecular weight, molecular shape, strength of intermolecular bonds, and other characteristics. At present, however, our limited knowledge of the physicochemical properties of natural gums prevents formulation of an adequate hypothesis.

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Repeatability Estimates in Sensory-Panel Selection

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SUMMARY

Repeatability estimates applied to panel selection enable the experimenter to predict the proportion of judges whose sensitivity can satisfy established specifications. Repeatability estimates of different test designs indicated paired comparison to be more sensitive than the 3-sample binomial design. Studies on sessions of extended length indicate that protracted testing may provide a more reliable basis of panel selection than short-period testing does.

INTRODUCTION

Methodological problems in sensory evaluation of foods have received close scrutiny in recent years. Many procedures adopted on the basis of pioneer studies, however, have proved to oversimplify the difficulties inherent in this method of investigation. A further complication is that testing conditions and procedures vary considerably in different laboratories (Boggs and Hanson, 1949; Dawson and Harris, 1951).

Success in any sensory test depends largely on the capabilities of the panel members. Their selection, however, is usually based on relatively few brief screening tests and on checks during the study. A variety of short-term selection techniques have been used (Boggs and Hanson, 1949; Dawson and Harris, 1951; Schlossberg, 1954), but few have been subjected to comparative evaluation. In spite of the opinion of Schlossberg (1954) that "the usual conception of selection as something you can do quickly by simple sensory tests is fallacious," the need remains for reliable short-term selection methods.

Methods used to solve problems in genetics in the prediction of performance ability suggest a possible approach to prediction of the outcome of selection of sensory test panels (Kempthorne, 1957; Kempthorne, 1960; Lush, 1945). Repeatability (R), the intraclass correlation of repeated measurements (measure of the constancy of repeated observations by a given judge), is a point estimate and may be estimated directly from variance analysis of discrimination test data, as follows:

Source of variation	Degrees of freedom	Mean square	Expectation of mean square
Judges	$j-1$	Vj	$\sigma e^2 + k\sigma j^2$
Sessions within judges	$j(k-1)$	Ve	σe^2

and $$R = \frac{\sigma j^2}{\sigma j^2 + \sigma e^2} \qquad [1]$$

where k = number of session replicates, σe^2 = error component of variance, and σj^2 = judge component of variance. Now, if the average of n tests is used for each judge's future score, then:

$$R_1 = \frac{\sigma j^2}{\sigma j^2 + \frac{\sigma e^2}{n}} \qquad [2]$$

since $\frac{\sigma e^2}{n}$ is the variance of the average of n intrajudge scores. R is then estimated by substituting these results in equation 1 (which are the best available estimates of σe^2 and σj^2) to give the working formula:

$$\hat{R} = \frac{(Vj - Ve)/k}{(Vj - Ve)/k + \frac{Ve}{n}} \qquad [3]$$

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Eq. 3 thus permits prediction of R, for test methods with increasing session length, based upon data from a preliminary trial.

An indication of the reliability of sample estimates of repeatability may be obtained by calculation of the variance of R according to an approximation suggested by Osborne and Paterson (1951-52), as follows:

$$S_R^2 = \frac{2(\sigma e^2)^2 \quad (\sigma e^2 + k\sigma j^2)^2}{(\sigma e^2 + \sigma j^2)^4 \quad k(k-1) \quad (j+1)} \qquad [4]$$

where k = number of session replicates and σe^2 is the estimate of error of repeat performances regardless of n. This measurement of the variance of R assumes the data to be normally distributed.

Repeatability estimates permit selection of a panel of judges that has an average level of performance "better" by any specific degree than the average performance of the population from which it was selected. The average expected gain in performance (ΔP) of the selected panel may be determined as follows (Lush, 1945):

$$\Delta P = R \times S \qquad [5]$$

where R = repeatability, and S = selection differential.

The selection differential measures difference in performance between the selected group and the whole population, in terms of the actual units of measurement. It represents the amount of gain attained by selection. Values of S corresponding to different proportions of those selected from the total population under selection may be obtained from Fisher and Yates (1953) (for a normal distribution only) and an estimate of the standard deviation, σe.

These calculations permit selection of a sensory discrimination panel with a specified performance ability, or, knowing the panel size, the performance of a hypothetical panel can be predicted, as compared to the unselected population.

The above method was used to evaluate various procedures in selecting judges.

EXPERIMENTAL

All sensory-difference tests were conducted under a carefully controlled environment in a room equipped with air conditioning, isolation booths, controlled lighting, and rinsing fountains. The test medium was reconstituted nonfat dry milk solids (10% w/v) with added vanillin (Fisher Scientific Company). To ensure flavor stability of the milk solids, a freshly processed commercial lot of nonfat dry milk solids (Western Condensing Company, Petaluma, California) was packed in 211 × 304 cans under nitrogen and stored at 32°F for use in daily preparation of test materials.

The test samples were prepared by a standardized procedure. A weighed amount of powder was reconstituted in distilled water under slow agitation with a Waring blender and divided into two parts. To one was added vanillin from a 95.5% ethanol stock solution, and to the other (control) was added the same amount of ethanol but without vanillin. Samples, served at room temperature, consisted of 25-ml portions of each mixture in covered 10-oz straight-tapered clear glass tumblers. Panel members were instructed to sniff samples in a defined sequence and to indicate the presence of the additive. In all studies, samples were presented in random order and with equal frequency. Each observer was advised of the correctness or incorrectness of his judgment immediately after his test session.

RESULTS

Before the use of repeatability estimates in panel selection could be investigated, it was necessary to know the maximum number of tests that could be useful in a single judging session. The "triangle" tests (Helm and Trolle, 1946) consisted of 2 to 36 tests per session. Vanillin was used in one or two of the samples at a concentration of 15 × 10^{-6} g/100 ml. Before the test proper, each panel member underwent a training schedule for seven days. This consisted of four paired-comparison tests per session at an additive level twice that used in the experiment proper. Ten judges participated in the study, but not all completed every test. Table 1 shows the pooled data of each session.

Panel performance, as represented by percent

Table 1. Panel performance in odor-discrimination sessions with various numbers of triangle tests per session.

No. of triads per session	No. of session replications	Total correct judgments possible	Total correct judgments observed	Percent correct
2	6	106	57	53.8
6	2	102	58	56.9
12	3	324	184	56.8
24	3	624	331	53.0
36	2	648	362	55.9

Table 2. Distribution of total correct judgments by thirds of session for panel in triangle odor-discrimination sessions of extended length.

No. of triads per session	No. of session replicates	Total correct judgments possible	Correct judgments observed by thirds			χ^2 of 1st third vs. 3rd third
			1st	2nd	3rd	
24	3	624	103	108	120	2.5[a]
36	2	648	124	123	115	0.6

[a] χ^2 at 5% level of significance (1 d.f.) = 3.84.

correct judgments, was essentially independent of session length. The possible occurrence of fatigue or learning during the latter stages of extended judging periods was investigated by tabulating in thirds of sessions all data obtained in triad series of 24 and 36 judgments (Table 2). These results indicate no significant fatigue or learning effects.

The reliability of sample estimates of repeatability was tested with different binomial designs and under conditions of extended session length.

In the first study, the effectiveness of panel selection was predicted by comparing R values calculated from data obtained under three different test designs (the paired-comparison, the 3-sample "triangle," and the 9-sample test). Lockhart (1951) has discussed the characteristics of these and other binomial test systems. Fifteen judges participated, and the conditions of testing were identical in all respects other than binomial design. The preliminary training was three judging periods of 7 triangular tests per period.

