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Letters *TO THE SCIENTIFIC EDITOR*

Letter to the Editor

May 7, 1961

Dear Sir:

The article by Jorge Chirife on "Diffusional Process in the Drying of Tapioca Root," J. Food Sci. 36:327 (1971), has several gross misrepresentations that many people make in calculating drying coefficients. The use of the resistance ratio S

$$\text{where } S = \frac{D_e \rho_s}{k_g a}$$

is not justified, as this is not a dimensionless unit as is used in heat transfer. It has units of grams solids per gram water which cannot be cancelled out. The term, in fact, must be multiplied by the instantaneous slope of the moisture sorption isotherm (dm/da) which occurs at the drying interface. This would account for the non-constant diffusivity found. There is no way, however, to understand what experiments the author is referring to in Figure 2 to calculate his diffusivities, or why he has three curves with a 3× variation in diffusivity.

The final argument that drying is occurring solely by liquid diffusion is very weak. In fact, an inspection of the literature of Kirscher and our previous work [Salas and Labuza, Food Technol. 22 (1968); Labuza and Simon, Food Technol. 24 (1970)] suggests that liquid diffusion only predominates in the constant rate period of drying and that vapor diffusion becomes more limiting as time increases. He states that the tapioca reaches constant diffusivity as temperature increases, but this cannot be found in his paper, and I don't see how this applies to extraction of seed with oil. The mechanisms are different.

His argument that Eq. 11 doesn't hold is erroneous. The reason one finds an exponential of D vs 1/T is that the limiting step is the vaporization of water or diffusion to the drying front. In fact, using the initial differential equation (1) is also erroneous as it does not account for a phase change. It can be used, however, to make gross comparisons but not to predict molecular mechanisms unless the D_{eff} is expanded to include the heat transfer effects.

Eq. 14 also should not hold since at 1 atm the mean free path of water vapor is at least 2 orders of magnitude smaller than the average pore diameter of a food.

After this paragraph (page 329) the author says, Therefore diffusion is by liquid flow. There is no way this argument holds. I don't see how this paper was allowed to be published in its present form as it is more of a lecture than a scientific paper.

THEODORE P. LABUZA
Massachusetts Institute of Technology

Letter to the Editor

July 23, 1971

Dear Sir:

Dr. Labuza is right when he criticizes the use of the resistance ratio $S = D_e \rho_s / k_g a$, without taking into account the slope of the sorption isotherm. However, it is unlikely that this factor could change the conclusion of solely internal mass transfer control in this particular experimental situation.

Temperature references in Figure 2 are missing. The curves correspond to experiments performed at, respectively, 55, 84 and 100°C, which can be inferred from the observation of Figure 3.

In my opinion, the experiments of Salas and Labuza [Food Technol. 22 (1968)] and Labuza and Simon [Food Technol. 24 (1970)] do not constitute a definite proof that the predominant water transport mechanism is vapor diffusion. In these papers, effective diffusion coefficients D_{eff} are determined by means of the classical method of Sherwood. Incidentally, the Salas and Labuza effective diffusion coefficients were evaluated by using the boundary condition $C = C_o$ for all X at t = 0, which cannot be used here since the drying curves show an appreciable constant rate period.

On the other hand, in Simon and Labuza, the assumption of unidirectional diffusion does not seem to be justified in view of the sample dimensions ratio. The vapor diffusion mechanism is assumed a priori, and perhaps an experimental demonstration of this behavior would have been to study the temperature coefficient of the D_{eff}.

If the drying mechanism is assumed to be some kind of activation liquid diffusion through the tapioca matrix, this can certainly be compared with the oil migration through a similar natural system. Of course, this reasoning has no sense for Dr. Labuza because he assumes a priori a water vapor diffusion. In Figure 2 it can be seen that the experimental points corresponding to higher temperatures show a better agreement with the theoretical curves for constant diffusivities. This behavior has been also observed in the oil extraction aforementioned.

King [Food Technol. 22:509 (1968)] recently made a theoretical study of the drying mechanism in dried foodstuffs, for the limiting case in which vapor diffusion is the principal mode of transport and where the moisture content corresponds to equilibrium with relative humidities less than 100%. He showed that the D_{eff} can be expressed as:

$$D_{eff} = \frac{M_W D' P_W^o}{\rho_S R_V T} \cdot \frac{P}{P - p_W} \cdot \left(\frac{\delta r}{\delta X}\right)_T \left(\frac{a}{1+a}\right) \qquad [1]$$

where

$$C \cdot D = D'$$

and

$$C = \text{constant less than unity}$$

In this case the most temperature-sensitive factor in Eq. 1 is P_W^o; hence, an exponential temperature dependence of D_{eff} can also be expected. However, the predicted activation energy should be close to the heat of vaporization of water, which is about 10 kcal/gmol. The experimental value for the tapioca root was 5.4 kcal/gmol, which is not in agreement with the former value.

In the work of Salas and Labuza, a constant D_{eff} was observed. According to King's ideas, this can only be explained if the factors $(\delta r/\delta X)_T$ and C in Eq. 1 vary in a compensating fashion. Therefore, as in Figure 6 (Salas and Labuza) there is no shrinkage below $X \cong 1$, the experimental constancy of D_{eff} cannot be explained in terms of that analysis.

Haque and Eubank [Food Technol. 18:390 (1964)] suggested a different hypothesis. They studied the drying of copra and found that the experimental

water diffusion coefficient was very close to that of the liquid water self-diffusion coefficient. According to the Eyring formula (Bird, Stewart and Lightfoot. 1962. "Transport Phenomena."):

$$\frac{D_{AA}\mu_A}{kT} = \frac{1}{2\pi}\left(\frac{N}{V_A}\right)^{1/3} \quad [2]$$

where D_{AA} is the self-diffusion coefficient of liquid water. The temperature dependence of D_{AA} should be mainly related to that of the viscosity, which in turn is exponential, with an activation energy of about 4.2 kcal/gmol. This value compares quite well with that found for tapioca root.

Although there are many studies which deal with the drying of food materials, I do not think that any of the many suggested mechanisms can be taken for granted, in view of the complex situations that usually appear in food dehydration.

JORGE CHIRIFE
University of Buenos Aires

Letter to the Editor

May 1971

Dear Sir:

I have a few comments about an article entitled "Alternate Storage Systems for the Production of Canned Black Ripe Olives" by J.W. Ralls et al., J. Food Sci. 36:408 (1971).

1. It is surprising that in a research article published in 1971 of the work that was carried out in the 1965–67 period, the authors did not review at all what has happened in the last six years. During these last six years, Dr. Reese Vaughn and his co-workers at the University of California at Davis have not only developed a salt-free storage of olives and other vegetables (in acidified sodium benzoate solution) but also have tested successfully this system on a commercial scale at the Oberti Olive Company (Div. of Tri-Valley Growers), Madera, Calif.

2. The research of Vaughn et al., on this subject has been reported at several technical meetings of the California Advisory Board to the entire olive industry. Incidentally, one paper was published in *Food Technology* itself.

3. Personally, I feel that in a research article (published in a professional journal such as ours), particularly so, when it is titled "Alternate Storage . . ." has to give due merit to other alternate systems already existing, i.e., at least a brief reference to the work of Vaughn et al. at U. C. (Davis).

4. Furthermore, it is difficult for me to believe that reviewers on your editorial board could let pass a paper without any proper introduction of chronological events that have already occurred.

5. And last, but not least, any scientific paper that is published by a reputable organization such as National Canners Association should be instrumental in setting ideal examples to the rest of the scientific community by giving proper reference in a paper.

I wanted to bring these points to the attention of all concerned.

B.A. DAVÉ
Glendora, California

Letter to the Editor

June 30, 1971

Dear Sir:

Thank you for sending me a copy of Dr. B.A. Davé's letter of May 1971. I regret that we did not include a reference to the important work of Vaughn and co-workers on salt-free storage of olives and other vegetables in the J. Food Sci. 36 (3):408 (1971) article.

The original manuscript for this article was written before any of Professor Vaughn's work was published (see 47th Annual Technical Report of the California Olive Association, San Francisco, Calif., June 1968, pp 24–30).

Unfortunately, the introductory section of the article was not updated. The update would have cited the publication of Vaughn and co-workers, as well as the related work of Rudolf A. Kellerman, U.S. Patent 3,397,999.

The co-authors and I apologize to the Institute of Food Technologists, former-Scientific Editor Dr. E.J. Briskey, the U.S. Department of Agriculture, and the National Canners Association for the omission and any embarrassment that it may have caused.

JACK W. RALLS
National Canners Association

Letter to the Editor

May 17, 1971

Dear Sir:

The paper entitled "Chemical Composition and Amino Acid Contents of Brazilian Beans (*Phaseolus vulgaris*)" by Roberto M. deMoraes and Eidiomar Angelucci, J. Food Sci. 36:493 (1971), contains questionable amino acid data.

Tryptophan values as published range from 100 to 138 mg/g protein which is equivalent to 10.0–13.8 g/100 g protein or %. The values are in the range of those for aspartic acid and second only to glutamic acid in magnitude! The statement in the summary that "it (the product) was poor in methionine, isoleucine, valine, tryptophane and leucine" is not supported by the tryptophan data. Is there not an error of a factor of ten, so that the values should be 1.0 to 1.4 g/100 g protein? The sums of the amino acids as published range from 99.7 to 140.4 g/100 g protein for the twelve varieties. When tryptophan values are reduced to one-tenth of the published figures, the sums of the amino acids range from 88 to 128%. Reasonable values would be from 105–115% if all of the nitrogen (Kjeldahl determination?) was from amino acids. Percentages greater than 100% are to be expected due to the addition of water in the hydrolysis of the protein.

However, the wide range of values suggests analytical errors, probably in hydrolysis. The sum of the amino acids is low for the varieties Goiano Precoce and Preto G-1: 88 and 92%. Values for methionine and cystine are very low for Carioca.

The description of the method is inadequate. Several of the amino acids, particularly tryptophan and cystine, are very labile in acid hydrolysis, yet no mention is made in the description as to any special treatment such as enzymatic hydrolysis for tryptophan or conversion of cystine to cysteic acid before chromatography. And was the hydrolysis time really only two hours, or was there a printing error and it should have been 22 hours? And to write that the hydrolysis was carried out "according to the procedure outlined in the instruction manual which accompanied the amino acid analyzer" is naive. My impression is that the amino acid analyses are the work of technicians, not of scientists.

BARBARA M. KENNEDY
University of California
(Berkeley)

Letter to the Editor

July 7, 1971

Dear Sir:

We wish to acknowledge receipt of the letter from Professor Barbara Kennedy. In her letter she raised some questions regarding our manuscript.

With reference to the above:

1. We agree with Professor Kennedy that the tryptophan data are ten times less; that is, they are in the range of 1.0 to 1.4 g/100 g protein and not of 10.0 to 13.8 as printed. That is the reason for the statement in the summary that "it (the product) was poor in methionine, isoleucine, valine, tryptophane and leucine." Unfortunately the data in our paper showed a tenfold higher content in tryptophan due to a lapse which we did not see.

2. As for the question raised about the sums of amino acids, we obtained the best values for nine of the varieties, whereas not so good figures were ob-

tained for Preto G-1, Goiano Precoce, and Chumbinho Opaco, in spite of the repeated tests performed.

3. Concerning the Caricoa variety, the value found for methionine is really very low. On the other hand, the correct value for cystine is 39.8 and not 9.8 as stated in the manuscript. This was a typing mistake.

4. Regarding special treatments for tryptophan, we used the Miller method, published in *Journal of the Science of Food and Agriculture*, 18, 1967. We did not make the conversion of cystine to cysteic acid because both cystine and cysteine were determined as half cystine.

5. The hydrolysis time was really 22 hours and not only 2 hours; this was a printing error.

6. As for the procedure used for the hydrolysis, our paper shows the concentration of HCl, time, and temperature; the other steps were the same as normally used for this type of analysis.

We are most grateful to you for this opportunity to answer the questions raised by Professor Barbara M. Kennedy and wish to thank her for her interest in clarifying these points.

ROBERTO MACHADO DE MORAES and EIDIOMAR ANGELUCCI
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ABSTRACTS:
IN THIS ISSUE

COMPARISON OF THE PROTEIN NUTRITIONAL VALUE OF TVP, METHIONINE ENRICHED TVP AND BEEF AT TWO LEVELS OF INTAKE FOR HUMAN ADULTS. C. KIES & H.M. FOX. *J. Food Sci.* **36,** 841–845 (1971)–The objective of the study was to compare the protein nutritive value for adult men of beef, an extruded soybean product resembling beef (TVP) and a 1% DL-methionine-fortified TVP product at two levels of nitrogen intake. Experimental diets were maintained adequate in calories, vitamins and minerals. Mean nitrogen balances of subjects fed 8.0g nitrogen per subject per day from beef, TVP or methionine-fortified TVP were +0.74, +0.78 and +0.72g nitrogen per day, respectively, indicating that at this level of protein intake all three sources equaled or exceeded the protein requirements of adult men. Mean nitrogen balances of subjects fed 4.0g nitrogen per subject per day from beef, TVP or methionine-fortified TVP were −0.30, −0.70 and −0.45g nitrogen per day, respectively. While none of the test protein sources fully met the protein needs of the subjects at this level of nitrogen intake, beef was superior to TVP on the basis of nitrogen balance data. DL-methionine fortification at the 1% level of the TVP was demonstrated to be partially effective in improvement in nitrogen balance.

MEASUREMENT OF THE RELATIVE TASTE INTENSITY OF SOME L-α-AMINO ACIDS AND 5′-NUCLEOTIDES. S. YAMAGUCHI, T. YOSHIKAWA, S. IKEDA & T. NINOMIYA. *J. Food Sci.* **36,** 846–849 (1971)–Intensity of glutamate- and/or inosinate-like taste (umami in Japanese) of various flavor amino acids and flavor nucleotides was studied using sensory analysis and always found proportional to that of monosodium glutamate (MSG) and disodium 5′-inosinate (IMP), respectively. Applying this fact to a previously obtained equation expressing the relationship between the taste intensity of MSG-IMP mixture and that of MSG alone, the intensity of umami of the mixture of any flavor amino acids and nucleotides could be expressed as: $y = u + \gamma uv$, where u and v are the concentrations of amino acids and nucleotides in terms of MSG and IMP, respectively, in the mixture; y is the equivalent concentration of MSG alone; and γ is a positive constant. Interrelationships within each group of substances were additive.

REACTION AT LIMITED WATER CONCENTRATION. 2. Chlorophyll Degradation. F. LaJOLLO, S. R. TANNENBAUM & T. P. LABUZA. *J. Food Sci.* **36,** 850–853 (1971)–Chlorophyll degradation was studied as a function of water activity (A_w) in freeze-dried spinach and model systems. Above A_w equal to 0.32, chlorophyll is converted mainly to pheophytin in a predictable manner. Below this A_w, pheophytin formation is low, but other interfering substances are formed. It was shown in model systems that the basic reaction is hydrolytic removal of Mg from the ring even at limited water concentration.

PATTERNS OF SOLUBLE PEROXIDASE IN RIPENING BANANA FRUIT. N.F. HAARD & C.L. TOBIN. *J. Food Sci.* **36,** 854–857 (1971)–Total peroxidase activity and the spectrum of isoperoxidases solubilized from the pulp of banana fruit were relatively constant when the extraction procedure was most efficient. When casein dispersion was present during extraction of green fruit, high levels of activity were detectable. Other scavengers of condensed polyphenols were less effective in yielding activity in the soluble and particulate fractions. When collagen fibers were included with buffer during tissue maceration, no peroxidase was solubilized at all stages of ripening; however, the particulate fraction contained very high levels of peroxidase. Previous reports showing a concomitant change in peroxidase activity with the respiratory upsurge of climacteric fruit may be anomalous because of differential phenol inhibition.

ANALYSIS OF THE PUNGENT PRINCIPLES OF Capsicum annuum BY COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY. Y. MASADA, K. HASHIMOTO, T. INOUE & M. SUZUKI. *J. Food Sci.* **36,** 858–860 (1971)–The pungent principles of *Capsicum annuum* include at least five closely related compounds. This paper describes a procedure whereby four of these five pungent compounds were separated and identified by combined gas chromatograph and mass spectrometer. Separation of these compounds into oleoresin capsicum by gas chromatography is also reported.

PROPERTIES OF BIXIN AND NORBIXIN AND THE COMPOSITION OF ANNATTO EXTRACTS. J.F. REITH & J.W. GIELEN. *J. Food Sci.* **36,** 861–864 (1971)–The melting point and $E_1^{1\%}{}_{cm}$-values of α-bixin, β-bixin and α-norbixin were determined and the stability of α-bixin examined under different conditions. Total pigment content of butter color was determined. α-Bixin was the principal pigment; in addition, at least eight different pigments were present. The content of α- and β-bixin in butter colors was also determined. Because of complex formation of bixin and norbixin with a yellow pigment, the simple chromatographic method became rather laborious. This complex formation did not occur in the annatto suspensions in oil and fat. These preparations were more concentrated, while the amount of decomposition products of α-bixin was relatively slight. The stability of the pigments in butter colors appeared to be great. Investigation of annatto cheese colors showed that these extracts contain at least seven color components. The total amount of red pigment and the principal pigment α-norbixin were determined.

COMPOSITION OF THE LIPIDS OF CUCUMBER AND PEPPERS. J.E. KINSELLA. *J. Food Sci.* **36,** 865–866 (1971)–Samples of commercial cucumbers and green peppers contain 103 mg and 400 mg total lipid per 100g raw vegetable tissue. The neutral lipids, phospholipids and glycolipids comprise 39, 49 and 15% of the cucumber lipids and 82, 2 and 16% of the pepper lipids respectively. The neutral lipids of both were composed mainly of glycerides. In the peppers the triglycerides accounted for 60% of the total lipids. Cucumber, had less glycerides but contained several unidentified sterols. Phosphatidylcholine was the major class in both vegetables accounting for 46 and 76% of the phospholipids of cucumber and pepper respectively. Palmitic, linoleic and linolenic acids were the principal fatty acid components. The lipids of peppers were very rich in linoleic acid (70%) whereas those of the cucumbers contained relatively more linolenic acid.

FLAVONOL GLYCOSIDES OF THE CULTIVATED STRAWBERRY. J.J. RYAN. *J. Food Sci.* **36,** 867–870 (1971)–After extraction and separation by thin-layer chromatography (TLC) on cellulose, nine flavonol glycosides were detected and purified in the fruit of strawberry from various sources. The structures of these were investigated by TLC, spectral properties and several types of hydrolysis. Four of them were completely identified as the -3-β-monoglucosides and -3-β-glucuronides of quercetin and kaempferol. Three others were characterized less fully as kaempferol-7-monoglucoside, a kaempferol-7-glucoside and an unreported quercetin-3-glucoside which was not the -3-β-monoglucoside or a diglucoside. A comparison between the flavonol glycosides and anthocyanins of this fruit was made with respect to the amounts and types present.

ABSTRACTS:
IN THIS ISSUE

PHENOLIC AND INDOLE CONSTITUENTS OF EDIBLE PEAS. J. MÉNDEZ & M. I. LOJO. *J. Food Sci.* **36,** 871–872 (1971)–p-Hydroxybenzoic, ferulic and vanillic acids were isolated from hydrolyzed and nonhydrolyzed extracts of seeds of *Pisum sativum* L. var. Feltham and var. Lincoln. In the Feltham variety syringic acid was only present as the free compound but p-coumaric acid occurred in both the free and bound forms. Indole-3-acetic and, tentatively, indole-3-carboxylic acids were identified in the Lincoln variety. It is very doubtful if phenols present in the minimal concentration found could play any role in the edible characteristics of the peas.

CHEMICAL COMPOSITION OF THE SEED OF SUNFLOWER HYBRIDS AND OPEN POLLINATED VARIETIES. J.A. ROBERTSON, J.K. THOMAS & D. BURDICK. *J. Food Sci.* **36,** 873–876 (1971)–The seed of high oil hybrids and open pollinated sunflower varieties from experimental plantings at nine locations in six southern states in 1969 was analyzed for moisture, crude protein, total oil and fatty acid composition. Total oil content ranged from 28.8–44.7% with an average of 35.3% for hybrids and 39.5% for open pollinated varieties. The crude protein ranged from 16.9–25.1%. The average oleic and linoleic acid content of the open pollinated varieties at the nine locations was 46.6 and 41.6%, respectively, as compared to 49.4 and 39.6% for the hybrids. The linoleic acid content of the sunflower oil varied inversely with temperature during development of the seed. A small number of confectionery and birdfeed sunflower varieties from four locations also were analyzed.

IDENTIFICATION AND QUANTITATION OF SEVERAL NONVOLATILE ORGANIC ACIDS OF COCOA BEANS. W. WEISSBERGER, T.E. KAVANAGH & P.G. KEENEY. *J. Food Sci.* **36,** 877–879 (1971)–Organic acids precipitated as lead salts from water extracts of cocoa beans were converted to trimethylsilyl ethers. TMS ethers were then separated and identified using a combined gas chromatograph-mass spectrometer. Citric, phosphoric, lactic, oxalic, malic, tartaric, succinic and gluconic acids were present in every sample examined, irrespective of geographic origin. Five of the acids were quantitated using a GLC procedure which involved the preparation of methyl esters from freeze-dehydrated, water extracts. Concentration ranges found, g/100g beans, were: lactic, 0.11–0.71; oxalic, 0.24–0.43; succinic, 0.02–0.07; malic, 0.02–0.10; citric, 0.56–1.32. Concentrations of lactic acid were lowest and citric acid levels tended to be highest in commercial samples from countries where cocoa beans usually are not subjected to a planned fermentation. These trends were confirmed in an experiment involving beans from Trinidad which had undergone a carefully controlled fermentation. Only minor differences were found in organic acid concentrations between roasted and unroasted cocoa beans.

STUDIES OF A METHOD FOR DETERMINING ORGANOCHLORINE PESTICIDE RESIDUES IN ANIMAL TISSUE. J.C. MILLER, J.D. SINK, G.W. SHERRITT & J.H. ZIEGLER. *J. Food Sci.* **36,** 880–882 (1971)–Three different studies were conducted to evaluate the accuracy and reliability of a widely accepted method for determining organochlorine pesticide residues in animals' tissues. These investigations involved laboratories of both state and federal regulatory agencies. Various adipose tissues, heat-extracted and non-heat-extracted, spiked and unspiked samples were analyzed by these laboratories. Results of these studies demonstrated many inaccuracies and inconsistencies associated with using the current method. The laboratory performing the analysis, as well as the tissue sample used, was shown to significantly ($P < .05$) affect results. Further, the heat-extraction phase, as well as the subsequent analytical phase of the method, was observed ($P < .05$) to present problems of repeatability.

ON THE NATURE OF COOKED MEAT HEMOPROTEIN. D.A. LEDWARD. *J. Food Sci.* 36, 883–888 (1971)–The hemoproteins present in mixtures of horse heart metmyoglobin (Mb) and bovine serum albumin (BSA) in 5.5 M urea were complexes of hematin and either denatured BSA or aggregates of denatured apomyoglobin (ApoMb). The affinity of hematin for BSA was greater than for ApoMb. These complexes were spectrally similar to those formed on heating meat, aqueous muscle extracts and BSA-Mb mixtures. It is suggested that the complexes present in cooked meat are also denatured hemoproteins, where the protein may be any of several of the denatured proteins found in cooked meat. The nature of possible bonding in the complexes is discussed.

A COMPARISON OF SALMONELLA EXCRETION BY STRESS-SUSCEPTIBLE AND STRESS-RESISTANT PIGS. R.Y.T. TSAI, J.M. GOEPFERT, R.G. CASSENS and E.J. BRISKEY. *J. Food Sci.* **36,** 889–891 (1971)–A relatively high percentage of apparently healthy pigs were Salmonella carriers and excreted salmonellae in the feces. Stress imposed by trucking did not significantly alter the excretion of salmonellae by the pigs. The incidence of salmonellae in fecal samples from 32 stress-resistant and stress-susceptible animals was investigated. There was a high incidence of salmonellae in cecum and colon contents, but salmonellae were not isolated from various muscles or from spleen, liver and kidney samples. Three of eight pigs were positive for *Salmonella* in the mesenteric lymph nodes. There was no apparent pattern of salmonella detection in regard to the stress-susceptible condition of the animal.