The 9-sample system had a specified distribution AAAAA-BBBB, judges were requested to separate the samples into two groups, dissimilar in odor characteristics but identical within the group, and discrimination only was at issue. Results from the 9-sample test were transformed to scale values according to the completeness of separation of A and B, as shown in Table 3. Scoring the data of the individual judges for all three odor discrimination tests is reported in Table 3-A.

Table 3. Scale values according to the completeness of separation of A and B in a 9-sample binomial odor discrimination test.

Possible sample arrangement	Chance probability	Scale value
AAAAA-BBBB	1/126—0.008	1.0
AAAAB-ABBB	20/126—0.16	0.5
AAABB-AABB	60/126—0.48	0
AABBB-AAAB	40/126—0.32	0.5
ABBBB-AAAA	5/126—0.04	1.0

The number of sessions conducted was three in the 2- and 3-sample tests, and five in the 9-sample test. Six consecutive pairs were presented in the 2-sample, 7 consecutive triad sets in the 3-

Table 3-A. Summary of individual judge performance data in 2-, 3-, and 9-sample binomial odor discrimination tests.

	2-sample test			3-sample test			9-sample test				
	Session			Session			Session				
Judge	1	2	3	1	2	3	1	2	3	4	5
1	1.00[a]	1.00	1.00	0.714	1.000	1.000	1.0[b]	1.0	1.0	1.0	1.0
2	1.00	0.83	0.83	.286	0.571	0.429	1.0	0.5	0.5	1.0	1.0
3	0.50	0.50	0.50	.143	.571	.143	0	0	0.5	0	0
4	1.00	1.00	1.00	.571	.714	.286	1.0	1.0	0.5	1.0	0.5
5	0.50	0.83	1.00	.571	.143	.286	1.0	0.5	0	0.5	0.5
6	1.00	0.50	1.00	.571	.714	.714	1.0	0.5	0	0.5	0
7	0.67	0.67	0.83	.429	.286	.143	0.5	0.5	0.5	1.0	0.5
8	1.00	0.83	0.83	.286	.286	.429	0	0	1.0	0	1.0
9	0	0.17	0.33	.286	.286	.143	1.0	0	0.5	0.5	0.5
10	0.50	0.50	0.33	.571	.429	.571	1.0	0.5	0	1.0	0
11	1.00	0.83	1.00	.429	.571	.857	0.5	1.0	0.5	1.0	1.0
12	0	0.67	0.83	.286	.286	.714	0.5	0	0	0	0
13	0.67	0.33	1.00	.857	.286	.286	0.5	0.5	1.0	0.5	1.0
14	0.67	0.50	0.33	.286	.429	.143	0	0.5	0.5	0	0.5
15	1.00	0.67	1.00	.714	.429	.286	1.0	1.0	0.5	1.0	0.5

[a] Ratio of correct judgments to total judgments.
[b] See Table 3 for definition of score values.

Table 4. Estimates of repeatability-of-performance data from the same odor-discrimination panel.

Test design	Repeatability R	Standard deviation of R
2-sample (6 pairs)	0.55	0.145
3-sample (7 triads)	0.36	0.170
9-sample (one group of 9)	0.32	0.130

sample, and one in the 9-sample test. χ^2 analysis of the data for "goodness of fit" indicated that day-to-day judge performance did not vary unduly (Goulden, 1952). Table 4 summarizes estimates of repeatability and their standard deviations. The results indicate that the 2-sample design provides for greater estimate of repeatability of performance data.

Agreement between direct and indirect estimates of repeatability was evaluated by comparing indirect estimates of R with direct estimates of R from three-sample tests of varying session size. The judging sessions consisted of 6, 12, 24, or 36 odor discrimination tests with two replications. The indirect estimates of R (Table 5) were calculated from the analysis of variance of the 6-triad session data:

Test method	Source of variation	Degrees of freedom	Sum of squares	Mean square
3-sample (2 reps)	Total	19	1.2115	
	Judges	9	0.7581	0.0842
	Sessions	1 } 10	0.0352 }	0.0453
	Error	9 }	0.4182 }	

The session term was combined with the error term since it was small and of no practical significance in this case. From Eq. 3, $\hat{R}_n = \frac{(Vj - Ve)/k}{(Vj - Ve)/(k + Ve/n)}$, $k = 2$ (number of session replicates), and $n = 1$, for the number of tests per judge (6 triads per session is equivalent to 1 test). Thus $n = 2$ for 12 odor tests per session, $n = 4$ for 24 odor tests per session and $n = 6$ for 36 odor tests per session. With the indirect testimates in Table 5 are the direct estimates of R calculated from the analysis of variance (Table 5-A) of each panel session (12, 24, and 36 odor discrimination tests).

The direct estimate of R for the 36-test session length agreed fairly closely with the indirect value. In each other case, deviations were large and probably attributable to the size of experimental error.

To compare repeatability estimates of 2-sample and 3-sample binomial methods under test conditions of extended session length, four odor-discrimination sessions consisting of 36 consecutive paired-comparison tests per session were accomplished in daily succession with a panel of 12 subjects. Reference samples (control and vanillin) were presented with each pair of experimental samples during two of the four sessions.

Observers were advised that one member of

Table 5. Direct and indirect estimates of repeatability for triad odor-discrimination sessions of extended length.

No. of triads per session	Indirect estimate of R	Direct estimate of R	Standard deviation of R
6	0.30	0.30	0.30
12	0.46	0.24	0.32
24	0.63	0.49	0.24
36	0.72	0.80	0.14

Table 5-A. Summary of individual judge performance data in three-sample binomial odor discrimination tests in judging sessions of varying length.[a]

Judge	Number of triad tests per session							
	6		12		24		36	
1	0.67[b]	0.83	0.42	0.25	0.42	0.54	0.36	0.47
2	0	.50	.75	.58	.42	.58	.39	.53
3	.33	.83	.58	.75	.63	.54	.58	.50
4	.83	.50	.67	.67	.63	.71	.92	.94
5	.17	.50	.58	.50	.75	.58	.86	.72
6	.33	0	.25	.58	.29	.38	.50	.28
7	.50	.50	.83	.42	.38	.63	.61	.50
8	.33	.17	.83	.67	.38	.42	.44	.47
9	.67	.67	.75	.50	.63	.67	.42	.55
10	.33	.50	.42	.42	.38	.54	....	....

[a] Analysis of variance was carried out on combined session replicates.
[b] Ratio of correct judgments to total judgments.

each pair contained additive, and each reference sample was identical to one unknown. Concentration of vanillin was 13×10^{-5} g/100 ml. When reference samples were provided, judges were requested to refer to both control and vanillin samples at least once for each judgment throughout the entire series of 36.

Direct estimates were made of repeatability for both paired-comparison methods for the entire 36 tests in the judging periods and for the first 18 tests of the 36 (Table 6).

Table 6. Comparison of direct estimate of repeatability from panel performance data in 2-sample tests with and without reference samples in sessions of extended length.

Test method	No. of tests per session	Repeatability	Standard deviation of R
No reference	36	0.89	0.06
References	36	.82	.10
No references	1st 18 of 36	.86	.08
References	1st 18 of 36	.65	.17

Seven judges who participated in this investigation had taken part in the previous study (36 consecutive triad tests per session). This fact permitted comparison of repeatability estimates for the two different binomial methods under conditions of extended session length. Table 7 shows estimates of R for the two different test designs.

Table 7. Comparison of direct estimates of repeatability from panel performance data in 2- and 3-sample tests in sessions of extended length.

Test method	No. of tests per session	Repeatability	Standard deviation of R
2-sample			
No reference	36	0.97	0.02
References	36	.85	.12
3-sample			
No reference	36	.79	.13
2-sample			
No reference	1st 18 of 36	0.97	0.03
References	1st 18 of 36	.57	.28
3-sample			
No reference	1st 18 of 36	.66	.20

The data of Tables 6 and 7 indicate that panel selection could be effective with screening sessions of fewer than 36 tests. R values were lower in all tests with reference samples. Comparison of 2-sample and 3-sample R values indicates the former to be the more sensitive, as also evidenced by the data of Table 4.

To evaluate the usefulness of repeatability estimates derived from analysis of variance for prediction of gains in performance by selection, observed repeatability (R^1) of selected-panel performance data in screening test sessions was determined as follows:

$$R^1 = \frac{\text{superiority of selected panel in subsequent test}}{\text{selection differential in screening test}}$$

Such observed R^1 values were obtained from five separate screening tests with 38 judges evaluating odor in 2-sample tests with vanillin additive (4×10^{-5} g/100 ml) in milk-solids medium (4 replications per session). In each of the first four screening sessions, judges were ranked according to the number of correct judgments, and the top 20% (8 judges) and bottom 20% of judges were respectively selected to compose Panel I (good) and Panel II (poor). The composition of each selected panel varied according to rank orders obtained in different sessions, and when more judges qualified than were required, random selection was necessary. The performance increment of Panel I (selected from session I) over the total panel in session I was compared to the increment for the same selected panel members observed in session 2. Performance of Panel I, newly selected from Session 2, was compared to observed performance of the same panel in Session 3, and so on. The ratio of increments provided a measure of repeatability for performance of Panel I selected from Session 1, etc. Thus, for the five screening sessions, four independent measures of R were obtained for upper-20% panels. Repeatability values were determined in the same manner for the lower-20% panels. The average R for each group of four measured was compared to the repeatability calculated by analysis of variance of the first four screening-test sessions combined. Table 8 summarizes the

Table 8. Summary of measured repeatability[a] values of odor-discrimination-test data from selected panels in screening-test sessions.

Screening session	Measured repeatability of selected panels	
	Panel I[b]	Panel II[c]
1 vs. 2	0.38	0.10
2 vs. 3	0.54	0.14
3 vs. 4	0.38	0.41
4 vs. 5	0.52	0.31
R′ average	0.48	0.24
Standard Deviation of R′	0.09	0.14

[a] R (by analysis of variance) = 0.29.
[b] Top 20% of ranked judges.
[c] Bottom 20% of ranked judges.

Table 9. Example calculations for the measured R values as reported in Table 8.

Session	Av. no. correct judgments per test session							
	1 vs. 2		2 vs. 3		3 vs. 4		4 vs. 5	
Panel I selected	4.00[a]	3.50	4.00	3.63	4.00	3.50	4.00	3.50
Total panel unselected	2.76	3.03	3.03	3.11	3.11	3.16	3.16	3.06
Differential	1.24	0.47	0.97	0.52	0.89	0.34	0.84	0.44
R	$\frac{0.47}{1.24} = 0.38$		0.54		0.38		0.52	

[a] Total possible correct judgments = 4.

results of the calculations. The measured R values reported for Panel I (and similarly for Panel II) were calculated from the data of Table 9-A in the manner outlined in Table 9. Average repeatability measured for Panel II performance data showed close agreement with R calculated by analysis of variance, but the average R for Panel I was somewhat larger. This discrepancy may be due to deviations from normality in the experimental data.

DISCUSSION

Results of the preliminary study on the effect of session length on panel performance agree with those reported by others. Pfaffmann *et al.* (1954), in tests with orange drink, brown bread, and other products, found that panel efficiency did not decline during 40-min taste sessions involving 18 triangle tests per session. Sather and Calvin (1960) recently reported similar results with flavor-preference tests (green beans, hamburger, peaches, and tomato juice) in which up to 20 samples were tested in one judging period. Those authors concluded that pre-tests would be most useful in determining a limit to the number of trials that may be conducted. The results of this study are consistent with those of Pfaffmann and Sather and indicate that, with reconstituted dry milk solids, as many as 36 odor discrimination tests per session could be accomplished without loss of panel efficiency.

Estimates of the repeatability of judges' performance were found to be of somewhat greater magnitude for the paired-comparison odor discrimination method than for the triangle and 9-sample binomial methods. This relation was particularly apparent in judging sessions of extended length. The observations lend support to evidence (Byer and Abrams, 1953; Gridgeman, 1955; Pfaffmann *et al.*, 1954; Filipello, 1956) that, under some conditions, the 2-sample test method may be more sensitive than the 3-sample binomial design.

In triangle tests in which the same panel of judges participated in judging sessions of varying length, values of repeatability estimated from performance data were not in close agreement with indirect estimates of R except in the case of 36-tests-per-session data. Since indirect estimates of R were calculated from performance data obtained in 6-test sessions, the observed discrepancy may be attributable to experimental error or interaction between judge performance and number of samples. Discrepancies of estimates of R probably indicate fatigue or other non-additive effects on judge performance. The standard deviations of direct estimates of R for sessions involving 6, 12, and 24 consecutive tests were large as compared to that for 36.

In paired-comparison tests with sessions of extended length, panel performance was essentially identical whether or not reference samples were provided. The observation, however, that one part of performance, repeatability of data, was lower with reference samples than without suggests the possibility that certain judges may benefit from a reference, thus obscuring true discrepancies between judges, and hence reducing R. The fact that repeatability of performance data in the first 18 tests of 36 in sessions of protracted testing were essentially identical to those for the entire series of tests combined suggests that, for this particular test system, effective panel selection could be achieved with screening sessions of fewer than 36 tests. The relatively high values of R observed in data from sessions of extended length confirm the predicted hypothesis that judging periods

Table 9-A. Summary of panel odor-discrimination performance data in 2-sample screening tests with vanillin additive in milk-solids medium.

Judge	Session number 1	2	3	4	5	Total
1	4[a]	4	4	4	4	20
2	4	4	4	4	4	20
3	4	4	4	4	4	20
4	4	4	4	4	4	20
5	4	1	2	2	2	11
6	4	4	3	4	4	19
7	4	4	3	4	4	19
8	4	3	3	4	4	18
9	4	4	3	3	4	18
10	4	3	3	4	3	17
11	3	4	4	4	4	19
12	3	4	4	3	2	16
13	3	3	4	3	1	14
14	3	3	2	4	3	15
15	3	4	4	3	2	16
16	3	4	4	2	2	15
17	3	2	3	3	3	14
18	3	4	3	3	3	16
19	3	4	4	4	4	19
20	3	2	2	4	4	15
21	3	4	4	2	4	17
22	3	3	2	3	2	13
23	3	3	3	4	4	17
24	3	1	3	3	4	14
25	3	3	4	3	4	17
26	2	2	2	4	2	12
27	2	2	1	2	3	10
28	2	1	3	2	3	11
29	2	3	4	4	2	15
30	2	3	4	4	3	16
31	2	3	0	2	3	10
32	2	1	3	2	3	11
33	1	4	2	2	3	12
34	1	3	4	1	2	11
35	1	3	4	4	3	15
36	1	1	3	3	2	10
37	1	4	1	1	2	9
38	1	2	4	4	2	13

[a] Total possible correct judgments per session per judge = 4.

involving protracted testing may provide a more reliable basis for panel selection than periods of short duration.