MODE OF ACTION OF ETHYLENE OXIDE ON SPORES OF Clostridium botulinum 62A. F.G. WINARNO & C.R. STUMBO. *J. Food Sci.* **36,** 892–895 (1971)–Death of spores of *Clostridium botulinum* when exposed to gaseous ethylene oxide followed first order kinetics. Supplementation of a synthetic medium with the purine and pyrimidine bases of DNA and RNA indicated, as judged by outgrowth from spores which had received sublethal ETO treatments, that the lethal action of ETO on the spores was through alkylation of the guanine and adenine components of DNA. Observed impairment of RNA and protein synthesis was considered an indirect effect resulting from alkylation of DNA components; however, additional evidence bearing on this point is needed to support a more definite conclusion.

EFFECT OF MONOVALENT AND DIVALENT IONS UPON THE GERMINATION OF BACILLUS SPORES IN THE PRESENCE OF NISIN. K.G. GUPTA, R. SIDHU & N.K. YADAV. *J. Food Sci.* **36,** 896–898 (1971)–The effect of Ca, Mg, Fe, Cu, Mn and Na ions on the germination of spores of *Bacillus stearothermophilus, B. cereus* and *B. megatherium* both in the presence and absence of nisin was investigated. Ca and Mg were found to reverse the inhibition of germination by nisin. This was correlated with the release of DPA. There was more DPA release by nisin-treated spores in the presence of Ca or Mg than in their absence. Mg was also more or less beneficial for germination in the absence of nisin. The rest of the ions were more or less inhibitory for germination both in the presence and absence of nisin.

SPIKED-PLATE FREEZE DRYING. J.D. HATCHER & J.E. SUNDERLAND. *J. Food Sci.* **36,** 899–905 (1971)–The acceleration of freeze-drying rates using spiked plates was observed experimentally and found to reduce overall drying times by as much as 24 hr. Experiments were conducted using different spike diameters, sample thickness, spike materials and heater plate temperatures. Weight loss and temperature profiles were obtained as a function of drying times. These experimental results were used to develop a simplified mathematical model for predicting drying rates which will be of use to the designer of spiked-plate freeze-

drying systems. Results obtained using the mathematical model indicate spike diameter, spike spacing and plate temperature have significant effect on the drying rate, whereas sample thickness and spike material are not significant.

VACUUM-PUFF FREEZE DRYING OF TROPICAL FRUIT JUICES. J.H. MOY. *J. Food Sci.* **36**, 906–910 (1971)–Guava and mango puree, passion fruit and pineapple juice were dehydrated by a vacuum-puff freeze-drying process. Sample preparations, freeze-drying conditions, process variables and the effect of loading density (0.25–1.0 lb/ft^2) on drying are discussed. Freezing temperatures of various juice-sucrose mixtures are measured. Sucrose in amount (25–40%) approximating nectar formulations was added to the juice or puree prior to drying to control puffing, to reduce product hygroscopicity and probably to retain flavor. Typical sample compositions are given which contain low concentrations of permissible calcium salts to improve the free flowing property of the finished products.

EFFECT OF RADIO-FREQUENCY ENERGY AT 60 MHz ON FOOD ENZYME ACTIVITY. A. LOPEZ & N.A. BAGANIS. *J. Food Sci.* **36**, 911–914 (1971)–The effect of radio-frequency (R-F) energy at 60 MHz on peroxidase, polyphenolase, pectinesterase, catalase and alpha-amylase activity was determined in water solutions of purified enzymes as well as in foods containing those enzymes. The enzyme systems were exposed to R-F energy at temperatures of 20°C (68°F) and 70°C (158°F) to differentiate enzyme inactivation caused by the heat effect of R-F energy from that which might be caused by R-F energy per se. Results showed that R-F energy per se did not affect the activity of the enzymes studied. The results further showed that, within the limits of the experimental conditions, enzyme concentration and pH of enzyme system were not factors in relation to lack of R-F energy effect on enzyme activity.

PREDICTION ON THE RELATIVE DIELECTRIC LOSS FACTOR IN AQUEOUS SOLUTIONS OF NONFAT DRIED MILK THROUGH CHEMICAL SIMULATION. R.E. MUDGETT. A.C. SMITH, D.I.C. WANG & S.A. GOLDBLITH. *J. Food Sci.* **36**, 915–918 (1971)–Relative dielectric loss factor, an important property in microwave heating of foods, has been studied in reconstituted dried milk by means of chemical simulation based on proximate analysis. This study shows that milk dielectric loss cannot be predicted by its chemical composition alone because of binding and nonbinding effects which may include interactions involving dissolved and colloidal milk particles in an aqueous solvent. These effects were studied close to a frequency of major commercial interest.

INVESTIGATION INTO THE THEORIES OF DESORPTION ISOTHERMS FOR ROUGH RICE AND PEANUTS. K.K. AGRAWAL, B.L. CLARY & G.L. NELSON. *J. Food Sci.* **36**, 919–924 (1971)–Several existing mathematical models of desorption isotherms are investigated with respect to their suitability and range of applicability to biological materials. It is found that a logarithmic plot of vapor pressure of water in the material versus vapor pressure of pure water can best describe the sorption phenomenon. The general form of the equation is: $\ln P = f_1(W) \ln P_s + f_2(W)$. The functions $f_1(W)$ and $f_2(W)$ may differ significantly from material to material. The method of determining these functional relationships is described in detail for rough rice and peanuts. The comparison of experimental and computed values for peanuts shows that this method can predict the required sorption isotherm data within 2% deviation from the experimental values.

EFFECT OF DIETARY FATS ON THE FATTY ACID CONTENTS OF CHICKEN ADIPOSE TISSUE. J.J. JEN, W.P. WILLIAMS JR., J.C. ACTON & V.A. PAYNTER. *J. Food Sci.* **36**, 925–929 (1971)–Broiler-type chicks were reared from hatching to 4 wk of age on a low-fat ration and then fed diets containing 10% of either corn oil, lard, beef tallow or hydrogenated coconut oil. The fatty acid content of extracted total lipids was characteristic of the dietary fats, and dietary fatty acid patterns were incorporated into the adipose tissue within 2 wk after the experimental diets were fed. The total lipids when separated into solid fats and liquid oils also reflected the fatty acid pattern of the experimental diets. Neutral triglycerides from the adipose tissue contained less linoleic acid and more palmitic acid and oleic acid than the total lipid fraction. High amounts of palmitic and oleic acids were observed in all of the fractions analyzed. Cooking losses, TBA values and taste panel studies did not reveal any significant effect of dietary treatment on 10 wk-old broiler carcasses.

FOOD PROTEIN FROM MEAT AND BONE MEAL. H.A. NASH & R.J. MATHEWS. *J. Food Sci.* **36**, 930–935 (1971)–Methods were developed for improving the nutritional quality and flavor of meat and bone meal. After concentration of protein by flotation in carbon tetrachloride, it was subjected to either size fractionation or extraction with 0.1N base. The finer fractions had a more favorable amino acid balance than coarser fractions. Base extraction removed material of a collagen-like amino acid pattern and removed flavors as well. Both the fine fraction and the base-extracted fraction gave considerably better growth and protein efficiency ratios in rat feeding tests than did the unfractionated materials. Effects on growth rate and protein efficiency as a supplement to wheat flour or corn were more than additive.

USE OF GAS-LIQUID CHROMATOGRAPHY FOR QUALITY EVALUATION OF DRY EGG PRODUCTS. K. LORENZ & J. MAGA. *J. Food Sci.* **36**, 936–939 (1971)–Changes in aroma and flavor of dry egg products stored at different temperatures have been recognized for many years. These flavor changes, which are indicative of egg product quality, can be followed with GLC headspace techniques and correlated with baking performance of the ingredient. Egg white solids and dried whole eggs were stored at 5, 24 and 43°C and analyzed and evaluated at regular intervals. Total peak area increases with time and temperature of storage while baking performance decreases. Changes in carbonyl composition as well as free fatty acid composition have also been investigated and found to be indicative of baking performance.

RETARDATION OF OXIDATIVE COLOR CHANGES IN RAW GROUND BEEF. B.E. GREENE, I-M. HSIN & M.W. ZIPSER. *J. Food Sci.* **36**, 940–942 (1971)–Meat pigments and the effect lipid oxidation has on their color are of paramount importance to meat packers, as is the relationship between objective color measurements and acceptability of meat to consumers. This study showed that ascorbic acid plus either BHA or PG effectively retarded both lipid and pigment oxidation in raw ground beef for up to 8 days of refrigerator storage in oxygen-permeable film. Both trained and consumer-type panels judged the additive-treated meat to be significantly better than controls. Percent metmyoglobin was found to be a useful objective measurement of color in raw beef, but total pigment concentration did not provide an adequate evaluation of color.

ABSTRACTS:
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DISCOLORATION OF SAUERKRAUT PROBABLY CAUSED BY A LEUCOANTHOCYANIDIN. N. GORIN & J.A. JANS. *J. Food Sci.* **36**, 943–947 (1971)–A series of experiments was conducted to determine what kinds of pigments bring about discoloration of sauerkraut. UV light altered the color of potential discolored sauerkraut but had no effect on normal or discolored sauerkraut. Spectrophotograms of HCl extracts of potential discolored and discolored sauerkraut peaked at 540 nm whereas normal sauerkraut had an extinction at 540 nm but no peak. A specific profile for discolored sauerkraut was determined via thin-layer chromatography on silica gel. The compound causing the discoloration is apparently a leucoanthocyanidin.

EVALUATION OF FROZEN BOILED PEANUTS AS AFFECTED BY COOK TIME, COOL TIME AND SALT CONCENTRATIONS. G.R. AMMERMAN, R.C. GLENN & C.R. OWENS. *J. Food Sci.* **36**, 948–950 (1971)–Cooking time of 15 min or less resulted in peanuts which were too firm, based on shear press data. Peanuts cooked 20–25 min had acceptable texture. The best boiled frozen product resulted when peanuts were cooked 10–30 min at 212°F in brine containing 6–14 oz of table salt per gallon of water and cooled 9–29 hr in the same brine. Samples of boiled peanuts were frozen at −20°F in a conventional blast freezer, vacuum sealed in 1.5-mil polyethylene pouches and stored at −20°F. No detectable degeneration of quality resulted from holding the frozen product for 8 months.

EFFECTS OF A CRYOGENIC AND THREE HOME FREEZING METHODS ON SELECTED CHARACTERISTICS OF PORK LOIN CHOPS. M.A. BANNISTER, D.L. HARRISON, A.D. DAYTON, D.H. KROPF & H. J. TUMA. *J. Food Sci.* **36**, 951–954 (1971)–Thirteen pairs of pork loin chops from each of four carcasses were assigned randomly to 13 treatments to study effects of freezing by liquid nitrogen vapor and by three home-freezing methods, and the effects of 1 and 4 wk of storage in three types of home freezers. Liquid nitrogen freezing produced superior appearance, but "quality" of the cooked product from home freezing was comparable to that of the liquid nitrogen-frozen product. All frozen chops were stored in home freezers at −18°C for 4 wk and at −9°C for 1 wk without loss of quality.

MICROBIOLOGY OF BEEF SHELL FROZEN WITH LIQUID NITROGEN. C.R. REY, A.A. KRAFT & R.E. RUST. *J. Food Sci.* **36**, 955–958 (1971)–The effect of freezing primal cuts of beef with liquid nitrogen on the subsequent microbiological quality of their retail cuts was studied. Cryovac-packaged loins were shell frozen and aged or cut into steaks without aging. Steaks from frozen and fresh loins were packaged and stored at about 5°C. Initial contamination on steaks increased with time of aging of loins at low temperature. High temperature-short time aging of loins promoted growth of some types of potential food poisoning organisms, but recovery of *Salmonellae* was more closely related to contamination of the fresh meat than to holding time and temperature of the loins.

COMPARISON OF TEXTURE OF CARROTS FROZEN BY AIR-BLAST, FOOD FREEZANT-12 AND NITROGEN VAPOR. E.R. WOLFORD & J.W. NELSON. *J. Food Sci.* **36**, 959–961 (1971)–Carrot sticks were frozen by immersion in Food Freezant-12, in vapor from liquid nitrogen, in a fluidized bed tunnel and on trays. Those frozen in Food Freezant-12 were the firmest and most crisp. Texture was related to time needed to freeze the carrots. Either a puncture-testing device or shear press can be used to test for firmness.

STABILIZATION OF ANTHOCYANINS IN FROZEN TART CHERRIES BY BLANCHING. A. SIEGEL, P. MARKAKIS & C.L. BEDFORD. *J. Food Sci.* **36**, 962–963 (1971)–Unpitted red tart cherries (*Prunus cerasus* L. cv. Montmorency) were blanched in steam (100°C) for 0, 30, 45 and 60 sec, then frozen at −20°C. The anthocyanin color of the fruit was determined periodically during frozen storage for 3 months in one experiment and 10 months in another. No anthocyanin color was lost during the frozen storage, whether the cherries were blanched or not. Upon thawing for 2 and 4 hr, the control cherries lost 14 and 25% of their color, respectively, while those blanched for 45 or 60 sec showed no appreciable color loss. Disintegration in a Waring Blendor for 30 min resulted in 70% color loss in the unblanched fruit, but practically complete color retention in the fruit blanched for 45 or 60 sec.

INFLUENCE OF EXTRACTING AGENTS DURING THE COLORIMETRIC DETERMINATION OF PECTIC SUBSTANCES. C.J.B. SMIT. *J. Food Sci.* **36**, 964–965 (1971)–Pectic substances present in plant tissues are often characterized by successively extracting with different solvent systems and determining extracted galacturonides colorimetrically. Sodium (tetra) ethylenediamine tetraacetate, when used in the extracting medium, interferes with the borate-sulfuric acid-carbazole reaction and may be responsible for serious errors. With 20 μg anhydrogalacturonic acid and a 25-fold concentration of sodium (tetra) ethylenediamine tetraacetate (technical grade) in the reaction mixture the error may be as high as 10%. At a 5-fold concentration, this is reduced to around 2%. On the other hand, ammonium oxalate in concentrations as high as 2.5 mg did not show any interference.

CELLULASE IN DATES AND ITS ROLE IN FRUIT SOFTENING. S. HASEGAWA & D.C. SMOLENSKY. *J. Food Sci.* **36**, 966–967 (1971)–Cellulase activity, absent at the green stage, began to develop as maturity progressed. There was a significant increase in activity during ripening. The activity reached its maximum at the late red stage and remained fairly constant during the softening stages. The possible role of cellulase activity in the softening process of dates was discussed.

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COMPARISON OF THE PROTEIN NUTRITIONAL VALUE OF TVP, METHIONINE ENRICHED TVP AND BEEF AT TWO LEVELS OF INTAKE FOR HUMAN ADULTS

SUMMARY—The objective of the study was to compare the protein nutritive value for adult men of beef, an extruded soybean product resembling beef (TVP) and a 1% DL-methionine-fortified TVP product at two levels of nitrogen intake. Experimental diets were maintained adequate in calories, vitamins and minerals. Mean nitrogen balances of subjects fed 8.0g nitrogen per subject per day from beef, TVP or methionine-fortified TVP were +0.74, +0.78 and +0.72g nitrogen per day, respectively, indicating that at this level of protein intake all three sources equaled or exceeded the protein requirements of adult men. Mean nitrogen balances of subjects fed 4.0g nitrogen per subject per day from beef, TVP or methionine-fortified TVP were −0.30, −0.70 and −0.45g nitrogen per day, respectively. While none of the test protein sources fully met the protein needs of the subjects at this level of nitrogen intake, beef was superior to TVP on the basis of nitrogen balance data. DL-methionine fortification at the 1% level of the TVP was demonstrated to be partially effective in improvement in nitrogen balance.

INTRODUCTION

SOYBEAN textured foods offer great possibilities for the nutriture of humans because of their adaptability for various forms of highly palatable, economical products (Noyes, 1969). Soybean protein is considered to be one of the most nutritious of the plant proteins; however, there has been little investigation of the nutritive value on textured products prepared from soybean isolates or extruded soybean mixtures. Variations in industrial processing may well alter the nutritive value of such products. Bressani et al. (1966, 1967) investigated the protein quality of a textured food simulating ground beef (made from isolated soybean protein with added egg albumin and wheat gluten) in experimental animals and in children. No significant differences were observed between the protein nutritive value of milk protein and the soybean isolated protein product when fed to young children to provide 2g of protein/kg of body weight/day. However, with decreasing amounts of these sources of dietary protein, it was found that 138 mg of nitrogen was required from the soybean textured food as compared with 97 mg from milk to maintain nitrogen equilibrium in young children (Bressani et al., 1966, 1967). Processes involved in preparation of extruded soybean products are considerably different from those involved in preparation of soy protein isolates (Noyes, 1969). Addition of extruded soybean products in the ordinary diets of low income families has been found to have beneficial effects on a wide range of physiological parameters (Simpson and Jones, 1969). However, little information based on controlled experiments is available on the comparative nutritive value of extruded soybean products and products which they can logically be expected to replace.

Protein is one of the principal nutrients provided by meat; therefore, investigation of the comparative nutritive value of extruded soy protein products and meat is of particular interest.

Comparison of the amino acid pattern of soy protein (Orr and Watt, 1957) with that of the Rose amino acid requirement pattern for adult men (Rose, 1957) suggests methionine as being the essential amino acid furnished in lowest amount in proportion to need. It may be that the protein nutritional value of soy textured foods may be enhanced via purified methionine fortification.

The general objective of the current study was to evaluate the protein value of one product produced from extruded soy bean protein processed to resemble chopped beef, termed in this study TVP—textured vegetable protein. The extruded soybean protein product processed to resemble chopped beef was prepared and furnished gratis by the Archer Daniels Midland Co., Decatur, Ill. The methionine-enriched TVP product was identical to this except that DL-methionine was added at the 1% level (dry weight of product). This test protein source was also prepared and furnished gratis by the Archer Daniels Midland Co. [TVP products are made by subjecting a plasticized mix of defatted soy flour (50% protein), water coloring and flavoring materials to high temperature and pressure in an extruder and extruding the mix through a die into a region of lower pressure and temperature. On emergence, the steam is flashed off and an expanded textured product results which is dried to about 5% moisture. Upon rehydration, one part of textured product absorbs approximately two parts water.]

The specific objective was to compare protein nutritive value of TVP, methionine-enriched TVP and ground beef at two levels of nitrogen intake in ability to meet the protein needs of adult men.

PROCEDURE

THE STUDY consisted of two parts, designated as Part A and Part B. Each 31-day part included an introductory 10-day adjustment period and three experimental periods of 7 days each arranged at random for each subject. The two parts followed one another directly with no lapse in time and used the same subjects. Subjects 451–454 first followed the regimen imposed by Part A followed by that imposed by Part B, while subjects 455–459 first followed the regimen of Part B followed by that of Part A.

Dietary nitrogen intake was maintained constant during the introductory nitrogen adjustment period and experimental periods of Part A at 4.8g per subject per day and during the similar periods of Part B at 8.8g per subject per day. During the two nitrogen adjustment periods, an isonitrogenous mixture of ground beef and TVP provided either 4.0g nitrogen (Part A) or 8.0g nitrogen (Part B). An additional 0.8g nitrogen per subject per day was provided by

Table 1–Experimental plan

Period[a]	No. of days	Source of dietary nitrogen[b]	Amount (g N/day)	Total dietary nitrogen[c] (g N/day)
Part A				
Adj. A	10	50% beef/50% TVP	4.0	4.8
Exp. 1	7	Beef	4.0	4.8
Exp. 2	7	TVP	4.0	4.8
Exp. 3	7	TVP + 1% methionine	4.0	4.8
Part B				
Adj. B	10	50% beef/50% TVP	8.0	8.8
Exp. 4	7	Beef	8.0	8.8
Exp. 5	7	TVP	8.0	8.8
Exp. 6	7	TVP + 1% methionine	8.0	8.8
	62 days			

[a]For subjects 451–454, Part A followed by Part B. For subjects 455–459, Part B followed by Part A. Experimental periods were randomly arranged for each subject within each part.

[b]Beef = ground lean beef, rump cut; TVP = textured vegetable protein, extruded soy products simulating ground beef; TVP + 1% methionine = TVP fortified with 1% DL-methionine.

[c]Total dietary nitrogen (amount per subject per day) figures include the 0.80g N/day provided by the basal diet. The basal diet consisted of 100g applesauce, 100g peaches, 100g pears, 100g green beans, 100g stewed tomatoes, 20g corn oil, 10g decaffeinated coffee and varying amounts of wheat starch bread and cake, butter oil, jelly, hard candy and carbonated beverages. A vitamin supplement provided each subject daily with 5000 IU of vitamin A (synthetic); 900 IU of vitamin D; and (in milligrams) thiamine, 2; riboflavin, 2.5; ascorbic acid, 50; pyridoxine, 1; niacin, 20; Ca pantothenate, 1; and vitamin B_{12}, 1 μg. Mineral supplements provided the following per subject per day (in grams): Ca, 1.00; P, 1.00; Mg, 0.199. Fe, 0.015; Cu, 0.002; K, 0.00005; I, 0.00015; Mn, 0.002; and Zn, 0.0009. NaCl was allowed ad libitum.

Table 2–Amino acid composition of ground beef, TVP and methionine-supplemented TVP

Amino acid	Amino acid composition of test products[a] (g AA/4g product N) Beef	TVP	TVP + Met.	Recommended amino acid intake pattern[b] (g AA/day)
Lysine	2.194	1.571	1.619	1.60
Histidine	0.857	0.668	0.677	–
Arginine	1.786	1.877	1.834	–
Aspartic acid	2.338	2.980	2.924	–
Threonine	1.154	1.079	1.047	1.00
Serine	1.063	1.408	1.368	–
Glutamic acid	4.317	6.538	6.431	–
Proline	1.740	1.457	1.371	–
Glycine	2.048	1.051	1.031	–
Alanine	1.688	1.086	1.063	–
Half cystine	–	0.203	0.237	–
Valine	1.327	1.300	1.275	1.60
Methionine	0.673	0.294	0.656	2.20
Isoleucine	1.193	1.225	1.204	1.40
Leucine	2.048	2.025	1.977	2.20
Tyrosine	0.821	0.803	0.800	–
Phenylalanine	1.015	1.293	1.268	2.20
Tryptophan	(0.292)	(0.344)	(0.344)	0.50
Moisture, %	69.0	07.45	07.90	
Fat,[c] %	9.16	0.69	0.93	
Protein,[c] %	21.58	49.70	50.38	
Misc.,[c,d] %	0.26	42.16	40.79	

[a]Values except tryptophan based on analyses via column chromatography. Handbook value is basis for listed tryptophan content (Orr and Watt, 1957). Listed values represent the intake of amino acids per day by subjects while receiving the lower protein diets; values on the higher protein diets can be obtained by multiplying each figure by 2.

[b]Rose recommended amino acid intake pattern for young men (double the minimum requirements of essential amino acids as estimated by Rose and his associates, Rose, 1957).

[c]Analysis of moisture, fat and protein expressed as percent of sample are on an "as is" basis.

[d]Calculated by difference. It is assumed that this is largely carbohydrate in the TVP samples.

the basal diet as described in Table 1. The main purpose of these periods was to allow subjects to adjust both to the level of dietary nitrogen and to the dietary composition similar to that used in the following experimental periods. In addition, the first of these periods also provided time for introduction of the subjects to their duties and responsibilities and for determination of individual subject's caloric requirements for weight maintenance.