The principal advantage to be derived from application of repeatability estimates to selection of judges is the ability of the experimenter to predict the proportion of judges whose sensitivity can be expected to satisfy established specifications. A simple ranking of judges may permit differentiation of individual capabilities but cannot ensure that the judges who are selected will perform at a specified level of proficiency. The evidence reported in this study, in which observed increments (and decrements) in performance of selected judges over the entire unselected panel averages were measured, indicates that average repeatability of performance was, in fact, equivalent to, or greater than, repeatability predicted by analysis of variance. Thus, estimates of intraclass correlation appear to provide a reliable basis for predictions in the selection of panels.

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All-Vegetable Protein Mixtures for Human Feeding VI. The Value of Combinations of Lime-Treated Corn and Cooked Black Beans [a, b]

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SUMMARY

Raw and lime-treated corn and raw and cooked beans were analyzed for nutrient content including essential amino acids. No major treatment changes were found. Eight different corn and bean combinations were tested isoproteically and isocalorically in both growing and protein-depleted adult rats. Corn protein alone gave better results than bean protein. Replacement of part of the lime-treated corn protein by cooked-bean protein improved growth up to the point where each dietary component contributed 50% of the total protein of the diet (72% corn and 28% beans by weight); thereafter growth was decreased with increasing amounts of bean protein in the diet.

When the diets were supplemented with the limiting amino acids found to be deficient by comparing their amino acid pattern with that of the FAO Reference Protein, methionine proved to be the most limiting, particularly in those diets with a higher content of bean protein. The addition of lysine and methionine together improved protein efficiency further. Whenever adequate growth was obtained, higher amounts of fat were found in the liver.

As previous INCAP publications have stressed, corn and beans are the most important staple foods consumed by the rural population of Central America (Castillo and Flores, 1955; Flores and Reh, 1955). The incidence of protein malnutrition among children in this area is high (Béhar *et al.*, 1958). It was hoped that a corn masa (Bressani and Scrimshaw, 1958; Bressani *et al.*, 1958a) and cooked black bean combination would be found that would be suitable for preventing this disease and also for treating mild cases of it.

Cereal grain–legume seed combinations are nutritionally better than is either ingredient alone (F.A.O., 1954; Baptist, 1956). However, optimum combinations of legume seeds and cereal grains have not been reported. Since corn is deficient in lysine and tryptophan (Sauberlich *et al.*, 1953; Bressani *et al.*, 1958b) but adequate in methionine, and beans are good sources of lysine and tryptophan and deficient in methionine (Russell *et al.*, 1946; Tandon *et al.*, 1957), the proteins of these two staples should complement each other efficiently.

Baptist (1956) found that rats fed a combination of cereal grain with legume seeds grew as well as those fed the stock ration of the colony. Tongur and Orlova (1956) obtained good results by mixing 60 parts of buckwheat, 20 parts of soybean, and 16 parts of rice. Desikachar *et al.* (1956) and Chitre and Vallury (1956) demonstrated that a legume and rice combination improved rat growth. More recently, Brock *et al.* (1955) reported that a combination of mealie-meal (ground

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[c] Part of the thesis by A. T. Valiente for a degree in Chemistry and Pharmacy from the Universidad Autónoma de El Salvador, El Salvador, C. A.

corn) with cowpea can initiate cure in children with kwashiorkor.

In view of these findings, it was of practical interest to study the nutritive value of several combinations of corn masa with cooked black beans. The best mixture was further evaluated by supplementing the diets with the limiting amino acids.

EXPERIMENTAL METHODS

One hundred pounds of white starchy corn *(Zea mays)*, cultivated in the highlands of Guatemala, were made into masa using the lime-treatment procedure described by Bressani and Scrimshaw (1958), a procedure involving cooking about 60 min at 96–98°C. A similar quantity of whole black beans *(Phaseolus vulgaris)* from Tecpan, Guatemala, were washed with water to remove foreign matter and cooked 60 min in the autoclave at 16 lb pressure and 121°C in the proportion of 100 g of whole beans to 2000 ml of distilled water. Both were then dried with hot air at 80°C and ground to pass 40 mesh. Representative samples of both raw and cooked material were analyzed by the A.O.A.C. method (1950) for moisture, nitrogen, ether extract, crude fiber, and ash. The carbohydrate content and metabolizable energy were obtained by calculation. The essential amino acid composition was determined by microbiological techniques with *Leuconostoc mesenteroides* P-60, using Difco media (Difco Laboratories, Detroit, Michigan) for lysine, methionine, leucine, isoleucine, arginine, cystine, phenylalanine, and tyrosine, and the media of Steel *et al.* (1949) for histidine and valine. The former medium was also used with *Lactobacillus arabinosus* 17-5 for the assay of tryptophan, and the latter with *Streptococcus faecalis* 8043 for the determination of threonine.

In evaluations of the nutritive value, both young and protein-depleted adult rats of the Wistar strain, obtained from the INCAP colony, were used. In three growth trials, 36, 48, and 48 weanling rats were distributed by weight, using 3 males and 3 females per experimental diet. The animals were placed in individual, all-wire screen cages with raised screen bottoms and provided food and water ad libitum, and weight gains and food consumption were measured every 7 days for 28 days.

At the end of the experiments, the animals in each group were sacrificed and their livers removed and analyzed for fat by ether extraction and for nitrogen by the micro-Kjeldahl procedure. A fragment of liver tissue was fixed in 10% neutral formalin solution for staining with hematoxylin-eosin, Gomori's reticulin fiber, and Sudan IV stains (Lillie, 1948). The amount of fatty change in the liver was estimated as follows: Grade I, no sudanophilic material seen; II, a few hepatic cells showing fat droplets (usually cells located in the periportal area of the hepatic lobule); III, same as Grade II but more severe; IV, fatty change diffuse and more severe.

In the depletion-repletion experiments, adult albino rats, weighing around 200 g, were depleted of protein by feeding a nitrogen-free diet made up of 86% cornstarch, 5% Hegsted mineral mixture (Hegsted *et al.*, 1941), 5% refined cottonseed oil (manufactured in Guatemala), 2% cod liver oil, and 2% cellulose (Nutritional Biochemical Corp., Cleveland, Ohio), supplemented with 5 ml per 100 g of a vitamin solution suggested by Manna and Hauge (1953). After losing 25% of their initial body weight, the rats were distributed by weight among the experimental groups and handled as previously described for the young rats. Two experiments with eight groups each were carried out in which body weight and food consumption were measured at 7 and 14 days.

In the growth studies, the animals were fed the experimental diets described in Table 1, which also shows the amount of cooked beans and lime-treated corn flour necessary to give isonitrogenous and isocaloric diets. The table also shows the protein percentage distribution from masa and cooked beans; the other ingredients of the diets were 2% cod liver oil (courtesy of Mead-Johnson & Co., Evansville, Indiana), 5% cottonseed oil, 4% Hegsted mineral mixture (Nutritional Biochemical Co., Cleveland, Ohio) (Hegsted *et al.*, 1941), 2% Alfacel, and cornstarch to adjust to 100%. All rations were further supplemented with a complete vitamin solution (Manna and Hauge, 1953).

Two amino acid supplementation studies were also carried out. In the first, each of the eight diets tested previously was supplemented with limiting essential amino acids to the levels indicated by the amino acid pattern of the FAO Reference Protein (1957). The second, the diet in which 50% of the protein came from beans and 50% from corn masa, was supplemented with methionine, lysine, isoleucine, and threonine for determination of the most limiting amino acid. In the amino acid supplementation studies, correction was made for the form of the amino acid added; but no attempt was made to make all diets completely isonitrogenous, because the quantities of nitrogen contributed by these additions were so small.

Table 1. Nitrogen distribution of experimental diets,[a,b]

Diet no.	Masa (% in diet)	Cooked beans (% in diet)	Nitrogen from: Corn masa (g)	Nitrogen from: Cooked beans (g)	Nitrogen distribution: Corn masa (%)	Nitrogen distribution: Cooked beans (%)
1	87.00	.....	1.15	.....	100	0
2	69.59	7.33	0.92	0.23	80	20
3	60.89	11.00	0.80	0.34	70	30
4	52.19	14.67	0.09	0.46	60	40
5	43.50	18.34	0.57	0.57	50	50
6	34.79	22.00	0.46	0.69	40	60
7	17.40	29.34	0.23	0.92	20	80
8	.....	36.67	.....	1.15	0	100

[a] % N in corn masa, 1.32; % N in cooked beans, 3.14.

[b] All diets were supplemented with 4% mineral mixture (Hegsted *et al.*, 1941); 2% cellulose; 2% cod liver oil; 5% cottonseed oil and with cornstarch to adjust to 100 g. All diets received 5 ml of a vitamin solution (Manna and Hauge, 1953) per 100 g diet.

RESULTS

Table 2 shows the chemical composition of the corn and the black beans in their raw and cooked states, and Table 3 their essential amino acid content. For comparison, the amino acid pattern of the FAO Reference Protein is also included in Table 3.

When the amino acids are expressed as g of amino acid per g of nitrogen, a change in arginine and tryptophan content is noted between the raw corn and masa. Other amino acids remain the same or are slightly higher in lime-treated corn than in raw corn. Small changes were found between the raw and cooked black beans in arginine, isoleucine, leucine, lysine, phenylalanine, and tryptophan content. The comparison of the amino acid pattern of lime-treated corn with that of the FAO Reference Protein indicated that the limiting amino acids were tryptophan, lysine, methionine, and isoleucine; in cooked black beans, methionine was most deficient, followed by tryptophan and possibly leucine.

Fig. 1 shows the results of the rat growth experiments. It can be seen that at the same protein percentage of the diet, growth in all experiments was better with lime-treated corn alone than with cooked beans alone. The maximum growth response was obtained when corn masa contributed 40–60% of the total protein with bean protein supplying the remainder.

Fig. 1 also shows the similar results of the protein depletion-repletion experiments. At equal protein levels, lime-treated corn as the sole protein source induced better weight gains during repletion than did cooked beans. Replacement of part of the lime-treated corn protein by cooked-bean protein improved repletion weight gains.

Fig. 1 also shows the fat content of the livers of the growing rats fed the several corn-bean combinations. It is evident that the fat content in the livers was greater when growth was better. Histopathological studies of the liver of the rats fed the several corn-bean combinations showed no consistent change in the hepatic structure. The hepatic cells were found to be normal, except for a variable degree of fatty change in the majority of the animals studied. The fatty change occurred mainly in the periportal areas, and the fat change in each cell varied from very slight (fine intracytoplasmic droplets) to severe (coarse

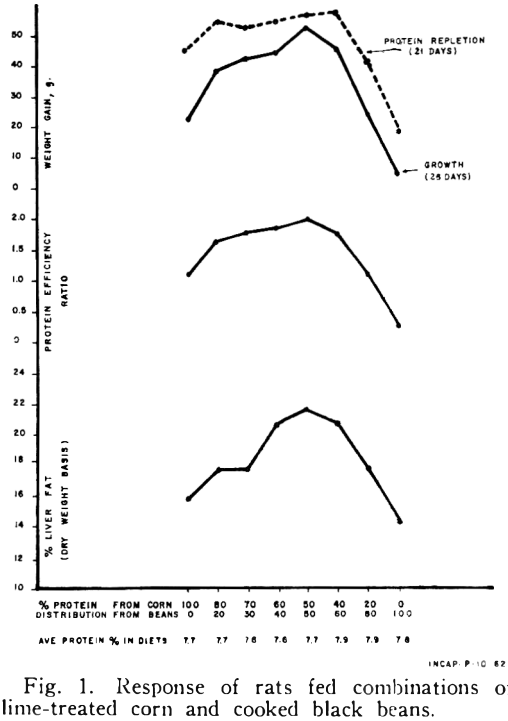

Fig. 1. Response of rats fed combinations of lime-treated corn and cooked black beans.

Table 2. Chemical composition of corn and black beans before and after cooking.

Nutrient	Raw ground corn	Lime-treated corn masa	Raw black beans	Cooked black beans
Moisture (g %)	14.20	11.50	12.40	10.50
Protein (g %)	9.18	8.25	18.50	19.62
Ether extract (g %)	4.00	3.30	1.80	0.50
Crude fiber (g %)	3.10	2.30	4.80	5.60
Ash (g %)	1.20	2.70	4.20	3.50
Carbohydrate (g %)	68.32	71.95	58.30	60.28
Metabolizable calories/100 g	346	351	323	324

unique drops). No pathological findings were encountered in the portal tracts, central veins, or hepatic lacunae.

Amino acid supplementation. The difference between the amino acid pattern of each combination of lime-treated corn and cooked beans and that of the FAO Reference Protein (1957) was calculated and the amount representing this difference was added to each combination of corn and beans. The only exception was methionine, which, on the basis of previous results (Bressani *et al.*, 1954; 1958), was added at a level of 180 instead of 270 mg/g N.

In the first of two experiments, 6 rats were used per group, and eight corn-bean combinations were supplemented with amino acids. Diets 1, 5, and 8 of the previous experiments served as negative controls. The results are in Table 4. The addition of lysine, tryptophan, and isoleucine to diet 1, in which all the protein was contributed by corn, improved growth and protein efficiency significantly. The supplementation of diets 2 and 6 resulted in similar growth responses; the amino acid addition to diet 5 gave only a small response in weight gain. Diet 7, with lime-treated corn contributing 20% and cooked beans 80% of the total protein, gave a poor growth response compared to the other dietary corn-bean combinations, even though it was supplemented with methionine and tryptophan. In the diet with all of the protein contributed by cooked beans, supplementation with methionine, tryptophan, and leucine improved growth response significantly. The weight gain, however, was much below that obtained when the diet contained only unsupplemented corn protein. Table 5 also shows the fat content of the liver of the rats in this experiment. The fat increased as the proportions of lime-treated corn decreased to 60% of the protein of the diet and the cooked beans increased to 40%. The fat decreased as the amount of cooked beans in the diet increased beyond 40%. In general, the amino acid supplements to the diets did not decrease the fat content of the liver of the experimental animals.

The second amino acid supplementation experiment was designed to determine the limiting

Table 3. Essential amino acid composition of corn and beans before and after treatment.