During the three experimental periods of each part, ground beef, TVP or methionine-enriched TVP were fed separately to provide 4.0g nitrogen (Part A) or 8.0g nitrogen (Part B). Composition of these products is given in Table 2. The basal diet as described in Table 1 provided an additional 0.8g nitrogen per subject per day during each of these periods. In order to effectively compare the protein nutritional value of these three protein sources, all subjects received all experimental treatments. Within each part, the order of the three experimental periods was randomly arranged for each subject in order to eliminate the variable of order of presentation.

Diets

The ground beef, TVP or methionine-fortified TVP allotments for each subject were fed in a casserole form. The casserole, consisting of one-half the daily allotment of the test protein source (kind and amount as determined by the experimental design), 100g green beans, 100g stewed tomatoes and 10g corn oil was served to each subject at the noon meal. This was repeated at the evening meal.

Additional calories were provided by a wheat starch bread and wheat starch cake prepared basically by the method of Steele et al. (1965), butter oil, carbonated beverages, hard candy, jelly and low-protein fruits (Table 1). Caloric intake was maintained constant throughout the study for each subject at that level as individually determined as being necessary for weight maintenance. The percent calories provided by fat was maintained constant for each subject (40%) throughout the study by altering intake of butter oil and corn oil as appropriate while receiving the various experimental products. At lower protein intake levels, sucrose (hard candy) was used to equalize caloric intake to higher protein intake levels as dictated by the experimental design. Vitamin and mineral supplements as described in Table 1 were also provided.

Subjects

Details regarding the nine men, inmates of the Nebraska Penal and Correctional Complex, who were subjects and satisfactorily completed the study are given in Table 3. Institutional health records, as well as physical examinations conducted at the beginning and end of the project by the institution's physician indicated all were in good health. Subjects were housed and ate meals together in a separate part of the institution; however, usual work assignments were maintained.

Analyses

The nitrogen balance technique was used as the principal method of evaluation. Urine, feces and food were collected and prepared for analysis as described previously (Linkswiler et al., 1958) and were analyzed for nitrogen content by the boric acid modification of the Kjeldahl method (Scales and Harrison, 1920). Urine samples were preserved under toluene and analyzed daily for 24-hr excretion of nitrogen and creatinine (Folin, 1914). Daily fecal nitrogen values were based on 7-day fecal composites.

Fasting venous blood samples were collected from each subject at the beginning of the study and at the end of each experimental period. These were analyzed by a hospital laboratory via routine clinical procedures as described previously (Kies et al., 1967) as a screening device for possible gross abnormalities in protein nutriture in particular and in normal health in general. Amino acid composition of the three test products, as shown in Table 3, were done by the Dept. of Agronomy, University of Nebraska, via auto analyzer methodology.

Statistical analyses of data, including analyses of variance and Duncan's Multiple Range Test, were by the Statistical Lab. of the Nebraska Agricultural Experiment Station, University of Nebraska.

Table 3—Characteristics of subjects

Characteristic[a] (start of study)	Subject number 451	452	453	454	455	456	457	458	459
Sex	Male	Male	Male	Male	Male	Male	Male	Male	Male
Weight (lbs)	180	170	147	190	157	182	140	153	180
Race/Nationality	White/ Am.	White/ Am.	White/ Am.	White/ Am.	Black/ Am.	Indian/ Am.	Mexican/ Am.	White/ Am.	Black/ Am.
Blood glucose	82	92	98	88	92	86	92	93	80
Blood urea nitrogen	8	11	11	8	11	9	14	13	8
Total blood plasma protein	6.3	7.1	7.5	7.4	6.9	7.0	7.0	7.4	7.5
Blood albumin	4.9	4.7	4.6	4.9	4.9	4.5	4.7	5.0	5.0
Blood globulin	2.4	2.4	2.9	2.5	2.0	2.5	2.3	2.4	2.5
Albumin/globulin ratio	2.0	2.0	1.6	2.0	2.4	1.8	2.0	2.1	2.0
Blood cholesterol	215	185	156	238	214	284	310	204	226
SGPT	43	35	43	35	39	35	43	46	48
Hematocrit	47	44	49	45	44	48	40	49	46
Hemoglobin	16.2	15.2	16.7	15.2	14.5	16.1	12.8	17.1	15.4
Blood pressure	121/80	118/74	124/84	121/78	136/84	121/82	118/78	108/68	122/82

[a]Determinations of blood constituents (fasting) were by standard laboratory procedures of the St. Elizabeth's Hospital Lab., Lincoln, Neb.

RESULTS & DISCUSSION

MEAN NITROGEN balances of subjects receiving the three protein sources at two levels of nitrogen intake are shown in Table 4.

At the 8.8g nitrogen intake level, mean nitrogen balances while receiving ground beef, TVP or methionine-fortified TVP were +0.74, +0.78 and +0.72g nitrogen per day, respectively. The lack of patterned trends among the subjects while receiving the three protein sources suggests that no true biological differences exist in protein nutritive quality of ground beef, TVP or methionine-fortified TVP for adult men when fed at the 8.8g nitrogen intake level. Analyses of variance indicated no differences due to treatment at this level of intake.

At the 4.8g nitrogen intake level, mean nitrogen balances of subjects while receiving ground beef, TVP or methionine-fortified TVP were −0.30, −0.70 and −0.45, respectively. Analyses of variance indicated differences in treatment to be present (⩾ 5% level of significance). Application of the Duncan's Multiple Range Test indicated results of each treatment to be different from each of the other two treatments (⩾ 5% level of significance). It is generally assumed that a decrease in body nitrogen loss denoted by a less negative, more positive nitrogen balance, denotes a more favorable protein nutriture (NRC, 1110). This assumption is the basis on which all data related to protein and amino acid requirements of human adults is based (NRC, 711). If one accepts this assumption, then the apparent order of protein worth from highest to lowest of the three tested sources is as follows: ground beef, methionine-fortified TVP and TVP.

At the 8.8g nitrogen intake level, TVP, methionine-fortified TVP and ground beef were seemingly of equal nutritional value while at the 4.8g nitrogen intake level, ground beef was seemingly superior to both methionine-fortified TVP and to the unenriched TVP. These results on first impression seem to be contradictory. However, it should be noted that differences in protein nutritive quality can only be ascertained when the test materials are fed in insufficient amounts to meet the organism's (human or other) needs (NRC, 1110). Similar nitrogen balance data would be obtained under conditions of feeding 100% of the need as under conditions of feeding 125% of the need if maximal nitrogen retention is used to define the meeting of protein requirement. Since most subjects were in apparent positive nitrogen balance at the 8.8g nitrogen level while receiving all of the

Table 4—Nitrogen balances of subjects maintained on beef, TVP and methionine-supplemented TVP[a]

Subject No.	Nitrogen balance (g N/day) while receiving: 4g N/day from[b] Beef	TVP	TVP + Met.	8g N/day from[b] Beef	TVP	TVP + Met.
451	0.06	−0.58	−0.07	1.24	0.92	1.08
452	−0.71	−1.45	−0.98	0.13	−0.04	−0.61
453	−0.67	−1.00	−0.83	0.61	0.43	−0.11
454	−0.52	−0.83	−0.66	0.16	1.52	1.84
455	0.21	0.04	0.02	0.74	0.82	0.99
456	0.04	−0.38	0.01	1.87	1.95	1.62
457	−0.63	−0.95	−0.89	0.11	−0.31	−0.17
458	−0.46	−0.81	−0.55	0.62	0.45	0.38
459	0.01	−0.30	−0.08	1.20	1.26	1.42
Mean N balance	−0.30	−0.70	−0.45	0.74	0.78	0.72
Crude protein digestibility	81.4%	79.4%	79.2%	82.7%	81.6%	80.1%

[a]Information supplied by the Archer Daniels Midland Co., Decatur, Ill., indicated rat PER values adjusted to casein (2.50) of 2.12, 2.82, 2.37, respectively, for the TVP product, the TVP + methionine product and the ground beef product used in this study.

[b]If the conversion factor of 6.25 is used for converting nitrogen intake to protein intake, protein intake from the three experimental sources were 25g and 50g/subject/day, respectively, at the two levels of nitrogen intake. Since Orr and Watt (1957) suggested the conversion factor of 5.71 for use with soybeans, 23 and 47g protein/subject/day might be better estimates of the protein intake from the soy products at the two levels of nitrogen intake.

three test protein sources, evidently protein needs were being met or exceeded at this level of intake. Subjects were primarily in negative nitrogen balance while receiving the 4.8g nitrogen diets indicating that dietary protein needs were not being met at this level. This, then, represents a much more critical level for evaluations to be made.

Evaluation of nitrogen balance data collected at the 4.8g nitrogen intake level indicates that ground beef is a superior protein source in comparison to TVP when fed at equal levels of nitrogen intake. Methionine fortification at the 1% level resulted in improvement in protein value but not to the extent of equaling beef. That methionine supplementation resulted in an apparent improvement is indicative that methionine is the first limiting amino acid in TVP. It may well be that methionine intake at this level was also inadequate for beef but to a lesser degree. (This point was not tested in the current project.) However, as shown in Table 2, a comparison of the essential amino acids provided by beef with the recommended essential amino acid intake pattern for young men (Rose, 1957) suggests that methionine intake at the lower intake level of beef might have been less than optimal. It is sometimes overlooked that animal products as well as plant products have "first limiting amino acids." It is unknown whether higher levels of methionine supplementation would have resulted in nitrogen balance data equaling (or bettering) that obtained with the beef diets. The 1% DL-methionine fortification level was used because levels higher than this have been found to adversely affect product palatability (Archer Daniels Midland Co., personal communication, 1970). The DL-form rather than the L-form was used since its lower cost makes its use more practical in industry situations at this time. The comparative effectiveness as an essential amino acid of D-form and L-form methionine for the human has not been extensively studied. For many amino acids only the L-form is biologically active for the human. If this is the case with methionine, it might be feasible that the effectiveness of the 1% fortification could be enhanced by straight L-methionine fortification rather than by use of the DL-mixture.

By making comparisons of the three test proteins on an equal nitrogen intake basis, the TVP products were actually placed at a disadvantage. This kind of comparison, the usual one, assumes that all test proteins are 16% nitrogen; thus, that protein content of a food can be found by multiplying the nitrogen content by the conversion factor 6.25 (Orr and Watt, 1957). Amino acids range in nitrogen content from 8–27% (NRC, 719). Proteins vary in their amino acid

Table 5—Effect of level and source of dietary protein on several blood constituents or characteristics

Blood constituent or characteristic[a]	Level of blood constituent[b] while receiving diet containing					
	4g N from			8g N from		
	Beef	TVP	TVP + Met.	Beef	TVP	TVP + Met.
Glucose, mg/100 ml	94 (79–109)	98 (84–108)	97 (84–112)	94 (83–108)	98 (80–114)	97 (85–110)
Phosphorus (inorganic) mg/100 ml	4.2 (3.6–4.7)	4.2 (3.6–6.0)	4.2 (3.8–4.7)	4.1 (3.3–5.2)	4.2 (3.8–4.8)	4.2 (3.9–4.7)
Urea N, mg/100 ml	8.6 (4–12)	9.0 (4–16)	9.1 (5–15)	12.4 (7–20)	12.5 (8–23)	13.6 (8–22)
Phosphatase (alkaline), units	31 (12–64)	32 (10–75)	35 (14–75)	31 (15–70)	27 (10–60)	31 (10–61)
Total protein, g/100 ml	7.0 (6.3–7.6)	6.9 (6.8–7.2)	7.0 (6.6–7.6)	7.1 (6.4–7.5)	6.9 (6.5–7.4)	7.0 (6.6–7.6)
Lactic dehydrogenase, units	252 (210–311)	250 (180–405)	270 (190–350)	232 (190–290)	241 (185–310)	237 (145–412)
Albumin, g/100 ml	4.5 (4.1–4.9)	4.5 (4.2–4.7)	4.6 (4.2–4.9)	4.6 (4.3–4.9)	4.6 (4.3–4.9)	4.6 (4.3–4.9)
Bilirubin, mg/100 ml	0.4 (0.1–0.8)	0.5 (0.2–1.0)	0.6 (0.1–0.9)	0.7 (0.4–1.2)	0.6 (0.2–1.3)	0.5 (0.1–1.1)
Globulin, g/100 ml	2.5 (2.2–2.9)	2.5 (2.1–3.2)	2.5 (2.2–2.8)	2.5 (2.1–2.8)	2.4 (2.0–2.8)	2.4 (2.0–2.8)
Uric acid, mg/100 ml	6.5 (3.7–10.1)	6.1 (4.8–7.8)	5.8 (4.1–7.7)	6.2 (4.4–9.1)	5.7 (4.4–8.0)	5.6 (3.8–7.8)
Albumin/globulin ratio	1.9 (1.6–2.4)	1.7 (1.5–2.1)	1.8 (1.6–2.1)	1.9 (1.7–2.2)	2.0 (1.6–2.3)	2.0 (1.7–2.3)
Calcium, mg/100 ml	10.1 (9.6–11.4)	10.2 (9.7–11.4)	10.0 (9.5–10.8)	10.3 (10.0–11.3)	10.0 (9.7–10.4)	10.1 (9.6–10.5)
Cholesterol, mg/100 ml	210 (144–266)	212 (138–301)	220 (175–319)	202 (155–255)	219 (145–290)	207 (132–270)
SGPT, Babson units	41 (30–75)	48 (28–103)	52 (28–166)	41 (31–58)	44 (32–62)	41 (21–55)
Hematocrit, ml/100 ml	44 (35–48)	46 (40–50)	45 (38–50)	44 (38–49)	44 (39–50)	45 (37–50)
Hemoglobin, g/100 ml	15.0 (11.3–16.6)	15.4 (12.7–16.6)	15.1 (11.9–17.7)	14.9 (11.9–17.5)	14.9 (12.0–16.5)	15.2 (11.5–17.2)
Blood pressure	114/72 (108/68–136/84)	120/80 (116/70–142/92)	116/76 (106/68–134/88)	115/73 (105/68–134/80)	117/73 (108/64–128/80)	115/74 (108/68–126/86)

[a]Determinations were by standard laboratory procedures of the St. Elizabeth's Hospital Lab., Lincoln, Neb. Additional information regarding methodology and quality control (within 1% for method used) is available from this laboratory.
[b]Venous blood samples were drawn on the morning before consumption of the first meal of the day.

composition. In addition, a food usually contains more than just one kind of protein. Also, the nitrogen content of a food is not necessarily all protein/amino acid nitrogen. In the case of soybeans, the raw material from which the TVP products were formulated, the conversion factor of 5.71 has been suggested (Orr and Watt, 1957). Thus, it could be assumed that at the lower nitrogen intake level of this study, the protein nutritional value of 25g of beef protein (the 6.25 figure conversion factor is fairly accurate for meat) was being compared against 22.84g of TVP protein.

The recommended level of protein intake for young men has been set at 65g by the Food and Nutrition Board, National Research Council. The results of the current study suggest that the minimum (not necessarily recommended level) requirement of protein from any of the three test proteins for most subjects was less than 55g of crude protein (N x 6.25) from any of the three test protein diets.

Mean crude digestibility figures of the beef, TVP and methionine-fortified TVP at the 8.0g nitrogen intake level were 82.7%, 81.6% and 80.1%, respectively; and at the 4.0g nitrogen intake level were 81.4%, 79.4% and 79.2%. The individual subject range was wide and followed no apparent trend.

Subjects completed check-list type questionnaires each day of the study pertaining to personal, mental and physical well-being. Data obtained from these questionnaires indicated no problems related to the possible flatulent nature of the TVP products.

Mean blood constituent data of subjects receiving the three protein sources at two levels of intake are shown in Table 5. All values for all subjects while receiving all diets were within normal range. Blood urea nitrogen values for all subjects while receiving the 8.8g nitrogen diet were higher than while receiving the 4.8g nitrogen diets; however, a reflection of dietary protein level in blood urea level has been found to be the usual response in this laboratory (Kies et al., 1967). Blood uric acid levels were slightly lower for subjects while receiving either methionine-fortified or unfortified TVP than when receiving the beef diets; however, all uric acid values were within "normal" range.

No effects on serum calcium levels were demonstrated, indicating no pronounced adverse affect of phytate content of the TVP products. Phytate content of the TVP products was not measured in this study. Since calcium supplements were included in the diet, effects of phytates may have been masked. In an earlier study (Simpson and Jones, 1969), the feeding of TVP products was found to have a lowering effect on blood cholesterol levels and on blood pressure levels. In the current study, the mean blood pressure of subjects was lowered while receiving the experimental diets in comparison to the pre-experimental value. No difference in effect was demonstrated relative to level of dietary protein or test protein source. No consistent effect on blood cholesterol levels was demonstrated. The lack of consistency in results between these two studies may be related to variation in experimental design, variation in experimental methodology and variation in experiment subjects used. In this context, particular note should be made that dietary fat level and source rather than dietary protein level and source are more likely important determinants affecting blood cholesterol and possibly blood pressure levels.

The current project was designed to investigate only one variable, i.e., that of variation in dietary protein (level and source). In order to reduce influence of other variables, a low-fat ground beef was used. The principal dietary fat for subjects receiving all three dietary protein sources was vegetable oil and percent calories furnished by fat was maintained constant at 40% in all experimental diets. In field situations, individuals receiving beef-based diets in comparison to TVP-based diets most probably would receive a diet both higher in total fat and higher in animal fat. Obviously, valid comparison of the multiple variables of level and source of dietary fat and level and source of dietary protein from beef and TVP diets within the same research design of a single study would lead only to confusion.

Both the beef-based and TVP-based experimental diets used in the current study were heavily fortified with vitamin and mineral supplements to insure adequate intake of these nutrients. Both meat and soybeans are rich sources of nutrients other than protein. Meat is an important source of dietary iron; soybeans contain high levels of various B vitamins. These nutrients are not only independently important but also affect protein nutriture as well. Processing of soybeans into TVP products may well adversely affect the final product's vitamin/mineral content due to water leakage or destruction by oxidation.

The current project was designed only to evaluate beef, TVP and methionine-fortified TVP as sources of dietary protein. Under the experimental conditions selected, adequate vitamin/mineral intake was insured. Quite different results pertaining not only to vitamin/mineral status but also to protein nutritional status as well might be demonstrated under different experimental conditions.

In summary, the results of the present study indicate that TVP, methionine-fortified TVP and unfortified TVP all meet the protein requirements of adult men when fed at the 8g nitrogen intake level. At the 4g nitrogen intake level, beef was found to be superior to a TVP product as a source of protein. However, this superiority could partially be overcome with 1% fortification of the TVP product with DL-methionine.

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MEASUREMENT OF THE RELATIVE TASTE INTENSITY OF SOME L-α-AMINO ACIDS AND 5′-NUCLEOTIDES

SUMMARY—Intensity of glutamate-like and/or inosinate-like taste (umami in Japanese) of various flavor amino acids and flavor nucleotides was studied using sensory analysis and always found proportional to that of monosodium glutamate (MSG) and disodium 5′-inosinate (IMP), respectively. By application of this fact to a previously obtained equation expressing the relationship between the taste intensity of MSG-IMP mixture and that of MSG alone, the intensity of umami of the mixture of any flavor amino acids and nucleotides could be expressed as an elementary equation: $y = u + \gamma uv$, where u and v are the concentrations of amino acids and nucleotides in terms of the concentrations of MSG and IMP, respectively, in the mixture; y is the equivalent concentration of MSG alone; and γ is a positive constant. Interrelationships within each group of substances were additive.

INTRODUCTION

IT IS well known that free amino acids and 5′-nucleotides, among the components of foodstuffs, contribute greatly to the flavor; however, much emphasis should be placed on the fact that a remarkable synergistic effect occurs between particular L-α-amino acids and 5′-nucleotides. The typical substances of these two compound series are monosodium glutamate (MSG) and disodium 5′-inosinate (IMP) or disodium 5′-guanylate (GMP), respectively. The characteristic taste of these substances is called "umami" in Japanese. In previous papers (Yamaguchi, 1967; Yamaguchi et al., 1968a), the authors investigated the taste of these typical substances in detail and reported that the synergistic effect between them could be expressed quantitatively by equation (1): i.e., if u g/100ml of MSG is mixed with v g/100ml of IMP or GMP solution, then the taste intensity of the resulting solution in terms of the concentration of MSG alone, y g/100ml, can be expressed as

$$y = u + \gamma uv \qquad [1]$$

where γ is a positive constant, 1.218×10^3 for IMP and 2.80×10^3 for GMP. If the concentrations are not expressed in terms of g/100ml but in terms of moles/liter, γ becomes 6.42×10^4 for IMP and 1.48×10^5 for GMP (Yamaguchi, 1967; Yamaguchi et al., 1968a).

However, it has already been found that the synergistic effect can also be caused by many pairs of undermentioned L-α-amino acids and 5′-ribonucleotides. Regarding the L-glutamic acid analogous substances, Akabori (1939) reported that the L-forms of the α-amino dicarboxylic acids with 4–7 carbon atoms have a similar kind of taste (umami) to that of L-glutamic acid. Kaneko et al. (1959) pointed out that the taste of L-hydroxy glutamic acid with –OH at the β-position is more intense than at the γ-position, and that the threo form is more intense than the erythro form. Kaneko et al. (1961) also discovered that L-homocysteic acid which is occupied by $-SO_3H$ at the γ-position of glutamic acid, has the same characteristic taste. Takemoto and Nakajima (1964) and Takemoto et al. (1964) found ibotenic acid and tricholomic acid as new umami substances.

As to flavor nucleotides, Kuninaka (1960) first investigated systematically the taste of the naturally occurring ribonucleotide derivatives and reported that the chemical structure, in which a purine nucleus has a hydroxy group at the 6-position and the 5′-position of ribose moiety is esterified by phosphoric acid, is required for the flavoring action. However, since the intensity of flavoring activity of 6-hydroxypurine ribonucleoside 5′-phosphate related compounds (such as 5′-inosinic acid, 5′-guanylic acid and 5′-xanthylic acid) differ considerably from one another, it was postulated that the group at the 2-position would affect the magnitude of the taste intensity: for example, the $-NH_2$ group for 5′-guanylic acid or the –OH group for 5′-xanthylic acid. Noting this fact, several 2-substituted ribonucleotides which have the umami taste were synthesized in our laboratories (Yamazaki et al., 1968a). Furthermore, since the 6-hydroxy group is capable of existing in enol-keto tautomers, the flavoring activity of 5′-nucleotides (in which the 6-position is fixed to keto form) was also taken into consideration and several N^1-methyl-2-substituted purine nucleotide derivatives were synthesized (Yamazaki et al., 1968b). Several 6-substituted ribonucleotides were also synthesized (Yamazaki, Kumashiro and Takenishi, unpublished data). Nakao and Ogata (1960) demonstrated that 5′-deoxy inosinic acid and 5′-deoxy guanylic acid also have similar flavoring action. Honjo et al. (1963) reported that the 2′,3′-O-isopropyliden-5′-inosinic acid has the flavoring activity and that the 2′ and 3′ – hydroxy groups are not essential for the flavoring action.

The purpose of this investigation was to determine the intensity of umami of various flavor amino acids and flavor nucleotides and to relate the findings to a previously obtained equation (expressing the synergistic effect of umami to other substances).

EXPERIMENTAL

Test stimuli

The test substances were monosodium salts of L-glutamate · H_2O (MSG), DL-threo-β-hydroxy glutamate · H_2O (MSO), DL-homocystate · H_2O (MSH), L-aspartate · H_2O (ASP) and L-α-amino adipate · H_2O (AA) and disodium salts of 5′-inosinate · $7.5H_2O$ (IMP), 2-methoxy-5′-inosinate · H_2O (2-MeO-IMP), N^1-methyl-5′-inosinate · H_2O (N^1-Me-IMP) and 2-methyl-6-mercaptopurine riboside 5′-phos-

Table 1—Relative umami intensity of various flavor amino acids

Test substance	Conc of test substance u′ g/100 ml	n	Equi-umami conc of MSG u g/100 ml	Std. dev.[a] (1 + Δu)	Ratio α = u/u′
MSO	0.1	25	0.089	1.06	0.89
	0.3	25	0.255	1.05	0.85
	1.0	25	0.860	1.07	0.86
MSH	0.1	50	0.079	1.04	0.79
	0.3	50	0.223	1.03	0.74
	1.0	50	0.783	1.05	0.78
ASP	1.0	50	0.073	1.07	0.073
AA	1.0	20	0.092	1.07	0.092

[a]In terms of the value of a ratio, i.e., the upper and the lower deviations of observed u are given as u × (1 + Δu) and u ÷ (1 + Δu), respectively.