Amino acid	Raw corn (g/g N)	Masa (g/g N)	Raw beans (g/g N)	Cooked beans (g/g N)	F.A.O. Ref. Prot [a] (g/g N)	Score [b] Masa (%)	Score [b] Cooked beans (%)
Arginine	0.262	0.242	0.408	0.387	....	....	....
Histidine	0.231	0.249	0.244	0.242	....	....	....
Isoleucine	0.213	0.227	0.366	0.350	0.270	84	....
Leucine	0.572	0.575	0.285	0.274	0.306	....	90
Lysine	0.126	0.138	0.584	0.567	0.270	51	....
Methionine	0.114	0.119	0.088	0.083	0.190	72	47
Cystine	0.075	0.076	0.045	0.043	0.080		
Phenylalanine	0.276	0.271	0.360	0.338	0.180	....	....
Tyrosine	0.199	0.195	0.181	0.171	0.180		
Threonine	0.214	0.228	0.296	0.331	0.180	....	....
Tryptophan	0.032	0.028	0.080	0.076	0.090	31	84
Valine	0.281	0.297	0.493	0.516	0.270	....	....
Nitrogen (%)	1.47	1.32	2.96	3.14	....	....	....

[a] Amino acid levels of F.A.O. Reference Protein.
[b] Amino acid adequacy in percent of masa and of cooked beans according to F.A.O. Reference Protein.

amino acids in a diet in which 50% of the protein came from cooked beans and 50% from corn masa. Amino acids were added as in the previous experiment. The results are shown in Table 4. Methionine addition alone improved weight gain and protein efficiency over the unsupplemented diet. Supplementation with methionine and lysine improved protein efficiency, whereas isoleucine and threonine added to the diet supplemented with methionine and lysine did not further improve growth or protein efficiency.

DISCUSSION

The high incidence of protein malnutrition in Central America is due to the low consumption of good-quality protein during the critical age of 2–5 years (Béhar *et al.*, 1958). Therefore, if a good corn and bean diet combination were fed during this period, prevention of protein malnutrition should be possible.

The amino acid patterns of the two foodstuffs under study complemented each other in the range of 80–50% of protein from lime-treated corn and 20–50% from beans. At isoproteic levels, lime-treated corn proteins are nutritionally better than cooked-bean proteins, even though the comparison of the amino acid pattern of each protein with that of the FAO Reference Protein suggests that corn proteins are low in at least three amino acids, while beans are low in only one or two.

The lesser growth observed with the diet in which all the protein was contributed by

Table 4. Response of rats to amino-acid-supplemented lime-treated-corn and cooked-black-bean diets.

Diet no.	Source of protein: Lime-treated corn (%)	Source of protein: Cooked beans (%)	Protein in diet (%)	Average weight gain [a] (g)	Feed efficiency [b]	PER [c]	Fat content of liver [d] (%)
Experiment 1 [e]							
1	100	0	9.25	29	8.21	1.05	16.34
1A	100	0	8.81	74	4.59	2.47	17.51
2A	80	20	9.00	58	5.26	2.15	20.28
3A	70	30	9.43	61	5.64	1.91	25.35
4A	60	40	9.06	61	5.33	1.89	20.69
5	50	50	9.25	51	5.76	2.10	21.14
5A	50	50	9.12	59	5.34	2.03	16.75
6A	60	40	8.94	61	5.26	2.10	21.58
7A	20	80	9.00	45	5.91	1.88	11.81
8	0	100	9.50	−3	.....	.....	16.03
8A	0	100	9.25	23	10.26	1.04	14.18
Experiment 2 [f]							
5-1	50	50	9.60	64	5.09	2.05	.....
5-2	50	50	9.30	71	4.92	2.18	.....
5-3	50	50	9.66	75	4.28	2.42	.....
5-4	50	50	9.60	72	4.67	2.26	.....
5-5	50	50	9.68	70	4.79	2.16	.....

[a] Average initial weight for Experiment 1, 62 g; and for Experiment 2, 49 g.
[b] Feed efficiency: average food consumed in g/average weight gain in g.
[c] Protein efficiency ratio: average weight gain in g/average protein consumed in g.
[d] Dry-weight basis.
[e] Diets with "A" were supplemented with amino acids as follows: 1A: L-lys. HCl, 0.19%; DL-tryp., 0.07%; DL-isoleu., 0.098%. 2A: L-lys. HCl, 0.07%; DL-tryp., 0.061%; DL-isoleu., 0.04%; DL-met., 0.016%. 3A: DL-tryp., 0.055%; DL-met., 0.023%; DL-isoleu., 0.01%. 4A: DL-tryp., 0.050%; DL-met., 0.032%. 5A: DL-tryp., 0.044%; DL-met., 0.039%. 6A: DL-tryp., 0.038%; DL-met., 0.047%. 7A: DL-tryp., 0.028%; DL-met., 0.064%. 8A: DL-tryp., 0.017%; DL-met., 0.080%; L-leu., 0.039%.
[f] The amino acid supplements for the diets in this experiment were: 5-1, none; 5-2, DL-met., 0.04%. 5-3 DL-met., 0.04% + L-lys. HCl, 0.07%. 5-4 DL-met., 0.04% + L-lys. HCl, 0.07% + DL-threo, 0.19%. 5-5, DL-met., 0.04% + L-lys. HCl, 0.07% + DL-threo., 0.19% + DL-isoleu., 0.18%.

beans may have been due to a poor over-all amino acid balance or to a lower digestibility of bean protein, resulting in a decreased availability of the amino acids for the growing rat.

Other investigators (FAO, 1954; Castro and Pechnik, 1951; Baptist, 1956; Tongur and Orlova, 1956; Desikachar *et al.*, 1956; Chitre and Vallury, 1956) have generally found that combinations of two sources of protein give a product of higher protein value than either alone. However, if the protein of lower nutritive value contributed 50% or more of the total protein of the diet, the net growth responses were similar to those obtained with the single protein of poorer nutritive value. Results were opposite when a protein of better nutritive value furnished 50% or more of the total dietary protein.

The amount of fat in the liver reflects the level of protein in the diet (Harper *et al.*, 1954, 1955). In the experiments reported in this paper, the accumulation of fat in the liver was higher when growth was better. This suggests that the proportions of amino acids in some of the diets were adequate for good growth but not for the mobilization of fat from the liver. If animal growth improves when diets with non-evident deficiencies and imbalances are fed, the effect of these soon becomes apparent in biochemical and pathological alterations. The results indicate that some of the diets were capable of inducing good growth but were still deficient in nutrients essential for the mobilization of fat from the liver. Harper *et al.* (1955) and other investigators (Deshpande *et al.*, 1955; Rosenberg and Culik, 1957) have found a similar effect in rice-fed rats.

The amino acid supplementation results showed that methionine was the most limiting amino acid in the diet in which the protein was half from corn and half from beans. Methionine improved both protein efficiency and growth; the latter was further improved by the addition of lysine. For practical reasons the most efficient diet combination contained 50% of protein from corn and 50% from cooked beans, which is equivalent to 72% corn and 28% beans by weight.

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All-Vegetable Protein Mixtures for Human Feeding VII. Protein Complementation Between Polished Rice and Cooked Black Beans [a, b]

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SUMMARY

Studies were carried out to determine the best combination of rice and cooked black beans when one partially replaced the other isonitrogenously. Experiments with young and with adult protein-depleted rats indicate that the best combination was one in which about 60% of the protein of the diet is from rice and 40% from black beans. The range of values was from 70 to 50% rice and from 30 to 50% cooked black beans. The median figures correspond to 80 g of rice and 20 g of beans. On an isoproteic basis, rice protein is superior to black bean protein, and supplementation of either protein source with known limiting amino acids improved the protein value. Cooked black beans supplemented with methionine and valine still did not induce as good growth as rice supplemented with lysine and threonine. When rice-black bean diets containing 50% protein from each source were supplemented stepwise with the limiting amino acids methionine, lysine, and leucine, the growth and feed and protein efficiencies of the rats improved; serum protein and liver fat tended to increase.

Rice is nutritionally better than most cereal grains, and is also one of the major staple foods contributing toward the nutrition of the world population. Large quantities are consumed throughout Central America, and in Nicaragua, Costa Rica, and Panama (Reh and Fernández, 1955; Sogandares and Barrios, 1955) it replaces corn as the major staple. Rice is deficient, however, in some of the essential amino acids and relatively low in total protein (Kik, 1956a). The most-limiting amino acids in polished rice are lysine and threonine (Deshpande *et al.*, 1955; Harper *et al.*, 1955; Hundley *et al.*, 1957; Kik, 1956b; Pecora and Hundley, 1951), and the addition of these two amino acids significantly improves its biological value. The nutritive value of rice in human diets is improved by consuming it with foodstuffs that have a complementary amino acid pattern.

In a previous study of corn masa flour and cooked beans, the best combination, as measured by rat growth, was that in which each contributed 50% of the total protein of the diets tested (Bressani *et al.*, 1962). Since beans are also a possible supplementary source of the limiting amino acids in rice and also of additional protein, the present work was designed to determine the relative quantities of rice and bean protein in a diet for optimum biological results.

EXPERIMENTAL METHODS

A 50-lb sample of polished rice from the lowlands of Guatemala was ground in a Wiley mill to pass a 40-mesh screen and stored at 4°C. The beans *(Phaseolus vulgaris)* were purchased in Tecpan, Guatemala, and cooked as previously described (Bressani *et al.*, 1962). Raw and cooked samples of each were analyzed for their essential amino acid content by microbiological methods referred to previously (Bressani and Rios, 1961; Bressani *et al.*, 1962). The protein content in the

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Table 1. Nitrogen distribution of experimental diets.[a,b]

Diet no.	Rice in diet (%)	Cooked beans in diet (%)	Nitrogen from		Nitrogen distribution	
			Rice (g)	Cooked beans (g)	Rice (%)	Cooked beans (%)
1	89.00	.....	0.98	.....	100	0
2	71.20	5.93	0.78	0.20	80	20
3	62.40	8.90	0.69	0.29	70	30
4	53.40	11.89	0.59	0.39	60	40
5	44.50	14.86	0.49	0.49	50	50
6	35.60	17.82	0.39	0.59	40	60
7	17.80	23.80	0.20	0.78	20	80
8	.....	29.70	.....	0.98	0	100

[a] Rice contained 1.10% of nitrogen; cooked black beans contained 3.50%.
[b] All diets were further supplemented with 4.0% mineral mixture (Hegsted *et al.*, 1941), 2.0% cod liver oil, 5.0% cottonseed oil, cornstarch to adjust to 100%, and 5 ml/100 g of a vitamin solution (Manna and Hauge, 1953).

materials and diets was determined by the macro-Kjeldahl method (A.O.A.C., 1950).

For evaluation of the best combination of rice and beans, both rat growth and rat protein repletion experiments were carried out on female albino rats of the CD strain from the Charles River Laboratories. In all experiments, the rats were distributed by weight, 6 animals per group, in individual all-wire cages with raised screen bottoms, and fed *ad libitum* the diets whose partial composition is shown in Table 1. These diets were supplemented with 2.0% cod liver oil (Mead Johnson, Evansville, Ind.), 5.0% refined cottonseed oil (manufactured in Guatemala), and 4.0% Hegsted *et al.* (1941) mineral mixture, with cornstarch added to 100%. All diets contained a complete vitamin supplemnt (Manna and Hauge, 1953).

Food consumption and weight gains were measured every seven days for 28 days. The rats were then sacrificed and their livers removed for fat and protein determination and microscopic examination. In one experiment, serum protein values were measured by the density-gradient method of Lowry and Hunter (1945). In the rat repletion studies, the experimental period lasted 14 days and records were made every seven days.

In the second growth study, three of the previous diets were further supplemented with the amino acids listed in Table 2. Lysine and threonine were added to diet No. 1; lysine, methionine, and threonine to diet No. 5; and methionine and valine to diet No. 8. These were then respectively designated diets 1A, 5A, and 8A. The 9% casein diet of Harper *et al.* (1954) was used as a control (Table 3).

The third growth experiment was planned to determine whether the nutritive value of diet 5 could be improved by the stepwise addition of methionine, threonine, lysine, and finally leucine. In this experiment the amino acids replaced an equal amount of cornstarch; the levels of methionine and leucine were adjusted to those of rice protein, and those of lysine and threonine to the levels found in black beans. The amounts of amino acids added were corrected for the inactivity of some of the D-forms. At the end of the 28-day experimental period, the animals were sacrificed to permit analysis of liver tissue.

RESULTS

Table 2 shows the essential amino acid composition of the sample of rice used. The composition of the cooked beans was described previously (Bressani *et al.*, 1961). Comparison with the amino acid pattern of the FAO Reference Protein (FAO, 1957) suggests that tryptophan is the first limiting amino acid and lysine the second.

Table 2. Essential amino acid composition of Guatemalan rice compared with that of the FAO Reference Protein.

Amino acid	Rice (g AA/g N)	FAO (g AA/g N)
Arginine	0.530	.....
Histidine	0.204	.....
Isoleucine	0.336	0.270
Leucine	0.493	0.306
Lysine	0.263 (97)[a]	0.270
Methionine	0.214	0.270
Cystine	0.079	
Phenylalanine	0.336	0.180
Tyrosine	0.487	0.180
Threonine	0.229	0.180
Tryptophan	0.078 (87)[a]	0.090
Valine	0.386	0.270

[a] Percentage of FAO value given in parentheses.

Table 3. Weight gains, protein and feed efficiency, and fat content of the livers of young rats fed several rice and cooked-bean combinations, and repletion weight gains of adult rats fed the same diets.

Diet no.	Source of protein		Growth [a]				Protein repletion [f]	
	Rice (%)	Cooked beans (%)	Av. weight gain (g)	Feed efficiency [c]	Protein efficiency [d]	Liver fat [e] (%)	Av. weight Initial (g)	Av. weight Gain (g)
1	100	0	40	6.95	2.3	23.0	170	53
2	80	20	51	5.63	2.7	24.3	170	62
3	70	30	52	5.69	2.7	24.1	171	66
4	60	40	53	5.92	2.6	25.6	170	68
5	50	50	51	5.86	2.6	21.8	171	67
6	40	60	38	6.61	2.3	14.2	170	57
7	20	80	18	11.22	1.3	15.9	171	41
8	0	100	−4	.....	...	16.2	171	23
9 [b]	.....	.....	79	3.61	3.4	23.7	.....	.....

[a] Average initial weight per group: 52 g. Duration of experiment: 28 days.
[b] 9% casein diet (Harper *et al.*, 1954).
[c] Feed efficiency: average feed consumed in g/average gain in weight in g.
[d] Protein efficiency: average gain in weight in g/average protein consumed in g.
[e] Dry-weight basis.
[f] Duration of repletion: 14 days.