Table 2—Relative umami intensity of various flavor amino acids in the presence of IMP

Conc of IMP v g/100 ml	Test substance	Conc of test substance u' g/100 ml	n	Equi-umami conc of MSG u g/100 ml	Std. dev.[a] (1 + Δu)	Ratio a = u/u'
0.0025	MSO	0.0125	25	0.0116	1.04	0.93
0.0400		0.0125	25	0.0109	1.06	0.87
0.0025		0.2000	25	0.1813	1.06	0.91
0.0400		0.2000	25	0.1421	1.05	0.71
0.0100		0.0500	25	0.0431	1.06	0.86
0.0025	MSH	0.0125	50	0.0100	1.04	0.80
0.0400		0.0125	50	0.0086	1.04	0.69
0.0025		0.2000	50	0.1538	1.04	0.77
0.0400		0.2000	50	0.1703	1.05	0.85
0.0100		0.0500	50	0.0366	1.04	0.73
0.0500	ASP	0.0125	50	0.00095	1.06	0.076
0.2000		0.0125	50	0.00107	1.04	0.086
0.0500		0.2000	50	0.01340	1.06	0.067
0.2000		0.2000	50	0.01515	1.05	0.076
0.1000		0.0500	50	0.00416	1.03	0.083
0.0500	AA	0.0125	20	0.00119	1.08	0.095
0.2000		0.0125	20	0.00123	1.07	0.098
0.0500		0.2000	20	0.02065	1.07	0.103
0.2000		0.2000	20	0.02175	1.06	0.109
0.1000		0.0500	20	0.00439	1.07	0.088

[a]In terms of the value of a ratio, i.e., the upper and the lower deviations of observed u are given as u × (1 + Δu) and u ÷ (1 + Δu), respectively.

Table 3—Relative intensity of umami of various flavor amino acids

Substance	Relative intensity of umami a (g/g)[a]	a' (moles/moles)[b]
Monosodium L-glutamate • H_2O	1	1
Monosodium DL-threo-β-hydroxy glutamate • H_2O	0.86 ± 0.06	0.92
Monosodium DL-homocystate • H_2O	0.77 ± 0.04	0.92
Monosodium L-aspartate • H_2O	0.077 ± 0.007	0.071
Monosodium L-α-amino adipate • H_2O	0.098 ± 0.008	0.10
L-Tricholomic acid (erythro form)	5–30[c]	4.3–26
L-Ibotenic acid	5–30[c]	4.2–25

[a]Concentration (g/100ml) ratio of MSG to other flavor amino acid at the point of subjective equality.
[b]Concentration (moles/liter) ratio of the same one as the above.
[c]Terasaki et al., 1965.

Table 4—Relative umami intensity of various flavor nucleotides in the presence of MSG

Conc of MSG u g/100 ml	Test substance	Conc of test substance v' g/100 ml	n	Equi-umami conc of IMP v g/100 ml	Std. dev.[a] (1 + Δv)	a = v/v'
0.100	2-MeO-IMP	0.0020	25	0.0080	1.06	4.00
0.100		0.0005	25	0.0021	1.06	4.20
0.025		0.0020	25	0.0079	1.05	3.95
0.025		0.0005	25	0.0023	1.04	4.60
0.050		0.0010	25	0.0040	1.05	4.00
0.100	N^1-Me-IMP	0.0100	20	0.0076	1.06	0.76
0.100		0.0025	20	0.0016	1.06	0.64
0.025		0.0100	20	0.0078	1.07	0.78
0.025		0.0025	20	0.0017	1.06	0.68
0.050		0.0050	20	0.0041	1.07	0.82
0.100	2-Me-6-MPRP	0.0020	25	0.0163	1.06	8.15
0.100		0.0005	25	0.0040	1.05	8.00
0.025		0.0020	25	0.0155	1.05	7.75
0.025		0.0005	25	0.0043	1.06	8.60
0.050		0.0010	25	0.0075	1.05	7.50

[a]In terms of the value of a ratio; i.e., the upper and the lower deviations of observed v are given as v × (1 + Δv) and v ÷ (1 + Δv), respectively.

phate • H_2O (2-Me-6-MPRP). All the samples (not less than 99% pure) were prepared in the laboratories or the factory of the Ajinomoto Co., Inc.

Sensory test panel and experimental conditions

Panel and experimental conditions were practically the same as reported previously (Yamaguchi, 1967; Yamaguchi et al., 1968a; b). The panel consisted of 100 people screened on the basis of their taste sensitivity from approximately 1,000 employees of the Ajinomoto Co., Inc.

Distilled water was used for preparing the solutions and for oral rinsing. The solutions were presented in 40ml portions in randomly-coded glasses. All the tests were conducted in individual sensory test booths. Temperature and humidity of the rooms were kept constant at 20°C and 60%.

Measurement of the relative taste intensity of flavor amino acids

The relative intensity of umami of various flavor amino acids, as compared with MSG, was measured by a two-part procedure.

(1) A single solution of each test substance was compared with single solutions of MSG. The method used was similar to that described previously (Yamaguchi, 1967; Yamaguchi et al., 1968a; b). A test solution (fixed sample) was paired with a set of MSG solutions (reference samples) consisting of five concentrations. The five concentrations were determined in logarithmically equal steps at 30% intervals. Concentrations of the test samples are shown in Table 1. Five pairs of samples in a randomized order were presented to each panelist. Panelists were asked to indicate which in each pair had a stronger umami. Numbers of replications were from 20–50.

(2) Similar comparisons between the test substances and MSG were conducted in the presence of IMP; i.e., a mixture of each test substance and IMP (fixed sample) was paired with a set of MSG-IMP mixtures (reference samples) which had IMP of the same concentration and constant as the fixed sample, but MSG of five different concentrations. The five concentrations of MSG in a set of reference samples were determined at 30% intervals calculated in terms of y in equation (1), which expresses the relationship between the taste intensity of the MSG-IMP mixture and that of MSG alone. Concentrations of the test samples are shown in Table 2.

In both cases, data were obtained as dichotomous judgments which were then converted into the proportion of judgments that the fixed sample had a stronger umami than a reference. Using the probit method we calculated the concentration of MSG required to attain the equivalent intensity of umami to that of the test substance.

Measurement of the relative intensity of flavoring activities of flavor nucleotides

As reported previously (Yamaguchi, 1967; Yamaguchi et al., 1968a; b), flavor nucleotides seem to give their taste only in the presence of flavor amino acids. Therefore, measurement of the taste intensity of any single solution was not derived, and the taste intensity or the intensity of tasting activities of the test substances was always measured in the presence of MSG.

In the experiment, IMP was selected as a standard substance of flavor nucleotides; i.e.,

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the relative intensity of flavoring activities of various flavor nucleotides, as compared with IMP, was measured. The procedure was similar to that of (2) above with one difference: in this case, MSG-IMP mixtures (reference samples) had MSG of the same concentration but IMP of different concentrations.

RESULTS & DISCUSSION

Taste intensity of various flavor amino acids

The data obtained are shown in Tables 1 and 2. The intensity of umami of all flavor amino acids with which we were concerned in this paper was always proportional to that of MSG, even though the concentration is varied. For example, the concentration ratio of MSG to MSO at the point of subjective equality was 0.85 to 0.89 for each level of fixed sample, and the variation to this extent was not significant (Table 1). From this fact, if u′ g/100ml of a substance is equivalent to u g/100ml of MSG in umami intensity, then $u = \alpha u'$ holds, where α is constant for any given flavor amino acid. The same relationship holds even when the mixture of any flavor amino acid and IMP is compared with that of MSG and IMP (Table 2). Hence, only a single numerical value, α, can adequately represent the relative intensity of umami of any flavor amino acid in this instance. In other words, as regards the intensity of umami u′ g/100ml of flavor amino acid is always replacable with $\alpha u'$ g/100ml of MSG. As described above, the relationship between the taste intensity (umami) of the mixture of MSG and IMP and that of MSG alone is expressed as equation (1). Therefore, by substituting $\alpha u'$ for u in equation (1), the intensity of umami of the mixture of any flavor amino acid and IMP can also be expressed in an equation substantially equal to equation (1) which expresses the synergistic effect between MSG and IMP. Furthermore, the α value was invariant not only for IMP but also for any flavor nucleotides. For example, even when GMP was used instead of IMP in the mixture, the α value for MSO was also 0.86 (Yamaguchi, unpublished data). The α values for various flavor amino acids are summarized in Table 3.

Taste intensity of various flavor nucleotides

The data obtained are shown in Table 4. The concentration of the test nucleotide with a particular MSG concentration was constantly proportional to the concentration of IMP with the same MSG concentration at the point of subjective equality, just as the above-mentioned flavor amino acid was constantly proportional to MSG. Hence, if v′ g/100ml of a substance is equivalent to v g/100ml of IMP, then $v = \beta v'$ holds, where β is constant for any given flavor nucelotide. Only the single numerical value, β, can adequately represent the relative flavoring activity of any flavor nucleotide also in this instance. Therefore, by substituting $\beta v'$ for v in equation (1), the intensity of umami of the mixture of MSG and any flavor nucleotide can also be expressed in an equation substantially equal to equa-

Table 5—Relative intensity of tasting activities of various flavor nucleotides

Substance (dosodium salt)	Relative intensity of tasting activity β (g/g)[a]	β' (moles/moles)[b]
5′-Inosinate · $7.5H_2O$	1	1
5′-Guanylate · $7H_2O$	2.3 ± 0.07[c]	2.3
5′-Xanthylate · $3H_2O$	0.61 ± 0.04	0.53
5′-Adenylate	0.18 ± 0.03	0.13
Deoxy 5′-guanylate · $3H_2O$	0.62 ± 0.07	0.52
2-Methyl-5′-inosinate · $6H_2O$	2.3 ± 0.16[d]	2.2
2-Ethyl-5′-inosinate · $1.5H_2O$	2.3 ± 0.14[d]	2.0
2-Methylthio-5′-inosinate · $6H_2O$	8.0 ± 0.97[d]	8.2
2-Ethylthio-5′-inosinate · $2H_2O$	7.5 ± 0.75[d]	6.9
2-Methoxy-5′-inosinate · H_2O	4.2 ± 0.33	3.5
2-Chloro-5′-inosinate · $1.5H_2O$	3.1 ± 0.25	2.7
2-N-Methyl-5′-guanylate · $5.5H_2O$	2.3 ± 0.15[d]	2.3
2-N-Dimethyl-5′-guanylate · $2.5H_2O$	2.4 ± 0.13[d]	2.2
N^1-Methyl-5′-inosinate · H_2O	0.74 ± 0.09	0.59
N^1-Methyl-5′-guanylate · H_2O	1.3 ± 0.13	1.1
N^1-Methyl-2-methylthio-5′-inosinate	8.4 ± 0.75	7.4
6-Chloropurine riboside 5′-phosphate · H_2O	2.0 ± 0.20	1.6
6-Mercaptopurine riboside 5′-phosphate · $6H_2O$	3.4 ± 0.35	3.3
2-Methyl-6-mercaptopurine riboside 5′-phosphate · H_2O	8.0 ± 0.83	6.7
2-Methylthio-6-mercaptopurine riboside 5′-phosphate · $2.5H_2O$	7.9 ± 0.69	7.5
2′,3′-O-Isopropylidene 5′-inosinate	0.21 ± 0.06	0.16
2′,3′-O-Isopropylidene 5′-guanylate	0.35 ± 0.06	0.28

[a]Concentration (g/100 ml) ratio of IMP to other flavor nucleotide at the point of subjective equality.
[b]Concentration (moles/liter) ratio of the same one as the above.
[c]Yamaguchi et al., 1968a.
[d]Yamaguchi et al., 1968b.

Table 6—Example showing the additive property of taste (umami) intensity among flavor amino acids (probit analysis, n = 25)

Mixture of flavor amino acids				Equivalent conc of MSG	
Substances	MSG +	MSH +	MSO	Calc	Obs
Concentrations (u_ig/100 ml)	0.1	0.1	0.1	0.26	0.25
$\alpha_i$values (from Table 3)	1	0.77	0.86	$\sum_i \alpha_i u_i$	

Table 7—Example showing the additive property of taste intensity among flavor nucleotides (probit analysis, n = 25) in solution of 0.05g/100 ml of MSG

Mixture of flavor nucleotides					Equivalent concentration of IMP	
Substances	AMP[a] +	GMP +	XMP[b] +	2-CH_3S[c] IMP	Calc	Obs
Concentrations (v_jg/100 ml)	0.01	0.001	0.005	0.0005	0.0112	0.012
β_j values (from Table 5)	0.18	2.30	0.61	8.0	$\sum_j \beta_j v_j$	

[a]5′-adenylate
[b]5′-xanthylate · $3H_2O$
[c]2-methylthio-5′-inosinate · $6H_2O$

tion (1). Moreover, the β value was invariant not only for MSG but also for any flavor amino acids; for example, even when MSO was used instead of MSG in the mixtures, the β values for, say, 2-MeO-IMP, was also 4.20 (Yamaguchi, unpublished data). The β values for various flavor nucleotides are summarized in Table 5. Some data previously obtained are also included.

From the results obtained, a universal relationship expressed by equation (1) could be expected for any combination of all flavor amino acids and 5′-nucleotides; i.e., $\alpha u'$ for a particular concentration of any flavor amino acid can be calibrated as u for a particular amino acid, say, MSG. Also, a similar conversion from $\beta v'$ to v of particular nucleotides, say, IMP can be made. Since an additive property of taste was recognized among flavor amino acids and flavor nucleotides, (as shown in Tables 6 and 7), in the case of the mixtures of two or more different flavor amino acids and two or more diffent flavor nucleotides, u and v in equation (1) must be calculated as product sums of the concentration of each flavor amino acid and its α value in Table 3, $\sum_i \alpha_i u_i$, and of the concentration of each flavor nucleotide and its β value in Table 5, $\sum_j \beta_j v_j$ respectively.

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REACTION AT LIMITED WATER CONCENTRATION. 2. Chlorophyll Degradation

SUMMARY—Chlorophyll degradation was studied as a function of water activity (A_W) in freeze-dried blanched spinach puree and in model systems containing cellulose-citrate buffer and chlorophyll a. At 37°C and A_W higher than 0.32, the most important mechanism of chlorophyll degradation is conversion to pheophytin. The rate of chlorophyll a transformation is 2.5 times faster than for chlorophyll b. The relation between A_W and log time for 20% chlorophyll degradation is linear and the chlorophyll/pheophytin ratio is useful for storage prediction as a function of A_W (down to A_W 0.32). At A_W lower than 0.32, the rate of pheophytin formation in spinach is low. New products are formed which can interfere with spectrophotometric determination of chlorophylls and pheophytins. The possibility of removal of Mg from the porphyrin ring of chlorophyll a was shown in model systems even at A_W below BET (Brunnauer-Emmet-Teller) monolayer coverage.

INTRODUCTION

IN DEHYDRATED FOODS, deteriorative reactions depend on the state of water present. At high water activities, enzymatic reactions and growth of microorganisms take place (Acker, 1969). Reactions such as nonenzymatic browning and lipid oxidation have their rates controlled by the water activity in the system (Labuza et al., 1970) and, even at water content below BET (Brunnauer-Emmet-Teller) monolayer coverage, model systems containing sucrose and organic acids undergo browning due to sucrose acid hydrolysis (Schoebel et al., 1969).

It is known that hydrogen ions can transform chlorophylls into corresponding pheophytins by replacing the Mg atom in the porphyrin ring. In solution, the reaction is first order with respect to chlorophyll concentration (Schanderl et al., 1962). Pheophytinization of chlorophylls causes discoloration in vegetable foods and may occur under many processing and storage conditions (Clydesdale et al., 1970; Buckle and Edwards, 1970) even when the water activity has been lowered by freezing (Olson, 1968). In freeze-dried blanched beans (3.5% moisture), Hamed (1967) found that storage under air in the presence of desiccant increased chlorophyll retention by 50%. However, his results did not indicate a parallel increase in the pheophytin content. Under nitrogen, the presence of water did not show much influence. In dried spinach with 18% moisture, Dutton et al. (1943) found pheophytinization of chlorophyll which did not seem to occur at 2.5% moisture. The type of storage atmosphere did not seem to influence the reaction. Bottrill and Hawker (1970) found that drying of sultana grapes in the dark causes a decrease in chlorophyll a and b, but the level of pheophytin a was constant and no pheophytin b was found, although the grapes were acidic.

Mechanisms other than pheophytinization can cause chlorophyll degradation in foods. The action of chlorophyllase and acid can lead to pheophorbide formation (White et al., 1963). Allomerized chlorophylls can be formed by oxidation at C_{10} on the isocyclic ring. Compounds without the characteristic chlorophyll spectra may be formed under lipoxidase activity and lipid oxidation (Holden, 1965; Buckle and Edwards, 1970). Walker (1964) showed that under anaerobic conditions pheophytinization of chlorophyll can also be related to lipid oxidation in beans.

The purpose of our work was to study the influence of water activity on chlorophyll degradation, since this was not considered in the above studies. Blanched, freeze-dried spinach was stored at 0.75, 0.62, 0.52, 0.32, 0.11 and $\simeq < 0.01$ A_W under air or nitrogen at 37 and 55°C; chlorophylls a and b were determined at various times. Studies were also conducted with pure chlorophyll a in a model system containing cellulose:citrate buffer:inert oil (Apiezon) with and without addition of oxidizable substrate (methyl linoleate).

MATERIALS & METHODS

Spinach system

Fresh, commercially available spinach was trimmed, washed, blanched 1 min in boiling water. The blanched spinach was comminuted in a blender to a puree, transferred to 50-ml flasks, frozen in liquid nitrogen and freeze dried. The flasks were then exposed for 24 hr to various relative humidities (saturated salt solutions; Rockland, 1960), after which they were capped, stored under nitrogen or air at the desired temperature (37 or 55°C) in the dark. Moisture content was determined for each relative humidity by weight increase of the bone-dry material, and is shown for each condition in Table 1.

Model system

Chlorophyll was dissolved in Apiezon oil and added to microcrystalline cellulose in a ratio of 1:6. To seven parts of this mixture was added 20 parts of 0.1 M citrate buffer, pH 5. The whole was mixed in a Sorvall Omnimixer for 20 min, with the mixing cup packed in ice. In one system 0.8 part methyl linoleate was added at the same time as the Apiezon oil. After being mixed, the samples were freeze dried and stored under air or nitrogen at 0 and 75% RH, in the dark, at 55°C.

Preparation of chlorophyll A

Fresh spinach leaves were placed in boiling water for 1 min, extracted in a blender either with acetone or methanol:petroleum ether and filtered under vacuum. $MgCO_3$ was added to reduce pheophytin formation. The pigments were transferred from the initial solvent to petroleum ether by adding water and 5% Na_2SO_4 solution and chromatographed on a dry packed sugar: 3% starch column after extraction of xanthophylls, according to Strain and Suec (1966). The column was developed by petroleum ether: 5% methanol under slight vacuum. The chlorophyll a band was cut out and rechromatographed by the same method. Finally, the pigment was eluted with acetone, transferred to petroleum ether, washed several times with water, treated with anhydrous Na_2SO_4 and evaporated to dryness under vacuum. The purity of the pigment was checked by thin-layer chromatography (method described below) and agreed with McKinney's (1940) criteria for pure chlorophyll a preparation.

Pigment analysis

Spinach system. Portions (70–90 mg) of the stored samples were added to $MgCO_3$ and extracted twice with 40 ml of 80% acetone solution for 15 min in stoppered flasks. After the extract was filtered under vacuum and the residue washed, the volume of filtrate was made up to 100 ml. From this solution, 10-ml aliquots were treated with 0.3 ml of saturated oxalic acid in 80% acetone solution to transform chlorophylls to pheophytins. After 3 hr, readings of the original and treated solutions were taken in a Hitachi-Perkin Elmer spectrophotometer at 645, 662, 655, 666 and 700 nm. The difference of the readings at 645 and 662, before and after acid treatment, was used to determine chlorophyll a and b concentration; absorbancies at 655 and 666 were used to calculate total pheophytin concentration according to Vernon's equations (1960).

The readings at 700 nm were used to correct light scattering of the solutions. Readings at

Table 1—Water sorption for freeze-dried spinach at 37°C

RH (%)	Moisture content (g H_2O/100g solids)
11	2.65
32	5.38
52	8.41
62	10.80
75	15.49

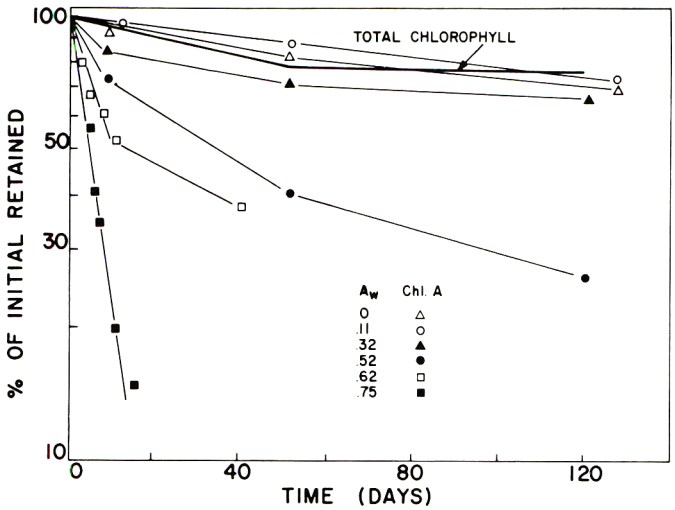

Fig. 1–Degradation of chlorophyll in spinach (37°C, air atmosphere). Total chlorophyll curve is average for all storage conditions.

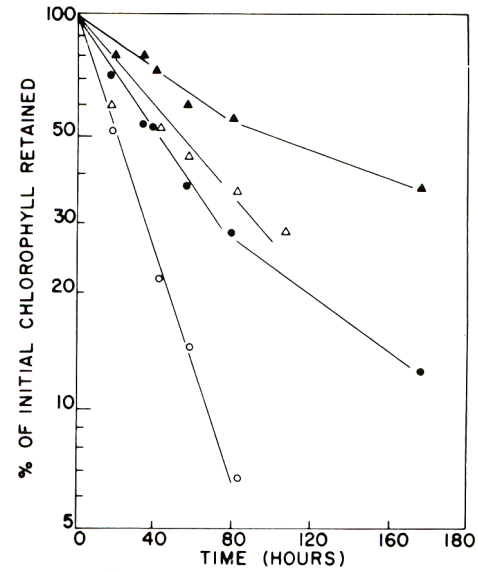

Fig. 2–Degradation of chlorophylls a and b in spinach (55°C, air atmosphere).
Chlorophyll a: △ 62%RH; ○ 75%RH
Chlorophyll b: ▲ 62% RH; ● 75% RH

558 nm (isobestic point) were also taken to check the composition of the samples.

Total chlorophyll was the sum of the respective chlorophyll concentration and the pheophytin concentration and thus shows the amount of product converted to nonchlorophyll-absorbing products by difference.

Model system. Analyses were made in pure acetone because of the low solubility of Apiezon oil in 80% acetone (used for spinach system).

The total content of the stored flasks (500 mg) was extracted twice with 20 ml of acetone, filtered, the filter washed to elute the pigment adsorbed on the paper and the volume made up to 50 ml. Appropriate dilutions were used when necessary.