Table 3 shows the gains in weight as well as the feed and protein efficiency in the first rat-growth trial, and the results from the depletion-repletion experiment. In both experiments there was an increase in weight gain until 60% of the protein of the diet was contributed by rice and 40% by cooked black beans. Feed and protein efficiencies followed the same trends as weight gains. Rice alone gave better growth and repletion gains than black beans at the same protein level in the diet. The fat content of the liver was high when rice contributed higher amounts of protein to the diet and decreased as bean protein replaced more than 50% of rice protein. The highest fat and lowest protein content of the liver occurred when weight gains were greatest. The opposite tendency was noted in the rats fed more than 50% black bean protein.

The upper part of Table 4 shows the results of the third biological trial. The average weight gains of the rats were similar to those of the previous experiment. Addition of 0.40% L-lysine HCl and 0.50% DL-threonine to the diet in which all of the protein was from rice (No. 1) induced a highly significant improvement in growth and feed and protein efficiency. There was also a marked decrease in liver fat and an increase in serum proteins. The addition of 0.25% L-lysine HCl, 0.14% DL-methionine, and 0.45% DL-threonine to the diet where rice and beans each contributed 50% of the total protein of the diet (No. 5), resulted in improved growth, increased feed and protein efficiency, and decreased liver fat. A small decrease in serum proteins was also observed. The levels of lysine, methionine, and threonine added were those of diet No. 1 supplemented with the levels of lysine and threonine described above. The growth response with the supplemented diet No. 5 was not, however, as high as that obtained with all-rice protein diet No. 1 supplemented with lysine and threonine. Finally, when a diet containing only black bean protein was supplemented with 0.22% DL-methionine and 0.30% DL-valine, there was a marked increase in weight gain and in feed and protein efficiency of the rats.

The lower half of Table 4 shows the results of the fourth growth experiment. In this trial, a diet containing 50% rice protein and 50% bean protein was supplemented stepwise with methionine, threonine, lysine, and leucine. Methionine addition alone, increased weight gains and both feed and protein efficiency; liver fat also increased, while serum proteins were unchanged.

The addition of 0.10% DL-threonine to the methionine-supplemented diet caused no further change in weight or liver fat. It did, however, improve feed and protein efficiency and increase serum proteins. The addition of 0.19% L-lysine further improved feed and protein efficiency and growth. Adding 0.21% L-leucine to the amino acid supplement increased not only weight gain and feed and protein efficiency, but also serum protein, although it seemingly decreased liver fat.

DISCUSSION

Nutritionists have always advocated the use of legume seeds as protein supplements

for cereal grains. In this study, cooked black beans improved the nutritive value of the diet when bean protein replaced rice protein isonitrogenously. The improvement effect reached a peak and was followed by a progressive decrease as bean protein became the major component of the diet. The maximum response occurred when rice contributed about 60% of the protein of the diet, with a range from 70 to 50%, and black beans about 40%, with a range from 50 to 30%. The 60/40 mixture corresponds to about 80 g of rice and 20 g of beans. These results are similar to those reported previously for the combination of beans and corn masa (Bressani *et al.*, 1961).

Measured on an isoproteic basis, the protein of rice is superior nutritionally to that of black beans. This may be due to a lower amino acid availability in beans as well as to the known deficiency of total sulfur-containing amino acids (Bressani and Elías, 1961; Russell *et al.*, 1946). It is also possible that the conditions of cooking beans further lowered the nutritive value of their protein. Most of the studies of essential amino acids in legume seeds of the species *Phaseolus* indicate sulfur-containing amino acids to be limiting (Bressani and Elías, 1961; Russell *et al.*, 1946).

Since the fat content of the liver is one indicator of the quantity and quality of the protein in a diet, the results suggest that, although replacement of rice by bean protein induced better growth up to a point, the amino acid balance was still not adequate for the effective mobilization of liver fat. The amino acid supplementation experiments indicate that this was due to amino acid deficiencies in the diets tested.

As reported by Harper *et al.* (1954, 1955), Rosenberg and Culik (1957), and

Table 4. Growth of rats fed different combinations of rice and black beans, and response to amino acid supplementation of diets.

Diet no.[a]	Protein distribution: Rice (%)	Protein distribution: Black beans (%)	Av. weight gain (g)	Feed efficiency [b]	Protein efficiency [c]	Liver fat [d] (%)	Serum proteins (%)
Trial no. 3 [e]							
1	100	0	38	7.50	2.21	24.5	5.14
1A	100	0	134	3.16	4.58	13.9	5.80
2	80	20	54	5.81	2.54	28.8	5.17
3	70	30	49	6.12	2.37	24.8	5.29
4	60	40	51	5.74	2.44	22.3	5.23
5	50	50	51	5.86	2.43	21.2	5.53
5A	50	50	95	3.93	3.38	16.4	5.30
6	40	60	54	5.83	2.24	26.5	5.13
7	20	80	18	10.67	1.08	21.2	5.00
8	0	100	0	.....	.....	18.0	4.47
8A	0	100	60	5.13	2.54	21.0	5.40
Trial no. 4 [f]							
5a	50	50	63	5.30	2.66	13.9	5.08
5b	50	50	73	4.90	2.87	18.4	5.12
5c	50	50	76	4.76	2.94	18.5	5.33
5d	50	50	97	3.82	3.32	14.5	5.20
5e	50	50	105	3.82	3.48	13.9	5.63

[a] Diets were supplemented with amino acids as follows: 1A: 0.40% L-lys. HCl, 0.50% DL-threo. 5A: 0.25% L-lys. HCl, 0.14% DL-met., 0.45% DL-threo. 8A: 0.22% DL-met., 0.30% DL-val., 5a: no supplement. 5b: 0.14% DL-met., 5c: 0.14% DL-met., 0.10% DL-threo., 5d: 0.14% DL-met., 0.10% DL-threo., 0.19% L-lys. HCl. 5e: 0.14% DL-met., 0.10% DL-threo., 0.19% L-lys. HCl, 0.21% L-leu.

[b] Feed efficiency: average feed consumed in g/average gain in weight in g.

[c] Protein efficiency: average gain in weight in g/average protein consumed in g.

[d] Dry-weight basis.

[e] Average initial weight per group: 48 g. Duration of experiment: 28 days.

[f] Average initial weight per group: 45 g. Duration of experiment: 28 days.

others (Kik, 1956b; Pecora and Hundley, 1951), growth improves significantly and liver fat decreases when a rice protein diet is supplemented with lysine and threonine. The results reported here also indicate that lysine and methionine are required to bring about a decrease in liver fat in those diets in which part of the protein is contributed by beans. Threonine is no longer limiting, for no response was observed when this amino acid was added to the diet (no. 5). The highest accumulation of liver fat and greater growth both resulted from the diets where most of the protein was contributed by rice. Presumably the amino acids in the diet were employed by the animal to synthesize body proteins for growth to a greater extent than for the mobilization of liver fat.

The amino acid pattern of each diet showed that beans contribute proportionately higher amounts of lysine and threonine, while there is a progressive decrease in methionine as bean protein replaces rice protein in the diet. The greater rat growth observed may be due to relative increases in lysine and threonine, an effect that is reversed as methionine becomes limiting. The point of maximum growth shows a lysine-to-threonine-to-methionine ratio of 1.4 : 1 : 0.83 when threonine is taken as a unit. Rosenberg and Culik (1957) also found that growth response from the amino acid supplementation of rice is maximum when the lysine-to-threonine ratio is 1.4 : 1.

For populations consuming most of their proteins from rice and beans, additional foods containing proteins of good quality are necessary to supply the needed amino acids. This can be done by adding protein concentrates such as to supply both the limiting amino acids of the mixture and extra protein.

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