Absorbancies of this solution at 662 and 410 nm corrected from readings at 700 nm were used to calculate chlorophyll a and pheophytin a concentration using the following equation:

$$\text{Chlorophyll } a \ \left(\frac{\text{mg}}{\text{liter}}\right) = \frac{A_{410} - 0.39\, A_{662}}{62.2}$$

$$\text{Pheophytin } a \ \left(\frac{\text{mg}}{\text{liter}}\right) = \frac{A_{410} - 0.75 \text{ chlorophyll } a}{131}$$

Using the extinction coefficient of chlorophyll a = 92.6 cm · liter · g^{-2} (Vernon, 1960), the above equations were used to calculate the appropriate coefficients at other wavelengths for chlorophyll a and pheophytin a.

Thin-layer chromatography. The pigments present were followed by TLC on Whatman CE 41 cellulose powder, 0.35-mm thick, dried 1 hr at 105°C, stored in a desiccator and activated 30 min before use. The developing solvents were acetone:hexane:n-propanol (90:10:0.45) or hexane:acetone (80:20) according to Bacon and Holden (1965).

RESULTS & DISCUSSION

1. Spinach

Water activity showed a definite influence on the rate of degradation of chlorophyll in dried spinach. The color of samples stored at 37 and 55°C showed a rapid change of color from bright-green to olive-brown, characteristic of the pheophytins. The visible absorption spectra of stored samples also showed changes in maxima corresponding to pheophytin formation. No other changes in absorbance occurred, which indicates a lack of significant amounts of other chlorophyll-derived pigments. These results were in turn confirmed by chromatographic analysis of extracted pigments on cellulose layers with a number of solvent systems after up to 40 days of storage. This confirms the earlier results of the investigations of Dutton et al. (1943) on total chlorophyll content of dried spinach. These results showed that even down to very low moisture contents where water is bound tightly, this water is available for acid-catalyzed reactions, as has been previously indicated for sucrose hydrolysis (Schoebel et al., 1969).

The complex kinetics of chlorophyll a degradation at 37°C are shown for the entire range of A_w investigated in Figure 1. The kinetics of degradation of both chlorophylls a and b at 55°C for the high range of A_w are shown in Figure 2. Under all conditions studied, only the degradation of chlorophyll a at A_w 0.75 showed simple pseudo-first-order kinetics over the entire range of concentration. This may indicate a compartmentalization of the chlorophylls at lower water activities, with the more firmly bound pigment being more resistant to conversion to pheophytin. On the other hand, other complex changes are also taking place in the spinach during storage, such as nonenzymatic browning, loss of volatile compounds and formation of organic acids. All of these tend to cause a change in the hydrogen ion activity of the system, with a shift toward more acid pH, as shown in Table 2. Although there is apparently no simple relationship between change in pH and storage temperature and water activity, these fluctuations undoubtedly influence the degradation kinetics of the chlorophylls.

The first-order rate constants for the initial degradation of chlorophylls a and b at 37 and 55°C and the calculated activation energies are shown in Table 3. The reaction is 2.5 to 3 times faster for chlorophyll a than for chlorophyll b, which is somewhat smaller than the fac-

Table 2–Change in pH of spinach during storage[a]

A_w	Storage time	Final pH (°C) 37	55
0.75	15 Days	6.00	–
	82 Hours	–	5.70
0.62	40 Days	6.55	–
	80 Hours	–	6.25
0.52	120 Days	5.90	–
0.31	120 Days	6.30	–
0.11	128 Days	6.40	6.00
Dry	128 Days	5.80	5.40

[a]Initial pH 6.70.

Table 3–Influence of A_w on rates of chlorophyll degradation

	Rate constant $k \times 10^{-2}$ (hr^{-1})				Activation energy E_a (kcal/g mole)	
	Chlorophyll a		Chlorophyll b			
A_w	37°C	55°C	37°C	55°C	Chlorophyll a	Chlorophyll b
0.75	2.74	14.7	0.92	5.4	19	20
0.62	1.12	7.2	0.42	3.3	21	23

Table 4–Effect of storage atmosphere on retention of chlorophyll and pheophytin at 55°C

		Chlorophyll[a]		Total pheophytin[b]	
A_w	Days	Air	N_2	Air	N_2
.11	0	100	100	100	100
	12	90.8	86.0	86.0	85.0
	40	61.7	60.3	66.4	63.4
	51	56.2	58.8	59.0	66.4
< 0.01	17	92.4	89.2	91.3	90.0
	40	54.5	54.1	54.0	65.8
	51	--	51.6	--	66.0

[a]Percent of initial.
[b]After acid treatment.

tor of 5.5 previously reported for acetone-water systems (Schanderl et al., 1962), and close to the factor of 2 reported by Gupte et al. (1964) for thermal processing of spinach. Since the activation energies are measured over only two temperatures, it is not possible to determine if the differences at different A_w are truly significant.

In samples stored at A_w less than 0.32, the conversion of chlorophyll to pheophytin is very slow. This cannot be ascribed primarily to a change in the pH of the system, because very low pH values were found for some of the driest samples. What is more likely is that at low activities the chlorophylls are strongly bound in nonreactive compartments, or that water is not available for the reaction to form pheophytins. What seems more significant is the indication from Figure 1 that the apparent loss of chlorophyll at low A_w is paralleled by a concomitant loss of total chlorophylls and pheophytins.

This latter effect is demonstrated even more strongly at 55°C and is not significantly influenced by storage atmosphere (Table 4). Although bleaching of the pigments to colorless products might have occurred, at least part of the effect seems to be due to an artifact of the spectrophotometric method. The absorption spectra of samples stored at low A_w and 55°C showed a spectral shift corresponding to pheophytin formation (to 410 and 667 nm). TLC also showed an increase in pheophytin in relation to other pigments, but new pigments were found and represented (after 50 days of storage) 20–30% of the total pigments present. In the sample stored at 37°C, this represented 10–15% of the total after 120 days of storage. In addition to pheophytin a and b, two more pheophytin-like spots were found, moving above and below the lutein zone. Chlorophyll a′ appeared just ahead of chlorophyll a and another blue-green pigment was seen just below chlorophyll b. Some of these new pigments were isolated and their UV-visible spectra recorded. They appear to have spectral characteristics similar to the modified chlorophylls isolated from processed pea puree by Buckle and Edwards (1969) and from legume seeds (Holden, 1965), but sufficient material could not be prepared for further characterization.

The storage life of dehydrated green vegetables is determined to a great extent by the rate of loss of chlorophyll under conditions of storage. If we assume that the product would be acceptable when 20% of the chlorophyll was converted to pheophytin (concentration at which gray color begins to predominate), the storage life of the product can then be determined as a function of A_w. The locus of points describing the acceptable storage life for freeze-dried spinach as a function of A_w is shown in Figure 3. This relation is valid only at A_w greater than 0.32, because at lower A_w the mechanism of deterioration changes, and the reaction-limiting storage life is no longer the conversion of chlorophyll to pheophytin. The chlorophyll/pheophytin ratio which is often used to assess quality would also not be a valid indicator for green vegetables stored at very low A_w.

In light of the difficulties of interpretation of pigment changes at low A_w in spinach, the investigations with model systems containing pure chlorophyll a were undertaken and are discussed in the following section.

2. Model systems

The most striking difference between spinach and the chlorophyll-cellulose model system is the latter's extremely rapid rate of pheophytin formation at all water activities. At A_w 0.75 and at 37°C, essentially all chlorophyll had reacted in 15 hr. As shown in Figure 4, and in contrast to spinach, even under dry conditions, the rate of formation of pheophytin was high; in addition the reaction was accelerated by conditions conducive to oxidation.

This observation again indicates that the location and form of chlorophyll in spinach must determine reactivity, and that even after freezing and drying there is some degree of protection due to association in a lipoprotein complex remaining from the original chloroplast structure. It is difficult to explain the effect of oxygen and oxidizing methyl linoleate in accelerating removal of Mg from chlorophyll, but both the accelerative effect as well as a bleaching reaction are known to be of significance in a number of natural systems containing unsaturated lipid and lipoxidase (Buckle and Edwards, 1970). In addition, Walker (1964) has shown similar effects in systems containing oxidized lipid resulting from nonenzymatic processes. If the influence of peroxides arises via formation

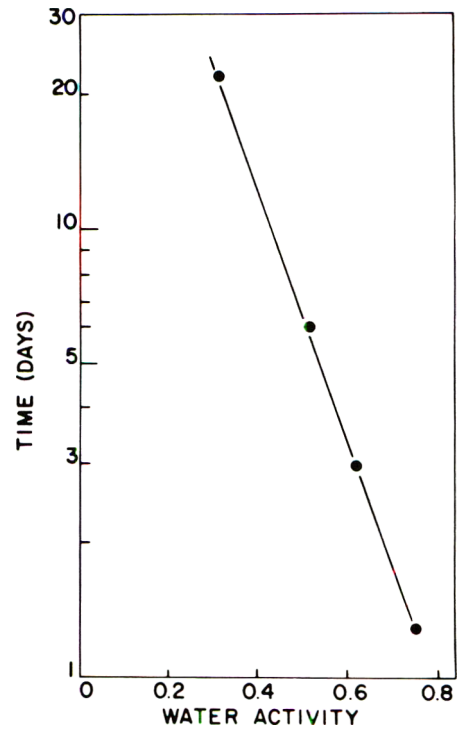

Fig. 3–Time required for 20% loss of chlorophyll in spinach at different water activities (37°C, air atmosphere).

Table 5–Characteristics of new pigments formed from chlorophyll at low water content

Model system					Spinach				
		Principal[b]					Principal[b]		
Zone	R_f[a]	Absorption maxima		Ratio	Zone	R_f[a]	Absorption maxima		Ratio[c]
1	.88	665.3	409.5	2.22	A	.93	666.0	410.0	2.21
2	.68	666.5	409.7	2.37	B	.87	666.5	410.0	2.41
3	.55	665.2	401.1	2.71	C	.74	666.0	410.0	2.83
4	.16	660.2	407.7	2.47	D	.67	665.5	402.0	3.14
5	.09	661.7	407.5	2.60	E	.0	662.0	400.0	3.30
6	.00	662.2	408.6	2.02					

[a]R_f on cellulose.
[b]Principal peaks in acetone solution.
[c]Ratio of absorbance red/blue.

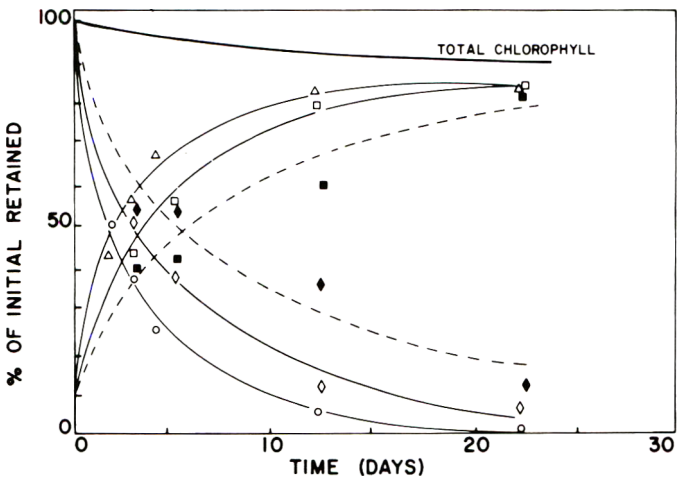

Fig. 4–Degradation of chlorophyll in a cellulose model system under conditions of bone-dry atmosphere (55°C, variable atmosphere).

Storage conditions:

Chlorophyll a: ◇ air; ◆ nitrogen; ⊙ air with methyl linoleate.

Pheophytin a: □ air; ■ nitrogen; △ air with methyl linoleate.

of a chlorophyll radical intermediate, it might be possible for oxygen alone to exert a similar action.

As can be seen in Figure 4, there appears to be a decrease in total chlorophyll and pheophytin under all conditions of storage, but chromatography of the extracted pigments indicates that as in spinach this may be an artifact. After 15 days of storage, new gray pheophytin-like bands appears in TLC. The new pigments were recovered and their spectra recorded and compared with similar bands found in spinach stored at 55°C and low A_w. Absorption maxima and chromatographic behavior of these pigments are given in Table 5. In both cases, at least part of the apparent bleaching could be explained on the basis of formation of pigments having shifted absorption maxima and different red/blue absorbance ratios. Although some of these pigments migrate on cellulose more slowly than the pheophytins, they do not spectrally resemble oxidation products of the purpurin type which have maxima at 680 nm. They are more similar to the pigments produced by alkaline treatment of peas (Buckle and Edwards, 1969). Since sufficient material could be recovered to record only visible spectra, no further identification was possible.

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PATTERNS OF SOLUBLE PEROXIDASE IN RIPENING BANANA FRUIT

SUMMARY—Total peroxidase activity and the spectrum of isoperoxidases solubilized from the pulp of banana fruit were relatively constant when the extraction procedure was most efficient. When casein dispersion was present during extraction of green fruit, high levels of activity were detectable. Other scavengers of condensed polyphenols were less effective in yielding activity in the soluble and particulate fractions. When collagen fibers were included with buffer during tissue maceration, no peroxidase was solubilized at all stages of ripening; however, the particulate fraction contained very high levels of peroxidase. Previous reports showing a concomitant change in peroxidase activity with the respiratory upsurge of climacteric fruit may be anomalous because of differential phenol inhibition.

INTRODUCTION

PEROXIDASES catalyze the oxidation of substrates such as phenols, aromatic-, primary-, secondary- and tertiary-amines, leuco-dyes, ascorbic acid and certain heterocyclic compounds such as indoles in the presence of H_2O_2. The enzyme appears to be ubiquitous to the living state, yet its physiological role(s) are ill-understood. Peroxidases have been implicated in ethylene biogenesis (Goldacre et al., 1953; Mapson and Wardale, 1968; Yang, 1967; Mattoo and Modi, 1969); hormone balance (Gortner and Kent, 1958; Schneider, 1970); membrane integrity (Dilley, 1970); and respiration control (Aylward and Haisman, 1969) and accordingly are implicated in plant senescence and physiological breakdown of fruits and vegetables. Recent investigations demonstrated that peroxidase activity increased with ripening of mango (Mattoo and Modi, 1969); grape (Ivanova and Ivanova, 1968); apple (Kuhive, 1969) and pear fruit (Ranadive and Haard, 1971). Changes in the patterns of peroxidase isozymes have also been shown to occur with the climacteric rise of pear (Frenkel, 1971; Ranadive and Haard, 1971) and tomato (Frenkel, 1971).

Here we show that peroxidase solubilized from the pulp of ripening banana fruit at low ionic strength remained relatively constant throughout ripening when appropriate scavengers of condensed polyphenols were incorporated into the assay medium.

MATERIALS & METHODS

Fruit ripening

Green (No. 2), preclimacteric banana fruit were generously supplied by the United Fruit Co. approximately 7–10 days postharvest under 13°C storage. Fruit were ripened at 20°C, 85–90% relative humidity in a ventilated incubator after exposure to 100 ppm ethylene for 2 hr. Fruit respiration was monitored by trapping carbon dioxide in 0.1N NaOH and estimating bicarbonate ion by a double end point titration (Haard, 1967).

Preparation of enzyme extracts

Banana pulp (20g) was prepared for extraction by one of the following methods: (a) 1 mm slices were frozen in liquid N_2 and powdered in a chilled blender; (b) 1 mm slices were frozen in liquid N_2, lyophilized and powdered in a blender; (c) preparation of an acetone powder (Nason, 1955); (d) extraction of 1 mm pulp slices after vacuum infiltration with isolation medium by intermittent evacuation and flushing in a desiccator attached to a water aspirator; and (e) extracting pulp directly by maceration in isolation medium. In all cases, the pulp was macerated in a Servall Omni-mixer blender for 1 min at 15 sec intervals of mixing and rest. All operations were performed at 4°C.

The only parameter of the extraction media varied in this study was the tannin scavengers. The basic extraction medium contained sodium phosphate (0.5 mM, pH 7.0) in 50 ml of distilled water. The following tannin complexers were incorporated into the medium at the indicated levels: polyethylene glycol carbowax 400 (0.2, 1.0, 2.0g); polyvinylpyrrolidone (Polyclar, GAF Corp., 0.2, 1.0, 2.0g); collagen dispersion (Hochstadt and Lieberman, 1960, 0.2, 1.0, 2.0g); and purified casein dispersion (Haard and Hultin, 1968, 0.2, 1.0, 2.0g). Collagen dispersion was dialyzed against 1000 volumes of 0.01M Tris for 3 days at 4°C prior to use.

Pulp slurries were immediately filtered through 4 layers of cheese cloth centrifuged for 15 min, 60,000 × G at 0°C. The resulting supernatants and pellets were retained at 4°C until assayed for peroxidase or electrophoresced.

Assay for peroxidase

Aliquots (0.010–1.0 ml) of extract were assayed for peroxidase at pH 7.1 in 0.03 mM sodium phosphate with o-dianisidine as H-donor and 0.001% H_2O_2 as oxidant. The oxidation of o-dianisidine was followed with a DU Beckman spectrophotometer at 460 mμ and 21°C (Guilbault, 1970).

Extracts were subjected to electrophoretic analysis on polyacrylamide gel with Tris (hydroxymethyl aminomethane)-barbital-sodium barbitol buffer (pH 8.8, 0.05 ionic strength) for 45 min. Gels were prepared from the following stock solutions: A (acrylamide, 30g; bisacrylamide, 0.8g; H_2O to 100 ml); B (Tris, 18.15g; 1N HCl, 12 ml; N,N,N'-tetramethylene-ethylenediamine 0.24 ml and H_2O to 100 ml); C (ammonium persulfate, 0.14g and H_2O to 100 ml). Stock solutions A, B and C were combined 1:1:2 (v/v/v) at approximately 25°C and pipetted into glass tubes (0.4 mm ID × 7.5 mm long). Electrophoresis was performed on a Buchler polyanalyst assembly at 4 milliamps per tube with cooling to approximately 10°C. Gels were incubated in the o-dianisidine-H_2O_2 assay solution for approximately 2 hr until all zones were visualized.

Centrifuge sediments of tissue macerates were incubated with the o-dianisidine-H_2O_2 medium and observed at 60X magnification with a binocular microscope to obtain a crude estimate of peroxidase activity in the pellets.

Protein was determined by a Biuret method described by Bailey (1967).

RESULTS & DISCUSSION

Fruit ripening

Fruit underwent a respiratory upsurge and ripened approximately 6 days after being gassed with ppm levels of ethylene and stored at 20°C and 85–90% relative humidity. The CO_2 production and peel color of ripening fruit are shown in Figure 1a. In later experiments, the peel color alone was used as an index to the ontogenic stage. Fruit which failed to ripen normally (such as those slightly chilled or desiccated) were not used in these studies since peroxidase activation has been associated with physical stress (McCown et al., 1969; Monseliser and Kahan, 1968).

Tissue maceration

Pulp samples (20g) were taken from the inner two-thirds of the banana length on same fruit for comparison studies. The total activity of peroxidase extracted from lyophilized powder, liquid N_2 powder, acetone powder, infiltrated pulp which was Omnimix blended and Omnimix-blended fresh pulp are shown in Table 1. All of these preparations contained excess casein (1g dry weight) in the isolation medium. Lyophilization of pulp prior to extraction and acetone powder had markedly lower levels of extractable peroxidase in both preclimacteric and postclimacteric fruit. Extraction of liquid N_2 powders from green and ripe pulp was effective although total activity was consistently lower than fresh pulp extraction. Previous study showed that the liquid N_2 powder was most effective

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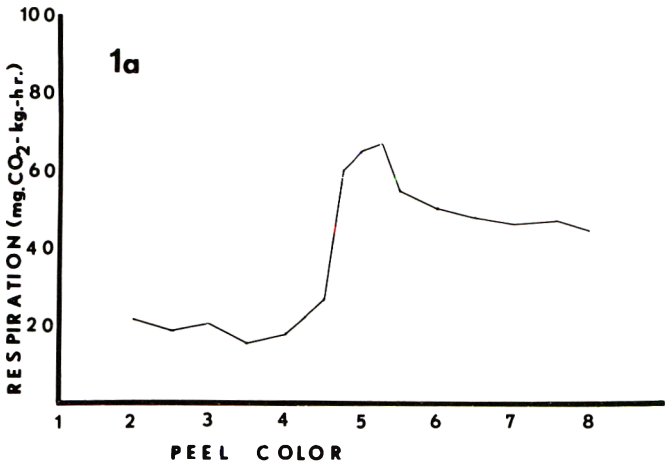

Fig. 1a—Carbon dioxide evolved from ripening banana fruit as a function of peel color: No. 2—solid green; No. 4—yellow flush; No. 6—full yellow; No. 8—speckled brown (color scale of von Loesecke, 1950).

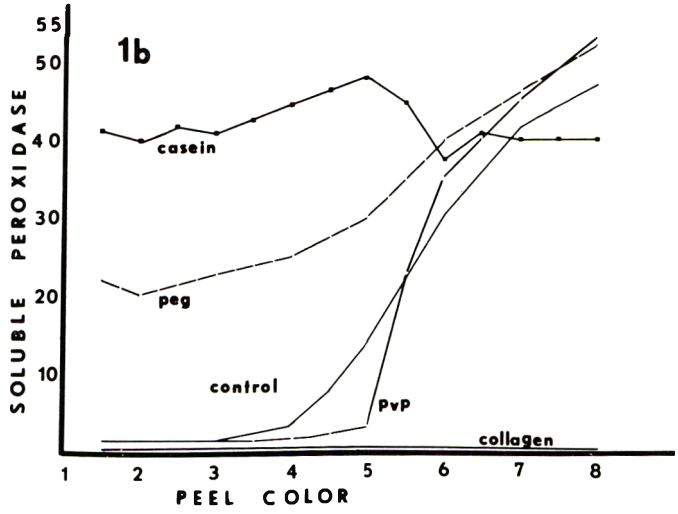

Fig. 1b—Peroxidase activity in supernatants of isolates as a function of peel color. Control was extracted with sodium phosphate (0.1M, pH 7.0); other samples contained 1g of the indicated scavenger of condensed polyphenols in the phosphate isolation medium. (Peg = polyethylene glycol; PVP = polyvinylpyrrolidone.) Peroxidase activity represents absorbancy change at 460 mμ for the total extract in 1 min at 21°C.

in isolating active mitochondria from banana fruit (Haard and Hultin, 1968). The lesser efficiency observed here may be due to lability of peroxidase to such treatment or to concomitant protection to peroxidase inhibitor such as that reported by Mattoo and Modi (1969) in mango fruit. Because of casein retention in the final preparation, meaningful indices of extractable pulp protein could not be obtained. When ripe fruit were extracted with an isolation medium devoid in casein, similar levels of peroxidase were observed as with casein (Table 1) and a measure of pulp extractable protein was obtained (Table 2). These results would suggest that the lyophilization and acetone treatments were specifically detrimental to peroxidase and did not decrease the efficiency of protein extraction generally.

Cytochemical assay of centrifuge pellet fractions which resulted from the above preparations (60,000 × G, 15 min) demonstrated measurable activity which was roughly parallel to that observed in the supernatant fraction. This supports the interpretataion that efficiency of extraction was not related to resulting localization in the particulate fraction but to minimization of peroxidase inactivation by condensed polyphenols.

Tannin scavengers in isolation media

The results reported in Tables 1 and 2 were obtained from preparations utilizing casein in the isolation medium as a tannin scavenger. Casein was previously reported to be a highly effective tannin scavenger for isolating mitochondria from banana fruit (Haard, 1967) when compared with other inert proteins such as bovine serum albumin, egg albumin, gelatin and other agents which prevent tannin inhibition of enzymes, notably polyvinylpyrrolidone, polyethylene glycol and caffeine. The effect of incorporating casein, polyethylene glycol (carbowax 400), polyvinylpyrrolidone (Polyclar, GAF Corp.), and collagen fibers into an isolation medium containing sodium phosphate (0.5 mM, 50 ml, pH 7.0) is summarized in Fig. 1b. When no tannin complexer was present

Table 1—Influence of pulp pretreatment on extractable peroxidase activity[a]

Treatment	Preclimacteric (no. 2) basic isolation medium[b]	Preclimacteric (no. 2) casein free isolation medium[c]	Postclimacteric (no. 6) basic isolation medium[b]	Postclimacteric (no. 6) casein free isolation medium[c]
Fresh pulp	44	~0	50	48
Infiltrated pulp	45	~0	48	48
Liquid N_2 powder	36	~0	38	31
Acetone powder	10	~0	8	11
Lyophilized powder	3	~0	4	2

[a]Total absorbancy change at 460 mμ in 1 min with o-dianisidine as H donor.
[b]Pulp was extracted with basic isolation medium containing 0.1M phosphate buffer (50 ml) and casein (1g). Results are average of four experiments.
[c]Pulp was extracted with 0.1M phosphate buffer (50 ml). Results are average of two experiments.

Table 2—Influence of pulp treatment on extractable protein from ripe fruit

Treatment	Protein in extract (mg)[a]	Peroxidase[b]
Fresh pulp	60	48
Infiltrated	65	51
Liquid N_2 powder	62	44
Acetone powder	48	18
Lyophilized powder	58	2

[a]Protein determined by Biuret method (Bailey, 1967). Results are an average of two experiments. Negligible protein was present in extracts from unripe (no. 2) banana fruit pulp because of co-precipitation with condensed polyphenols.
[b]Total absorbancy change at 460 mμ in 1 min for entire supernatant. Results are an average of two experiments.

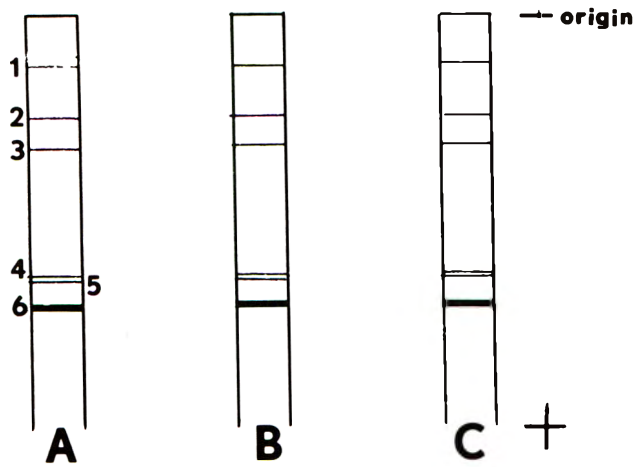

Fig. 2–Diagrams showing patterns of isoperoxidases as separated by polyacrylamide gel electrophoresis. Peroxidases were extracted from A (preclimacteric fruit); B (climacteric fruit); and C (postclimacteric fruit) with casein isolation medium.

Table 3–Relative extent of dye oxidation by particulate fractions[a]

Tannin Scavenger[b]	Relative peroxidase activity in pellets	
	Preclimacteric	Climacteric
–	–	++
PVP	–	++
PEG	++	++
Casein	++	++
Collagen	++++++	++++++

[a]Pellet fluff was incubated with excess o-dianisidine-H_2O_2 assay medium (see Experimental).

[b]Isolation medium containing 50 ml phosphate buffer (100 mM, pH 7.1) with the indicated tannin scavenger (1g).

during extraction, unripe fruit showed negligible peroxidase activity in the supernatant fraction until the fruit began to turn when peroxidase activity surged. The pattern of activity was nearly parallel to that of respiration during the climacteric. When casein was incorporated into the extraction media, the level of solubilized peroxidase was relatively constant throughout ripening and optimal at all stages except late postclimacteric when slightly higher peroxidase activity was observed with extracts devoid of casein as well as those containing polyethyleneglycol or polyvinylpyrrolidone. Peroxidase of extracts prepared with casein showed a slight, but consistent rise prior to the climacteric followed by a decline postclimacteric. The casein dispersion used in these experiments showed no measurable peroxidase activity when assayed alone.

When collagen was introduced as a tannin scavenger and pulp was macerated identically to the other preparations, no peroxidase activity was observed in the supernatant at all stages of fruit studied.

Polyvinylpyrrolidone was found to be least effective in facilitating peroxidase solubilization from preclimacteric fruit; however, optimal activity was present in preparations from postclimacteric fruit. Introduction of polyethylene glycol into the isolation medium resulted in preparations with intermediate activity preclimacteric and activity similar to PVP preparations postclimacteric. All extracts showed similar peroxidase activity at about the climacteric although differing pre- and postclimacteric. Polyethylene glycol (Badran and Jones, 1965) and polyvinylpyrrolidone (Hulme and Jones, 1963) have been found effective in preventing tannin inhibition of various enzymes in fruit tissue.

Interpretation of these results is complicated by the interplay of several factors which may be variant throughout ripening. The observed patterns of soluble peroxidase activity are certainly influenced by tannin (Goldstein and Swain, 1963) inactivation of the enzyme during extraction of unripe fruit. The influence of other factors such as in situ localization or binding at particulate loci, synthesis or activation of peroxidase with ripening, or the disappearance of a native inhibitor of peroxidase with ripening cannot be judged at this time.

We have examined the levels of peroxidase in the particulate fraction of tissue homogenates and the distribution of isoperoxidases during ripening to provide a better understanding of these different patterns of soluble activity.

Cytochemical analysis of centrifugal pellets

The homogenate sedimetable at 60,000 x G in 15 min were washed with isolation buffer, recentrifuged at 60,000 x G for 15 min and aliquots of pellet dispersed directly in o-dianisidine assay solution in a Petri dish. In all cases, only the lightest or fluff fraction of the pellets contained measurable activity and this was routinely separated from the heavy fraction which was predominantly starch. Table 3 shows the relative extent of dye oxidation by particulate fractions prepared with various tannin complexers. Clearly, peroxidase activity was lost from green fruit when no tannin scavenger was present. Similar levels of dye oxidation were retained by the particulate fractions when PEG or casein were present during extraction. It was particularly interesting that pulp homogenized with collagen, which contained no activity in the supernatant, contained markedly higher levels of peroxidase in the particulate fraction. While it is known that collagen tends to bind enzymes (Lieberman, personal communication), study of these homogenates with a binocular microscope clearly showed that the observed activity was not associated with collagen fibers but was associated with cell wall and smaller particles of disrupted cells. Preliminary results have shown that collagen-promoted binding of peroxidase to cell particulate matter was readily reversed by washing homogenates with gross levels of $CaCl_2$ (2M). It would appear that collagen acted to lower ionic strength during cellular disruption, perhaps with respect to specific ions; although further study will be necessary to conclude on this point.

Polyacrylamide gel electrophoresis of pulp extracts

Pulp extracts separated into six species of peroxidase on polyacrylamide gel (Fig. 2). In all cases the fastest moving, band six, was overwhelmingly predominant such that the other five zones were not readily visualized. The relative distribution of these isozymes was constant when the experimental procedures for pulp extraction were varied as described in this communication. Accordingly, there appeared to be no differential response of isozymes to tannin inhibition or to be a major change of these peroxidase species with ripening.

Species one to three were slightly more intense in extracts from ripe fruit than those from green fruit although species six was predominant at all stages. Other reports have noted changes in distribution of isoperoxidases with ripening of pears (Ranadive and Haard, 1971); mangoes (Mattoo and Modi, 1969); apples (Kuhive, 1969); and grapes (Ivanova and Ivanova, 1968).

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ANALYSIS OF THE PUNGENT PRINCIPLES OF Capsicum annuum BY COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

SUMMARY—The pungent principles of Capsicum annuum *includes at least five closely related compounds. Four of these pungent compounds—capsaicin, dihydrocapsaicin, nordihydrocapsaicin and homodihydrocapsaicin—were separated by gas chromatography with a 3% SE-30 column and identified by combined gas chromatography-mass spectrometry. Separation of these compounds into oleoresin capsicum by gas chromatography is also reported.*

INTRODUCTION

FRUITS OF *Capsicum annuum* or oleoresin capsicum are widely used in Japan for their marked pungency, notable as a crude drug, a traditional spice or as a food additive.

Early work on identification of the pungent principles of *Capsicum annuum* included such conventional means as infrared or ultraviolet examination (Kosuge et al., 1958a), thin-layer chromatography (Rangoonwala, 1969) and paper chromatography (Kosuge et al., 1959).

In 1968, Benett and Kirby reported that capsaicin, the pungent principle of *capsicum annuum*, was found to be a mixture of five closely related compounds, which were identified by mass spectrometric studies.

Analysis of these pungent principles by gas chromatography (GC) were studied by Todd and Perun (1961), Morris (1967) and Hollo et al. (1969). However, only one or two of the five pungent principles were isolated and identified.

Recent technique advances in combined use of GC-MS considerably improved the possibilities of obtaining separation and identification of the pungent principles. This paper describes the isolation and identification of four pungent principles in extract and oleoresin directly by GC-MS.

EXPERIMENTAL

Preparation of the acetone extract

Capsicum fruits were extracted with acetone, as described by Kosuge et al. (1958b). The detailed procedure is outlined, step by step, in Figure 1.

Apparatus and experimental conditions

A Hitachi K-53 model gas chromatograph equipped with a flame ionization detector was utilized for separation of the components. The following column conditions were employed:

Column (1): 2m × 3mm ID glass U-tube packed with 3% OV-17 on 60–80 mesh, acid washed, silanized chromosorb W; column temperature 210°C with an N_2 carrier gas flow rate 40ml/min.

Column (2a): 2m × 3mm ID glass U-tube packed with 3% SE-30 on 60–80 mesh, acid washed, silanized chromosorb W; column temperature 210°C with an N_2 carrier gas flow rate 40ml/min.

Column (2b): 2m × 3mm ID glass U-tube packed with 3% SE-30 on 60–80 mesh, acid washed, silanized chromosorb W, column tem-

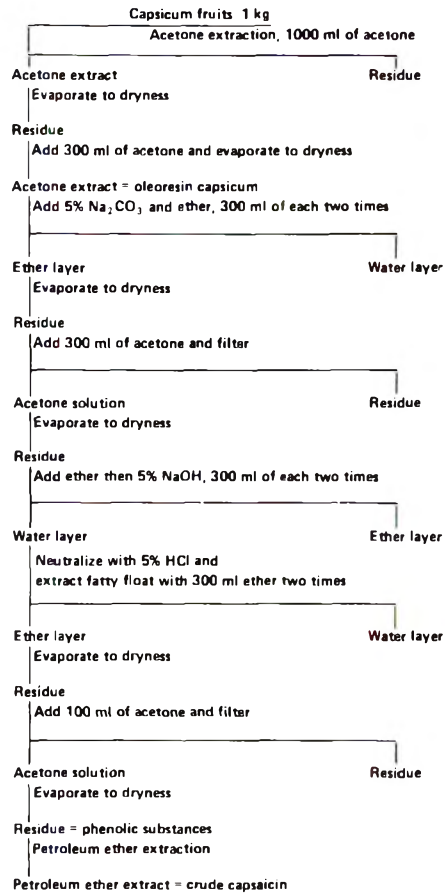

Fig. 1—Extraction of capsaicin from capsicum fruits.

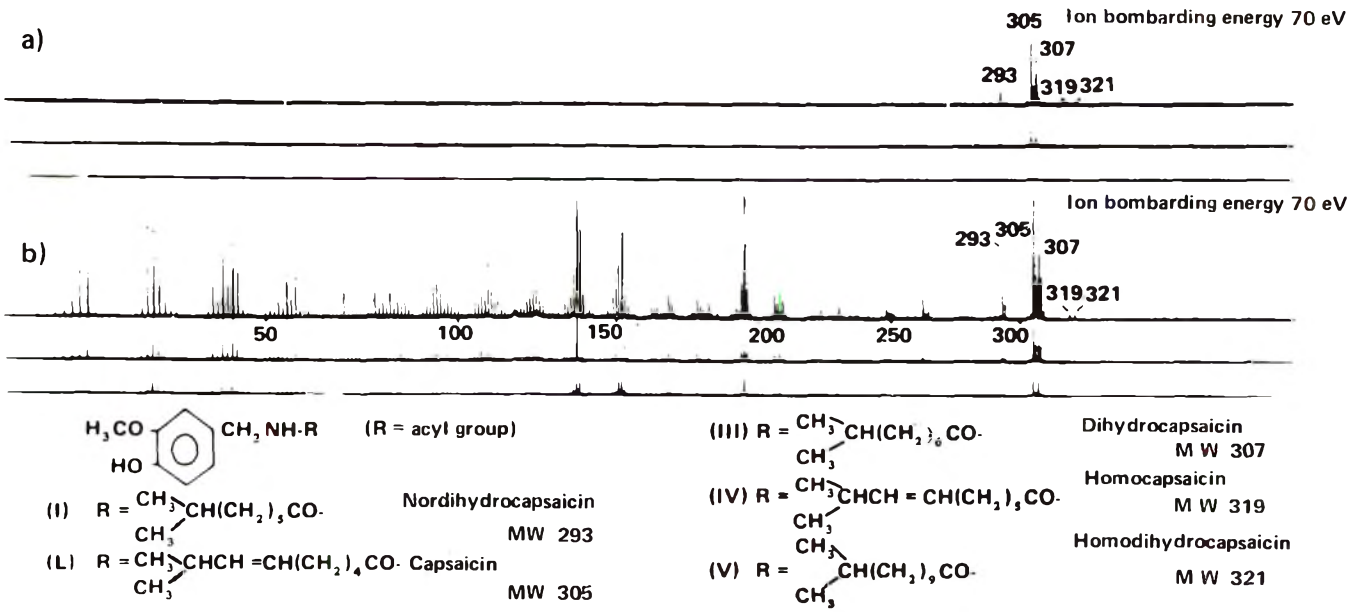

Fig. 2—Mass spectra of crude crystalline pungent principles obtained with electrons of different energy: (a) 17 eV; (b) 70 eV.

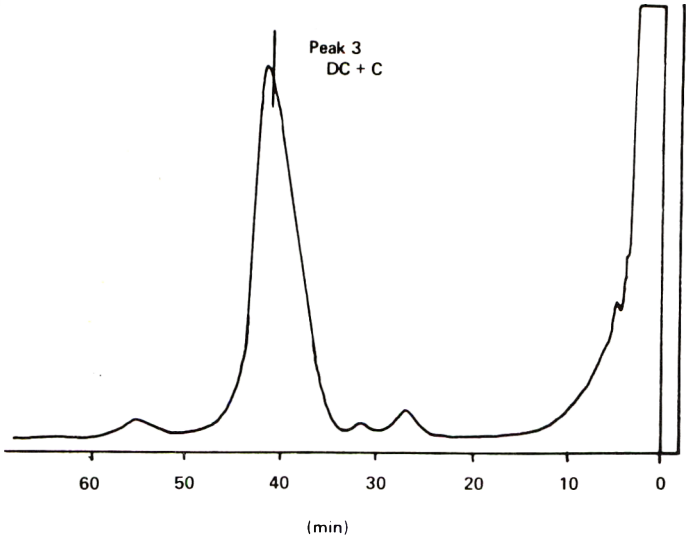

Fig. 3—Chromatogram of TMS der of crude capsaicin obtained using total ion current monitor of mass spectrometer. Column (1) apparatus and conditions. Sample volume 3μl. DC: TMS der of dihydrocapsaicin; C: capsaicin.

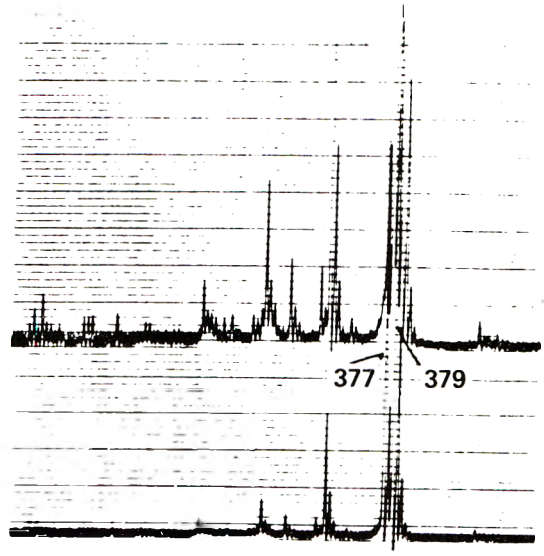

Fig. 4—Partial mass spectrum of peak 3 in Figure 3. (m/e 377, TMS der of capsaicin; m/e 379, TMS der of dihydrocapsaicin.)

perature programmed from 170–230°C at 2°C/min, with an N_2 carrier gas flow rate 40ml/min.

Column (3): 2m × 3mm ID glass U-tube packed with 3% SE-30 on 60–80 mesh, acid washed, silanized chromosorb W; column temperature 230°C with a He carrier gas flow rate 40ml/min.

Mass spectrometry was conducted utilizing a Hitachi RMU 6E single focusing mass spectrometer.

For GC-MS, a Hitachi K-53 gas chromatograph in conjunction with a Hitachi RMU 6E single focusing mass spectrometer directly connected with a Watson-Biemann type molecular separator was employed. The temperature of the column exit, connected to the ion source, was programmed 10°C higher than that of the column. The gas chromatograms were recorded by measurement of the total ion current. The scanning time over the mass range 2–500 was approximately 3 sec. Other analytical conditions were: acceleration voltage, 1800 v; ion bombarding energy, 70 and 17 eV; total emission current, 80μA; multiplier voltage, 3 kv; ion source temperature, 250°C.

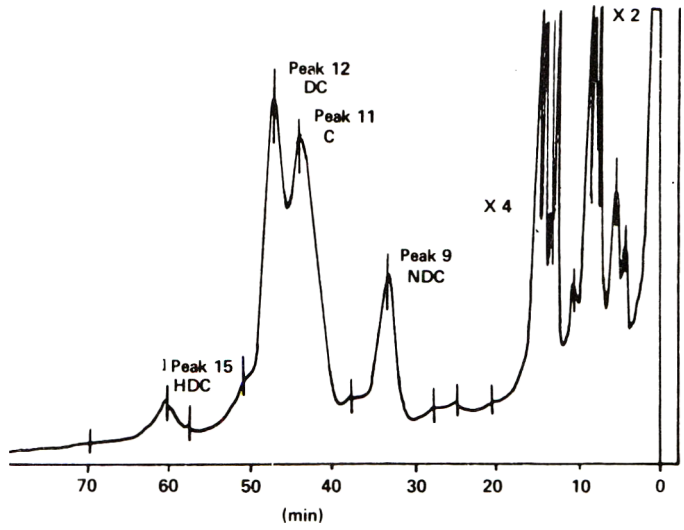

Fig. 5—Chromatogram of TMS der of phenolic substances of Capsicum annuum obtained using total ion current monitor of mass spectrometer; Column (3) apparatus and conditions. Sample volume 3μl. Peak 9, TMS der of nordihydrocapsaicin (NDC); Peak 11, TMS der of capsaicin (C); Peak 12, TMS der of dihydrocapsaicin (DC); and Peak 15, TMS der of homodihydrocapsaicin (HDC).

RESULTS & DISCUSSION

THE CRUDE crystalline pungent principles of *Capsicum annuum* (mp 63.0–64.5°C), extracted as in Figure 1, were analyzed by the direct inlet system of the mass spectrometer. The resultant mass spectrum (Fig. 2) shows the presence of peaks at m/e 293, 305, 307, 319 and 321. A second mass spectrum was obtained after reducing the ion bombardment energy to 17 eV. Since the above mentioned peaks appeared in the mass spectrum as relatively strong and distinct peaks, it may be assumed that this crystal is, at least, a mixture of nordihydrocapsaicin (mol wt 293), capsaicin (mol wt 305), dihydrocapsaicin (mol wt 307), homocapsaicin (mol wt 319) and homodihydrocapsaicin (mol wt 321).

For the gas chromatographic separation, the crude crystal was converted to trimethylsilyl derivative (TMS der) with bis-trimethylsilyltrifluoroacetamide (BSTFA) at room temperature. The gas chromatographic separation of TMS der with a 3% OV-17 column (Fig. 3) shows one major peak which, upon analysis of the mass spectrum (Fig. 4) can be seen to contain a mixture of capsaicin and dihydrocapsaicin.

When a 3% SE-30 column was utilized, however, the separation of capsaicin and dihydrocapsaicin was obtained, as indicated by GC-MS. Other pungent principles except homocapsaicin, which is a minor component, were also separated and identified by GC-MS.

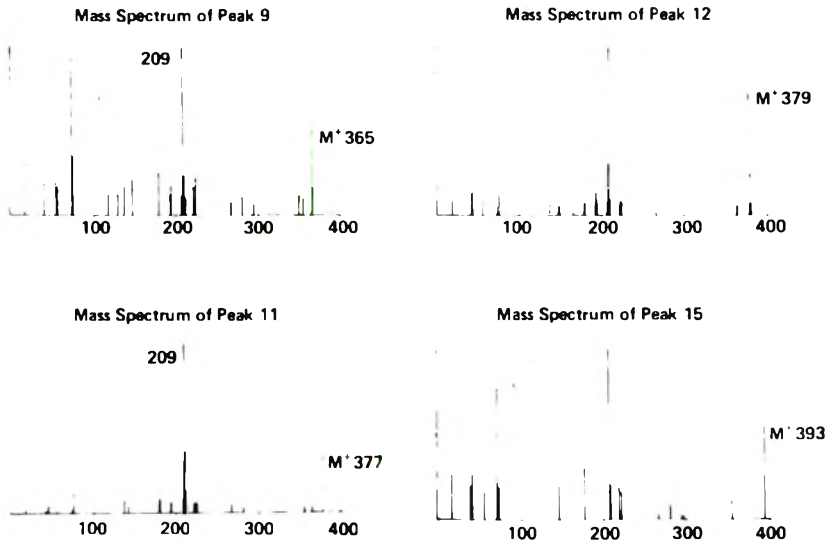

Fig. 6–Mass spectra of peaks 9, 11, 12 and 15 of gas chromatogram in Figure 5 by GC-MS. Peak 9, TMS der of nordihydrocapsaicin; Peak 11, TMS der of capsaicin; Peak 12, TMS der of dihydrocapsaicin; and Peak 15, TMS der of homodihydrocapsaicin.

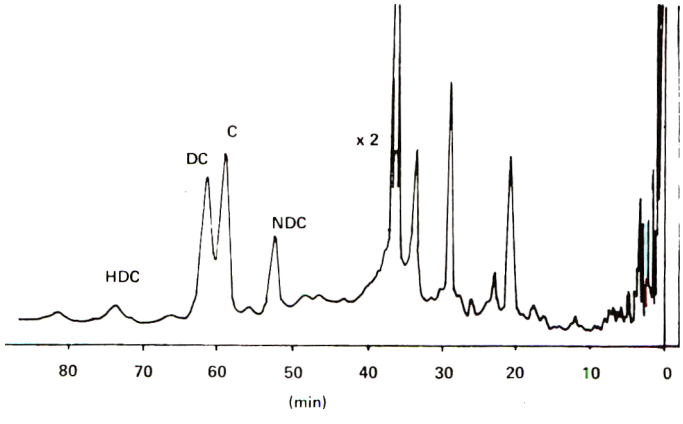

Fig. 7– Linear temperature-programmed gas chromatogram of TMS der of oleoresin capsicum. Column 2(b) apparatus and conditions. Sample volume 1 µl. NDC, TMS der of nordihydrocapsaicin; C, TMS der of capsaicin; DC, TMS der of dihydrocapsaicin; and HDC, TMS der of homodihydrocapsaicin.

Results of gas chromatographic analysis of the phenolic substances extracted from *Capsicum annuum* (see Fig. 1) are given in Figure 5. The peaks were identified by their mass spectra, obtained from the electron multiplier. These spectra are given in Figure 6.

From a review of the above results, it appears a 3% SE-30 column is the most optimal liquid phase for the gas chromatographic separation of the pungent principles of *Capsicum annuum*.

Investigations were also carried out on extracted and commercial samples of oleoresin capsicum. The oleoresin capsicum was converted to TMS der with BSTFA at room temperature. The gas chromatographic separation was carried out using a 3% SE-30 column. The typical gas chromatogram showing separation of the pungent principles of *Capsicum annuum* is presented in Figure 7.

These results confirm the fact that this method is both a rapid and a convenient way of analyzing the pungent principles of *Capsicum annuum*.

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PROPERTIES OF BIXIN AND NORBIXIN AND THE COMPOSITION OF ANNATTO EXTRACTS

SUMMARY—The melting point and $E_1^{1\%}{}_{cm}$-values of α-bixin, β-bixin and α-norbixin were determined and the stability of α-bixin examined under different conditions. The total pigment content of butter colors was determined. α-Bixin was the principal pigment; in addition, at least eight different pigments were present. The content of α- and β-bixin in butter colors was also determined. Because of complex formation of bixin and norbixin with a yellow pigment, the simple chromatographic method became rather laborious. This complex formation did not occur in the annatto suspensions in oil and fat. These preparations were more concentrated, while the amount of decomposition products of α-bixin was relatively slight. The stability of the pigments in butter colors appeared to be great. Investigation of annatto cheese colors showed that these extracts contain at least seven color components. The total amount of red pigment and the principal pigment α-norbixin were determined.

INTRODUCTION

ANNATTO food colors have been used for a considerable time in the food industry, mainly for coloring butter, margarine and cheese. The pigment preparations are obtained by extracting the seed of the tropical annatto tree (Bixa orellana). The pulp surrounding the seed is rich in pigment, consisting of more than 80% α-bixin (McKeown, 1961). (We think the notations α and β more correct than the often used terms labile and stable).

α-Bixin is the monomethylester of the dicarboxylic acid α-norbixin. The configuration (Barber et al. 1961; McKeown 1965) is shown in Figure 1.

To prepare butter and margarine colors from annatto, one commonly uses vegetable oil or propylene glycol as the extraction liquid and solvent. Since an elevated temperature is applied, the α-bixin can for the greater part decompose (Iversen and Lam, 1953; McKeown and Mark, 1962; McKeown, 1963). Suspensions of annatto in vegetable oil are commercially available; these are far more concentrated than the solutions mentioned.

Annatto cheese color is prepared by extracting the annatto seed with an aqueous alkaline solution. The α-bixin is thus hydrolyzed into the water-soluble salt of α-norbixin (Karrer and Jucker, 1948).

An accurate picture of the qualitative and quantitative compostion of annatto butter and cheese colors is necessary to enable drawing up specifications on which to base toxicological statements. This paper aims at contributing to this.

EXPERIMENTAL

α-Bixin and derivatives

The melting point apparatus was Gallenkamp type no. 889339 with an aluminium heater and calibrated thermometers. Measurements of optical density were made in a Zeiss UV spectrophotometer M4Q II 3093. Complete spectra were taken in a Zeiss UV spectrophotometer M4Q III 29272.

Use was made of glass and quartz cuvettes of 1 and 0.5 cm path length. The chloroform used was specially purified for UV spectrophotometry (J. T. Baker, no. 7019). The IR spectra were taken with a Perkin-Elmer infracord spectrophotometer (model 137E).

Butter color

The total pigment content of the different butter colors was determined from the optical density at 501 and 404 mμ in chloroform solution according to McKeown and Mark (1962). The pigments in butter color were separated by means of thin-layer chromatography:

plate:	cellulose, 20 × 20 cm (Eastman, chromagram sheet 6065)
eluent:	cyclohexane-chloroform-acetic acid (65:5:1).
chromatography chamber:	22 × 22 × 5 cm
elution time:	100–120 min

The chromatography chamber was provided with a strip of filter paper which had been moistened with the eluent to obtain a rapid liquid-vapor equilibrium (about 30 min). The cellulose plates were used without pretreatment. The butter colors A, B and C were applied undiluted to the plates by means of a capillary and eluted briefly with cyclohexane (front distance about 3 cm) to separate the oil from the pigments. If this were omitted, the pigments remained mixed with the oil too long during the normal elution, resulting in a less satisfactory separation. After air drying we eluted with cyclohexane, chloroform and acetic acid (65:5:1).

The suspensions D, E and F had to be diluted with chloroform before they could be chromatographed. Acetic acid being absent in the eluent, bixin and norbixin remained on the starting line (Francis 1965). Samples A, B, C, D, E and F were commercially available.

Determining α- and β-bixin

The determination of α- and β-bixin was carried out as follows: The cellulose plate was spotted with about 15 mg of butter color over the entire width (20 cm) by means of a capillary. By re-weighing the capillary the spotted amount was known accurately. After brief elution with cyclohexane, followed by elution with cyclohexane, chloroform and acetic acid (65:5:1), the α-bixin spot was scratched off and transferred to a glass filter (Gl, ϕ = 1 cm). The α-bixin was extracted with 15 ml chloroform-acetic acid (9:1) and filled up in a volumetric flask to 25 ml with chloroform. These amounts for β-bixin were 5 and 10 ml respectively. The optical density of the solutions obtained was determined at 505 mμ (α-bixin) and at 509 mμ (β-bixin) after which, with the aid of $E_1^{1\%}{}_{cm}$-values (2790 and 2970 respectively), we calculated the amounts of α- and β-bixin in the original sample.

On applying this procedure to our own solution of α- and β-bixin in known concentration in corn oil, it appeared that 93% of both α- and β-bixin was recovered via the TLC separation. In the above mentioned calculation the factor 100/93 was applied.

Particularly in other samples a disturbance could occur in the determination because of the complex formation of α-bixin, β-bixin and α-norbixin with a nonidentified yellow pigment with absorption maxima at 430 and 410 mμ. To be able to determine the α- and β-bixin content, the orange-colored complex with R_f = 0.49 (and which therefore arrived at a place other than that of α- and β-bixin), was scratched off and extracted as described for β-bixin. From the optical density at 505 mμ, the sum of α- and β-bixin content occurring in

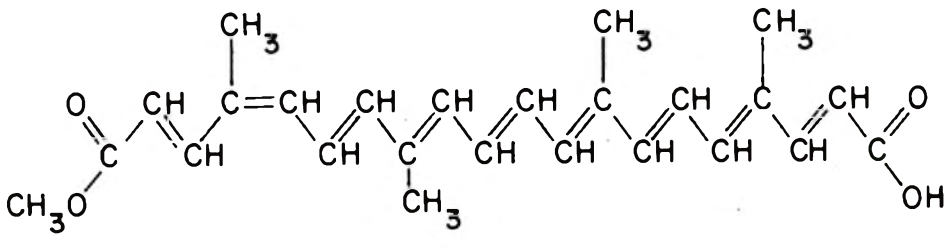

Fig. 1—Configuration of α-bixin.

the complex was determined (the amount of α-norbixin in the complex was negligibly small). Next the solution was evaporated to dryness on the rotary evaporator (max bath temp at 40°C) and the residue dissolved in acetone and removed quantitatively to a cellulose plate, after which we eluted again. The complex then for the greater part decomposed. After elution α- and β-bixin were again scratched off and determined spectrophotometrically. From the finally obtained ratio α/β-bixin and the sum of the α- and β-bixin content in the complex after the first elution, the amount of α-bixin and β-bixin present in the complex was calculated. By adding these amounts to those of the noncomplexed α- and β-bixin in the sample, the total contents of α- and β were obtained. The results are given in Table 1. In the suspensions D, E and F no complex formation occurred.

For the separation we initially used streaked silica gel plates according to Dendy (1966). However, the R_f-values of α- and β-bixin were then identical. A good separation was possible on manufactured silica gel plates (Merck) with different eluents. However, these plates were less suitable for butter colors containing many pigments. On streaked cellulose plates (Camag) satisfactory separation of α- and β-bixin was not possible.

Because of the oxidative decomposition of α-bixin on the thin-layer plates, especially after the elution on the dried plate, the time between the termination of the elution and removal of the components was kept as short as possible. It may be possible to prevent the decomposition by spraying the plate with an antioxidant or by adding an antioxidant to the eluent. However, attempts with hydrochinon appeared to be unsuccessful. To prevent photochemical decomposition of α- and β-bixin the plates were protected against UV light as carefully as possible.

Cheese color

The dry weight was determined by evaporating to dryness about 2.5g of accurately weighed cheese color on the rotary evaporator (bath temp 80°C; 20 mm Hg) and weighing the residue. The percentage of red pigment was calculated as α-norbixin ($E_1^{1\%}{}_{cm}$ (482 mμ) = 2550) from the optical density at 482 mμ of cheese color, diluted with 0.1N NaOH.

The pigments in cheese color were separated by means of thin-layer chromatography. The method was as follows:

plate:	silica gel (10 × 20 cm, Merck)
eluent:	chloroform-ethanol (abs)-acetic acid (68:2:1)
chromatography chamber:	22 × 22 × 5 cm with filter paper
elution time:	about 225 min

The plates were not pretreated. The undiluted cheese colors were spotted on the plates with the aid of a capillary. The acetic acid contained in the eluent transformed the norbixinate into norbixin.

To determine the α-norbixin content we spotted the plate over the entire width (10 cm) with 10–15 mg cheese color by means of a capillary. By weighing the capillary before and after spotting the amount applied was known. After eluting, the α-norbixin spot was removed from the plate, transferred to a glass filter (Gl, φ = 1 cm) and the α-norbixin extracted from the silica gel with 10 ml chloroform-acetic acid (9:1). A volumetric flask containing the extract was filled to 50 ml with chloroform, and the optical density measured at 503 mμ. In the calculation of the α-norbixin content use was made of $E_1^{1\%}{}_{cm}$(503 mμ) = 2290.

The whole procedure was applied to our solution of 1.11% α-norbixin in 0.1N NaOH. It appeared that 93% of the amount spotted was recovered. The values found experimentally were therefore multiplied by 100/93. Table 2 shows the results.

Table 1—Total pigment content and the content of α- and β-bixin of six samples of butter color

Sample	Total pigment content in % of weight	α-Bixin in % of weight	β-Bixin in % of weight
Solution A	0.447	0.133	0.042
Solution B	0.640	0.196	0.072
Solution C	0.258	0.095	0.037
Suspension D	2.40	1.75	0.30
Suspension E	4.26	3.80	0.0
Suspension F	4.26	3.50	0.0

Table 2—Dry weight, red pigment and α-norbixin contents of four samples of cheese color

Sample	Dry weight in wt. %	Red pigment in wt. %	α-Norbixin in wt. %
A	10.7	1.20	1.03
B	11.1	1.48	1.27
C	18.1	0.372	0.132
D	18.1	0.525	0.290

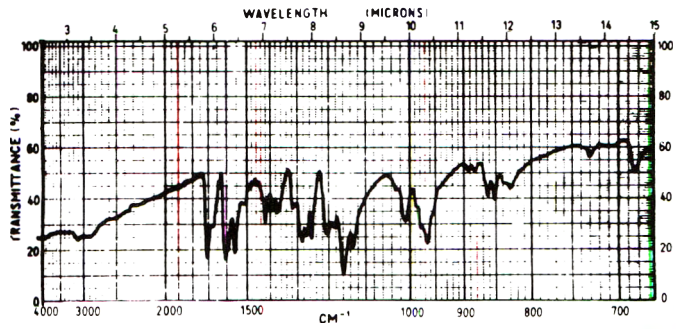

Fig. 2—IR spectrum of α-bixin (KBr disk) (recorded on Perkin-Elmer infracord spectrophotometer).

RESULTS & DISCUSSIONS

Properties of α-bixin and derivatives

α-Bixin. α-Bixin was obtained from the Nederlandse Borterkleurselfabriek L. van der Grinten. The substance was purified by recrystallizing four times from acetone. On further crystallization the melting point appeared to be unchanged, while thin-layer chromatography showed the substance to be pure. The melting point was 189.5–190.5°C (corr); the heating-up speed was 1.6°C per min in this region. The melting point appeared to be greatly dependent upon the heating-up speed, probably because α-bixin decomposes at the melting point. Presumably this is the reason for the differences in the melting point data in the literature.

The $E_1^{1\%}{}_{cm}$ values were determined in chloroform at the absorption maxima 501 and 470 mμ. We found: $E_1^{1\%}{}_{cm}$ (501 mμ) = 2880 ± 30; $E_1^{1\%}{}_{cm}$ (470 mμ) = 3230 ± 30. McKeown and Mark (1962) found $E_1^{1\%}{}_{cm}$ (501 mμ) = 2826 in chloroform. If the chloroform contained 2–4 vol % acetic acid, the maxima of α-bixin would shift to 505 and 474 mμ. The $E_1^{1\%}{}_{cm}$ values became 2790 and 3130 ± 30 respectively.

The stability of α-bixin is lower than one would expect from the literature. When the powdery α-bixin was stored in air (in darkness, at room temperature) the $E_1^{1\%}{}_{cm}$ (501 mμ) value regularly decreased by d $E_1^{1\%}{}_{cm}$ (501 mμ)/dt = 13 day^{-1}. Probably on account of this, the α-bixin contents of three trade preparations of α-bixin which we had obtained from another source, were only 21, 30 and 31%.

Also, α-bixin dissolved in chloroform and stored in the dark at room temperature appeared to be unstable. The rate of decomposition was greatly concentration dependent. With α-bixin in concentration of 3 μg/ml chloroform, the content of α-bixin after 90 days was 91.4% of the initial concentration. With an initial concentration of 150 μg/ml chloroform after 90 days, only 3.7% of the original amount of α-bixin remained. These decomposition phenomena are probably due to oxidation by oxygen from the atmosphere. When α-bixin was stored at −20°C in the dark, after 70 days no decrease in the $E_1^{1\%}{}_{cm}$ (501 mμ) was observed.

The IR spectrum of α-bixin is given in Figure 2.

β-Bixin. β-Bixin is a stereoisomer of

α-bixin; the double-bonded system is in all-trans configuration. It was prepared according to Kuhn and Winterstein (1932), starting from α-bixin. The β-bixin was once recrystallyzed from 96% ethanol. TLC-examination showed the substance to be pure. The melting point was 204.0–206.0°C (corr) with a heating-up speed of 2.2°C per min in this region. Karrer and Jucker (1948) mention melting point of 216–217°C (uncorr, heating rate not given). Since β-bixin also decomposes at the melting point, it is probable that the difference in melting point is due to a difference in rate of heating.

β-Bixin, dissolved in chloroform showed absorption maxima at 507 and 476 mμ. $E_1^{1\%}{}_{cm}$ (507 mμ) = 2970 ± 40 (in chloroform); $E_1^{1\%}{}_{cm}$ (501 mμ) = 2660 ± 40 (in chloroform); and $E_1^{1\%}{}_{cm}$ (476 mμ) = 3240 ± 50 (in chloroform).

The IR spectrum of β-bixin is given in Figure 3.

α-Norbixin. α-Norbixin, the dicarboxylic acid obtained by careful hydrolysis of α-bixin, was prepared according to Karrer et al. (1929). The end product was once recrystallyzed from glacial acetic acid. TLC-examination showed that the α-norbixin was very pure.

On heating of α-norbixin in a melting point apparatus no liquid phase occurred. Yellow coloring was observed at about 240°C, followed by black coloring (carbonization) from 280°C onward (rate of heating 5°C per min on an average).

α-Norbixin, dissolved in chloroform, had absorption maxima at 499 and 468 mμ. $E_1^{1\%}{}_{cm}$ (499 mμ) = 2200 ± 30 (in chloroform) and $E_1^{1\%}{}_{cm}$ (468 mμ) = 2470 ± 30 (in chloroform).

When the chloroform contained 0.5 vol % acetic acid, the absorption maxima of α-norbixin shifted to 503 and 473 mμ. The $E_1^{1\%}{}_{cm}$ values then became 2290 and 2620 ± 30 respectively.

α-Norbixin, dissolved in 0.1N NaOH, had absorption maxima at 482 and 453 mμ. $E_1^{1\%}{}_{cm}$ (482 mμ) = 2550 ± 40 (in 0.1N NaOH) and $E_1^{1\%}{}_{cm}$ (453 mμ) = 2850 ± 40 (in 0.1N NaOH).

The IR spectrum of α-norbixin is given in Figure 4.

β-Norbixin. β-Norbixin is a stereoisomer of α-norbixin; the double-bonded system is in all-trans configuration. The method of preparation of Karrer et al. (1929) was applied. The β-norbixin obtained was TLC pure. On heating β-norbixin to 300°C in a melting point apparatus, no liquid phase occurred; from 250°C on the red color became gradually darker.

β-Norbixin, dissolved in chloroform, had absorption maxima at 506 and 475 mμ. In 0.1N NaOH the absorption maxima were found at 486 and 457 mμ. Because of the very poor solubility of β-norbixin in these two solvents no $E_1^{1\%}{}_{cm}$ values were determined.

The IR spectrum of β-norbixin is given in Figure 5.

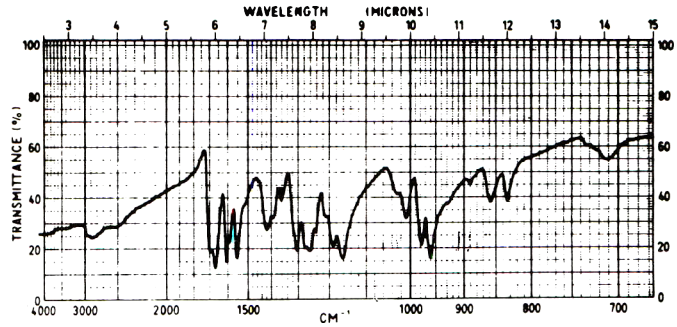

Fig. 3–IR spectrum of β-bixin (KBr disk) (recorded on Perkin-Elmer infracord spectrophotometer).

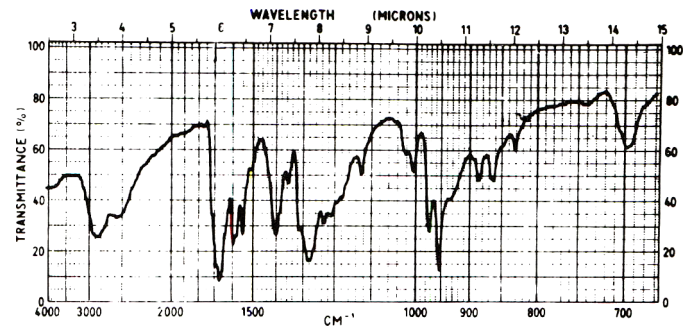

Fig. 4–IR spectrum of α-norbixin (KBr disk) (recorded on Perkin-Elmer infracord spectrophotometer).

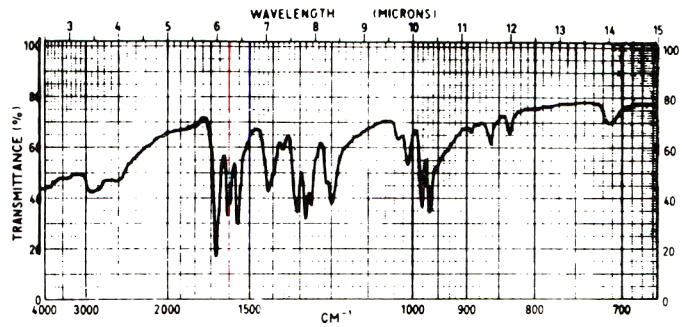

Fig. 5–IR spectrum of β-norbixin (KBr disk) (recorded on Perkin-Elmer infracord spectrophotometer).

Composition of butter color prepared from annatto seed

McKeown (1961; 1963; 1965) and McKeown and Mark (1962) investigated a number of trade preparations of annatto butter color by means of McKeown's paper chromatographic method. The total pigment content of butter color on the basis of vegetable oils varied from 0.250–0.687 weight %. The total pigment content of a propylene glycol solution was 1.45%. The pigment composition was as follows: α-bixin 12–59%; β-bixin 4–19%; α-norbixin 2–4%; 13-carbomethoxy-4.8-dimethyl tridecahexaeneoic acid 7–40%; and a pigment of a structure not yet determined to 57%. This unknown material consisted of at least seven components. The differences in composition were attributed to the solvent, extraction temperature, heating period and the origin of the seed.

In the samples A, B and C examined by us, α-bixin, β-bixin and α-norbixin were demonstrable on the ground of identical R_f values and Vis-UV absorption spectra. The R_f values of α-bixin, β-bixin, α-norbixin (all applied in chloroform) and

β-norbixin (applied in ethanol) were 0.39, 0.31, 0.20 and 0.0 respectively.

In these three samples at least six other nonidentified pigments were present. After scratching off and dissolving these components in chloroform-acetic acid (9:1), most of these pigments could be demonstrated to be carotenoids by means of Carr-Price reagent.

TLC-examination of annatto-suspensions in fat and oil (samples D, E and F) showed that a far lesser amount of yellow pigments (decomposition products) occurred than in the solutions mentioned above. The reproducability was good. The accuracy, however, depended strongly on the amount of pigment to be determined. Results of the quantitative measurements are given in Table 1.

Butter colors stored in the dark at room temperature were quite stable. In 219 days, the total pigment content of sample A had decreased by only 6.9%, which decrease was due exclusively to decomposition of the yellow pigments, for the optical density had not decreased at 501 mμ. The great stability is presumably to be attributed to the antioxidatively-acting tocopherols naturally occurring in vegetable oils.

The composition of cheese color prepared from annatto seed

Diemair and Zacharias (1951) determined spectrophotometrically, without preceding separation, the norbixin content of the precipitate resulting from acidifying the cheese cholor. They found a norbixin content of 0.3%; the dry weight of the examined sample was 12.33%.

Van Esch et al. (1959) demonstrated by paper chromatography that cheese color contained at least four color components. The total pigment content determined by spectrophotometry and calculated as norbixin was 0.15% in the examined sample.

By means of a thin-layer chromatography on silica gel plates we were able to show that four samples of cheese color each appeared to contain at least seven to nine color components. The principal pigment was always α-norbixin with R_f = 0.32. In all samples investigated, a component also occurred with the same R_f value as β-norbixin, namely 0.39. The Vis-UV spectrum, however, resembled that of α-norbixin, so that possibly another stereoisomer plays a part. The results of the quantitative measurements are given in Table 2. From the last two columns of Table 2 it appears that beside α-norbixin other pigments were also present.

The stability of cheese color is lower than that of butter color. In 35 days the red pigment content in sample B, stored in the dark at room temperature, decreased by 4%. This might explain why the α-norbixin contents of samples C and D were so low. These samples were about 2 years old, whereas A and B (at the time of determination) were only a few weeks old.

Cheese color contains 10–20% solid material, consisting of 15–30% alkaline (calculated as KOH) and up to about 11% α-norbixin. The rest is unknown material.

On acidifying cheese color with 0.1N HCl about half of the dissolved material precipitated. After filtering off the voluminous red-brown colored precipitate, a colorless filtrate remained.

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COMPOSITION OF THE LIPIDS OF CUCUMBER AND PEPPERS

SUMMARY—Cucumber and green peppers contain 103 mg and 400 mg total lipid per 100g raw vegetable tissue. The neutral lipids, phospholipids and glycolipids comprise 39, 49 and 15% of the cucumber lipids and 82, 2 and 16% of the pepper lipids respectively. The neutral lipids of both were composed mainly of glycerides. In the peppers the triglycerides accounted for 60% of the total lipids. Cucumber, had less glycerides but contained several unidentified sterols. Phosphatidylcholine was the major class in both vegetables accounting for 46 and 76% of the phospholipids of cucumber and pepper respectively. Palmitic, linoleic and linolenic acids were the principal fatty acid components. The lipids of peppers were very rich in linoleic acid (70%) whereas those of the cucumbers contained relatively more linolenic acid.

INTRODUCTION

THE QUANTITY and composition of lipid material consumed in regular diets is of nutritional and medical importance. Much of our dietary fat (60%) is invisible fat derived from meat, milk, eggs and to a lesser extent vegetables. While attempting to calculate dietary intake of essential fatty acids and other lipid materials we discovered that data on the lipid composition of several vegetables were not available in the literature nor in the Agricultural Handbook "Composition of Foods" (1963). Consequently we analyzed the lipid content and composition of two common vegetables, the cucumber and green pepper.

METHODS & MATERIALS

MATURE GOOD quality peppers and cucumbers obtained in the local supermarket were diced and homogenized in a Waring Blendor with twice their volume of chloroform methanol (2:1 v/v). The homogenate was transferred to separatory funnels and the lipids extracted by the method of Folch et al. (1957). The extracted lipids were dried under nitrogen, weighed and separated by silicic acid column chromatography according to the method of Rouser et al. (1967.) Silicic acid activated by the method of Hirsch and Ahrens (1958) was placed in 3 cm diam glass columns to a height of about 15 cm. These columns fractionated 100–200 mg of the lipid extract which was applied in 2 ml chloroform and washed into the column. 300 ml each of chloroform, acetone (containing 5% water) and methanol were used sequentially to elute the neutral lipids, glycolipids and phospholipids respectively. These solvents were removed by a rotary vacuum evaporator at 45°C. The lipids were then transferred to tared vials, dried under nitrogen and weighed. The lipid classes were subsequently separated by thin-layer chromatography using activated silica gel G coated plates for neutral lipids and glycolipids (Rouser et al., 1965). The phospholipids were separated in silica gel HR layers of 250μ thickness. Solvent systems used were: (1) for neutral lipids, petroleum ether:diethyl ether:acetic acid, 70:30:0.5 v/v; and (2) for glycolipids, chloroform:methanol:water, 75:25:4 v/v. The phospholipids were separated by two-dimensional chromatography (Parsons and Patton, 1967) using solvent systems of chloroform:methanol:water:28% aqueous ammonia, (65:35:4:0.2 v/v) and chloroform:acetone:methanol:acetic acid:water (50:20:10:10 v/v).

The approximate distribution of the lipids separated by one-dimensional chromatography was determined by densitometry following charring (Rouser et al., 1965). The phospholipids were quantified by determining phosphorus content of spots on the chromatograms (Parsons and Patton, 1967).

Aliquots of the lipids were saponified overnight at 60°C (5 mg lipid + 2 ml ethanolic KOH, 7% soln). Following drying, the nonsaponifiable materials were removed with hexane and the soaps were then converted to methyl esters by transesterification with boron-triflouride methanol. The methyl esters were twice extracted from the water/salt saturated esterification mixture with hexane. These were concentrated under a stream of nitrogen and immediately analyzed by gas-liquid chromatography.

A Barber-Colman Model 5000 gas chromatograph with a hydrogen flame detector was used. This contained a 1.8 meter U-shaped glass column packed with gas-chrom P which contained a 12% coating of high efficiency diethylene glycol succinate (Applied Science, State College, Pa.). Argon carrier gas was used (flow rate 60 cc/min) and the temperature programmed from 150–210°C at 10° per min. The fatty acids were identified by comparison with standard mixtures (Applied Science, State College, Pa.). The triangluation method was used to quantify the fatty acids.

The lipid components, separated by thin-layer chromatography, were identified by co-chromatography with pure standards (Applied Science, State College, Pa.; Supelco, Bellefonte, Pa.; Analabs, North Haven, Conn.) and from published R_f values (Lepage, 1967; Nichols 1964). In addition specific sprays were used to identify phospholipids (Nichols 1964) and lipids containing sterols (Galliard 1968a; b).

Chromatographic supplies and equipment were purchased from Brinkmann, (Westbury, N.Y. and Applied Science, State College, Pa.). Purified solvents were used.

RESULTS

TOTAL LIPID content of both vegetables was quite low i.e., 400 mg and 103 mg

Table 1—Composition of the neutral lipids and phospholipids of pepper and cucumber

Composition	Pepper %	Cucumber %
Neutral lipids		
Monoglycerides	2.1	4.1
Diglycerides	6.9	8.5
Triglycerides	73.2	54.6
Unesterified fatty acids	5.0	4.3
Sterols	6.7	16.7
Sterol esters	2.8	3.8
Pigments and waxes	1.8	1.2
Unknown	t	6.8
Phospholipids		
Phosphatidylcholine	75.8	45.6
Lysophosphatidylcholine	2.8	3.2
Phosphatidylinositol	4.1	5.0
Phosphatidylserine	2.1	2.2
Phosphatidylethanolamine	8.7	31.3
Lysophosphatidylethanolamine	t	1.0
Phosphatidylglycerol[a]	2.3	6.4
Phosphatidic acid	t	0.7
Unknown	4.2	4.6

[a]Tentatively identified from its R_f value.

Table 2–Percentage distribution of glycolipids of pepper and cucumber[a]

R_f value	Tentative identification	Approximate content Pepper %	Cucumber %
0.99	Pigments	15	13
0.92	Sterol glycosides	11	9
0.84	Monogalactosyl diglycerides	16	15
0.80	Unknown (glycoside)	4	Absent
0.70	Cerebrosides & digalactosyl diglycerides	28	31
0.61	Digalactosyl diglyceride	31	40
0.48	Unknown (sulfolipids)	20	15
0.30	Unknown	Absent	2

[a]Tentative identification from co-chromatographic R_f values of known standards of galactosyl diglycerides and from published R_f values of other components.

Table 3–Fatty acid composition of the major lipid classes from green pepper and cucumber

Fatty Acid	Neutral Lipids Pepper	Neutral Lipids Cucumber	Phospholipids Pepper	Phospholipids Cucumber	Glycolipids Pepper	Glycolipids Cucumber
C12:0	0.1	t	–	0.5	t	0.3
C14:0	0.4	1.0	t	0.8	0.4	0.4
C16:0	15.1	29.5	19.8	47.3	11.5	23.5
C16:1	0.1	t	–	0.4	0.5	t
C18:0	3.2	4.1	6.0	3.4	15.7	3.4
C18:1	8.6	5.4	1.8	1.2	5.3	3.1
C18:2	70.0	32.3	64.5	27.1	30.5	25.5
C18:3	2.5	27.7	7.9	19.3	36.1	43.8

per 100g (wet weight) of pepper and cucumber respectively. The composition of each extract as determined by column chromatography was markedly different. Thus neutral lipids phospholipids and glycolipids accounted for 82, 2 and 16% of the pepper lipids and 39, 46 and 15% of the cucumber lipids respectively.

Densitometric measurements showed that the neutral lipids were variable in composition (Table 1). In the pepper lipid extracts, triglycerides were the preponderant class comprising 60% of the total lipid extract; in cucumbers the triglycerides were much lower. The unknown spot had an R_f value similiar to that of ketoglycerides and from chromatographic behavior it appeared that some autoxidation of the cucumber lipids had occurred.

The cucumber lipids showed five distinct sterol spots (blue spots with sulfuric acid) excluding the sterol esters. The chromatograms of the pepper lipids showed only one free sterol and one sterol ester spot.

The peppers contained very small amounts of phospholipids i.e., 2% compared to 46% of the cucumber lipids. Phosphatidylcholine was the preponderant component in the peppers whereas the cucumbers showed a more usual distribution of phospholipids (Table 1).

The glycolipid fractions from both vegetables had very similar chromatographic patterns and distribution (Table 2). The peppers contained a compound (R_f 0.8) that migrated in the region of steryl glucoside. This was absent from the cucumber glycolipids.

The fatty acid composition of both vegetables was quite typical of plant tissue. The neutral lipids and phospholipids from the peppers were very rich in linoleic acid (C18:2) while the corresponding fractions of the cucumbers possessed more linolenic acid. The glycolipids of both contained greater quantities of linolenic acid (Table 3).

DISCUSSION

CUCUMBERS and peppers contained lipids that were qualitatively similar to those reported for other edible vegetables, potato and apple (Galliard, 1968a; b), turnip (Lepage, 1967) and for plants in general (Allen and Good, 1965). The composition and distribution of the neutral lipids and polar lipids of the cucumber were comparable to those of the potato as reported by Galliard (1968a). However the cucumber tissue contained a much greater quantity of sterols than potatoes but somewhat less than reported for apple tissues (Galliard 1968b). The cucumbers possess several unknown sterols which are presently being identified and from preliminary analyses β-sitosterol and stigmasterol appear to be the major components.

The triglycerides constituted the preponderant component of the lipids from whole peppers (about 60%). This may be attributed to the seeds which are abundantly present in the peppers. The pepper extract contained a remarkably low concentration of phospholipids of which phosphatidyl choline was preponderant and the other components were present in very small quantities.

The glycolipids of both vegetables were similar and mono- and digalactosyl diglycerides were apparently the major components. These are concentrated in the plastid membranes of the chloroplasts and are universal constituents of photosynthetic tissue (Allen and Good, 1965) which is present in abundance in both green peppers and cucumbers.

The fatty acids present in the lipid fractions of both vegetables were quite typical for plants. The lipids extracted from the peppers were very rich in linoleic acid. The glycolipids of both tissues contain a relatively greater concentration of linolenic acid. This acid usually occurs in high concentration in photosynthetic tissue.

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FLAVONOL GLYCOSIDES OF THE CULTIVATED STRAWBERRY

SUMMARY—After extraction and separation by thin-layer chromatography (TLC) on cellulose, nine flavonol glycosides were detected and purified in the fruit of strawberry from various sources. The structures of these were investigated by TLC, spectral properties and several types of hydrolysis. Four of them were completely identified as the -3-β-monoglucosides and -3-β-glucuronides of quercetin and kaempferol. Three others were characterized less fully as kaempferol-7-monoglucoside, a kaempferol-7-glucoside and an unreported quercetin-3-glucoside which was not the -3-β-monoglucoside or a diglucoside. A comparison between the flavonol glycosides and anthocyanins of this fruit was made with respect to the amounts and types present.

INTRODUCTION

THE RED PIGMENTS of the cultivated strawberry, *Fragaria ananassa*, the anthocyanins, have been well studied over the years, yet the related flavonol glycosides have received only minor attention. This paper is concerned with the isolation and identification of the flavonol glycosides of strawberries from several sources and relates these findings to those of previous investigations. This study of the phenolic composition of fruits (Ryan and Coffin, 1971) was initiated with the intention of using these constituents as monitors of food quality and adulteration.

The earliest examination of the flavonols from the cultivated strawberry was accomplished by Williams and Wender (1952), who characterized the aglycones kaempferol and quercetin. No flavonol glycosides were reported. In a study of the phenolic constituents of the family Rosaceae, Bate-Smith (1961) showed by paper chromatography the presence of quercetin in the leaves of the cultivated strawberry, *Fragaria chiloensis*, and both quercetin and kaempferol in the leaves of highbush strawberry. In an extension of the above work, Creasy et al. (1964) demonstrated the existence of at least four flavonol glycosides in the leaves of both wild and cultivated strawberries, two being quercetin and two kaempferol glycosides. In the fruit, Co and Markakis (1968) identified and characterized both kaempferol-3-monoglucoside and quercetin-3-monoglucoside. Recently, Duggan (1969) reported an extraction and purification method for the examination of flavonol glycosides in plants and showed by thin-layer chromatography (TLC) that strawberry contained at least five discrete glycosides, whose aglycones were quercetin and kaempferol. Hence, up to the present time, five glycosides have been indicated in strawberry; whereas, only two of these have been identified. The aglycones of the glycosides appeared to contain but two members, quercetin and kaempferol.

EXPERIMENTAL

Fruit

Mature 1970 locally grown strawberries were obtained at Ottawa markets, dehulled and stored at −5°C in polyethylene bags until used (1–2 wk). Other strawberries of different origin were purchased as unsweetened frozen packs at local supermarkets and kept at −5°C. The year of origin of these was uncertain.

Authentic flavonoids

A pure sample of quercetin-3-β-monoglucoside was supplied by Dr. S.H. Wender, University of Oklahoma, Norman, Okla. Quercetin-7-monoglucoside was provided by Dr. R. Horowitz, USDA, Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif. Dr. I.A. Pearl, Institute of Paper Chemistry, Appleton, Wis., contributed a liberal supply of quercetin-3-glucuronic acid. The aglycones quercetin and kaempferol and the enzymes, β-glucosidase and β-glucuronidase, were purchased from commercial sources.

Extraction and purification

The extraction, purification and chromatography of the flavonol glycosides from strawberries were similar to the methods described previously for raspberries (Ryan and Coffin, 1971). Some glycosides required as many as six separate elutions from TLC for purification starting from the crude extract; others needed only two. The amount of flavonol present either in a crude extract or purified fraction was estimated by its UV absorption in the range 345–375 nm, assuming that the molecular extinction coefficient for this peak was 18,000 corresponding to the actual value of quercetin-3-β-monoglucoside (isoquercitrin).

Absorption spectra

The absorption spectra of the glycosides were recorded in methanol solution using a Cary 14 Recording Spectrophotometer with the diagnostic reagents and methods for determination of substitution patterns as developed by Jurd (1962) and Mabry et al. (1970).

Acidic hydrolysis

Prior to hydrolysis, and because of the small amount of material being used, all samples were filtered as methanol solutions through Millipore Filters (Millipore Filter Corp., Bedford, Mass.). This removed particulate material as well as any finely divided cellulose which would interfere with the sugar analysis. The material (0.1–0.5 mg) was then refluxed and stirred for 90 min with a 5% aqueous solution of trifluoroacetic acid (TFA) according to the method of Wösel and Barz (1970). The aglycone was extracted with three equal portions of EtOAc, leaving an aqueous solution of pH 2–3. The evaporated organic phase was analyzed qualitatively by TLC in several solvent systems and quantitatively by either GLC or its UV spectrum.

The water phase from the hydrolysis was evaporated at room temperature either with a stream of nitrogen or under vacuum. The constituent sugars were silylated and determined by GLC. A reference experiment containing only aglycone and sugar was performed for every glycoside by measuring how much of these survived the experimental conditions.

Gas chromatography conditions

The sample (0.05–0.2 mg) was treated with Trisil (0.1–0.5 ml) at room temperature for at least 5 min.

A Hewlett-Packard 5750 GLC was used with a hydrogen flame detector and helium as the carrier gas (30 ml per min at room temperature). Stainless steel columns (4-ft by 1/8-in. od) were packed with either 3% OV-17 or 3% SE-30 on Chromosorb W/60–80 mesh. Sugar analysis was performed using a temperature program of 120–175°C at 4° min and flavonol analysis using a program of 230–260°C at 2° min. Such methods were sufficient to measure all known sugars and flavonols combined as flavonol glycosides.

Peroxide oxidation of -3-glycosides

The method of Chandler and Harper (1961) was used to remove sugars attached to the 3 position of flavonols by treating the glycosides with hydrogen peroxide from 8–24 hr at room temperature. Sugar analysis was accomplished by GLC, taking note that peroxide can oxidize sugars to the corresponding sugar acids.

Enzyme hydrolysis

The glycoside (0.1–0.5 mg) was dispersed in 1 ml of an acetate buffer (pH 4.6) containing the enzyme (0.5 mg). These were incubated with agitation at 37°C for 18 hr. The heterogeneous mixture was then extracted with EtOAc and the aglycone and sugars analyzed by GLC and TLC.

RESULTS & DISCUSSION

FROM 1.8 kg of strawberries, a crude extract was obtained which contained about 55 mg of flavonol glycosides. After TLC purification, only about 13 mg total of glycosides were recovered. These results are shown in Table 1 along with the number of flavonols isolated and their relative intensities. The values in columns 3 and 4 depend on the number of TLC elutions and are only a guide as to the amounts capable of being isolated. Column 5 gives an estimate of the relative amounts in a crude extract.

Some of these losses can be attributed to incomplete recovery of the glycosides from the cellulose layers, a loss accentuated by the numerous elutions necessary for ultimate purity. Other losses can be attributed to the instability and decomposition of the glycosides. An additional problem arises in the purification of flavonol glycosides as they chelate effectively and irreversibly with most metallic ions (Fe, Zn, Sn). These insoluble complexes are ultimately lost to the reaction pool. Thus, all solvents and absorbents must be free from these interfering ions. Moreover, on elution of flavonols from TLC plates with methanol, significant amounts of cellulose or its oligomers are extracted. These impurities cause a bias both in the actual weight of flavonol glycoside and in the sugar analysis after hydrolysis.

Glycoside 1 was believed to be the most plentiful component as estimated by TLC of a crude extract, but in the final purification was only the second most common component. When it was stored dry under nitrogen at 5°C for 1 month, the amount as determined by UV dropped from 3.0–1.9 mg. Nine glycosides were found in strawberry, although flavonols 3 and 6 could not be isolated in pure form, suggesting they were undergoing change. For this reason, their structures were not determined. Glycoside 4 was a nonseparable mixture of two components.

The TLC behavior of the glycosides afforded much information on the number and types in the crude extract. The R_f values of these are shown in Table 2, along with some representative standards. In addition, the two-dimensional chromatogram of the glycosides from strawberry (Fig. 1) shows a well-resolved pattern of spots whose most prominent members are the glycosides 1 and 4. This pattern is similar to one reported by Duggan (1969), except for the number and intensity of compounds. Comparison of R_f values of these compounds with those of known glycosides and reported values (Harborne, 1967) gave some indication as to the nature of the aglycone, the number of sugars and their position of attachment.

Glycosides 1 and 2

The UV spectra of glycosides 1 and 2 with the addition of diagnostic reagents (Table 3) pointed to a quercetin-3- and a kaempferol-3-glycoside. Hydrolysis or cleavage, or both, under a variety of conditions (Table 4) (e.g., TFA, H_2O_2 oxidation and enzyme hydrolysis) confirmed the structures of 1 and 2 as quercetin-3-β-glucuronide and kaempferol-3-β-glucuronide, respectively.

The hydrolysis with TFA proceeded smoothly to give a reproducible measurement of the aglycone to sugar ratio. TFA is a weaker acid than HCl and less destructive to sugars but strong enough to cleave flavonol glycosides. No matter what steps are taken for purification and filtration, traces of glucose are encountered in this hydrolysis. This has been noted previously by Ribereau-Gayon (1964) in a study of grapes. The peroxide oxidation of flavonol-3-glycosides is not as clean a method as acidic hydrolysis and sugar yields are lower, particularly in glucuronic acid. The enzyme cleavage of glycosides proceeded smoothly with both β-glucosidase and β-glucuronidase. Authentic samples of quercetin-3-β-monoglu-

Table 1–Amounts and relative intensities of isolated glycosides

Glycoside	No. of TLC elutions	Amount[a] (mg)	% of Total[b]	Visual est. of relative int.[c]
1	3	3.0	23	1
2	4	0.7	5	4
3	5	<0.2	<1.5	7
4	4	7.1	55	2
5	4	0.4	3	6
6	6	<0.1	<1	3
7	2	1.2	9	5
8	2	0.3	2	8

[a]From 1.8 kg berries; estimated by UV.
[b]By addition of column 3.
[c]After spraying plate of crude extract with $AlCl_3$.

Table 2–R_f Values of strawberry glycosides on cellulose layers

Glycoside	Solvent[a] A	B	C	D	E	F
1	.48	.76	.29	.47	.43	.60
2	.56	.84	.43	.63	.60	.69
3	.50	.14	.82	–	–	–
4	.58	.26	.58–.62	.08	.61	.73
5	.49	.19	.48	.06	.50	.67
6	.60	.88	.60	–	–	–
7	.21	.05	.58	–	.47	.56
8	.15	.03	.35	–	–	.48
Q-3-gl	.48	.21	.53	.05	.48	.65
Q-7-gl	.14	.04	.32	<.03	.17	.42
Q-3-ga[b]	.48	.78	.30	.49	.43	.60
K-3-ga[b]	.57	.82	.45	.59	.57	.67

[a]Cf. experimental.
[b]Obtained from raspberry; Ryan and Coffin (1971).

Q = quercetin, K = kaempferol, gl – monoglucoside, ga = glucuronic acid.

Solvent: A–30% HOAC; B–3% phenol in H_2O; C–80% phenol in H_2O; D–H_2O; E–n-butanol - HOAC - H_2O: 6-1-2; F–HOAC-conc. HCl - H_2O: 30-3-10 (Forrestal)

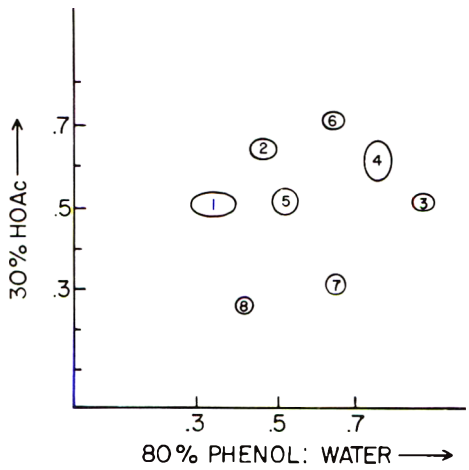

Fig. 1–Two-dimensional chromatogram of glycosides in strawberry.

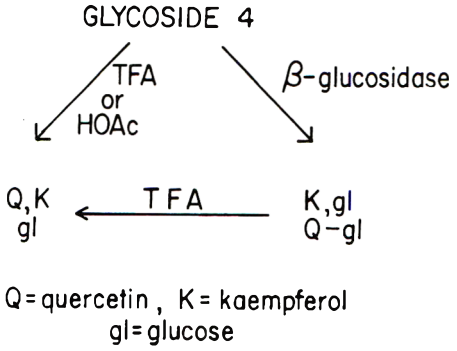

Fig. 2—Chemistry of glycoside 4.

Table 3—UV Spectra of isolated glycosides

Glycoside	Absorption max. in MeOH (nm)		Shift: Band II $AlCl_3$	Shift: Band II $AlCl_3$ HCl	Shift: Band II NaOAc H_3BO_3	Shift: Band I NaOMe	Shift: Band I NaOAc	Hydroxyl pattern: Free	Hydroxyl pattern: Subst.
1	255	355	50	30	28	50	17	7,5,3′,4′	3
2	262	348	45	45	0	50	10	7,5,4′	3
4	260	350	55	45	10	50	12	7,5	3
5	258	354	70	40	35	65	12	7,5,3′,4′	3
7	262	365	59	58	3	63	2	5,4′,3	7
8	268	325	58	58	–	55	0	5,4′,3	7
	370								

coside (isoquercitrin) and gentiobiose (6-0-β-glucosyl-D-glucose) were hydrolyzed by the former enzyme quantitatively.

Glycoside 5

In a similar manner, glycoside 5 from strawberry was proven to be quercetin-3-β-monoglucoside (isoquercitrin).

Glycosides 7 and 8

These components were obtained in small amounts from the fruit. Their UV and TLC indicated flavonol-7-glycosides. Acidic hydrolysis confirmed glycoside 7 as kaempferol-7-monoglucoside and glycoside 8 as a kaempferol-7-glucoside. Insufficient material was available for a detailed investigation of these compounds.

Table 4—Hydrolysis products of glycosides

Glycoside	Acidic hydrolysis: Aglycone	Acidic hydrolysis: Sugar	Acidic hydrolysis: Ratio	Peroxide oxidation	Enzyme hydrolysis	Conclusion
1	Q	ga	1:1	ga	ga	Q-3-β-ga
2	K	ga	1:1	ga	ga	K-3-β-ga
4	Q, K	gl	1:0.8	gl	gl, Q-gl	K-3-β-gl Q-3-glu
5	Q	gl	1:1	gl	gl	Q-3-β-gl
7	K	gl	1:1	NA	ND	K-7-gl
8	K	gl	ND	NA	ND	K-7-glu

Q = quercetin, K = kaempferol, gl = glucose, ga = glucuronic acid.
NA = not applicable, ND = not determined, glu = glucoside.

Glycoside 4

The identity of glycoside 4 was particularly difficult to resolve. Its TLC in various solvent systems showed a single spot except in 80% phenol, where there was a slight elongation of the pattern. The UV spectra was ambiguous, in that the test for an ortho-dihydroxy group in the B ring was inconclusive; the shift of the long wavelength peak after addition of $AlCl_3$/HCl or NaOAc/H_3BO_3 was only half that expected for an ortho-dihydroxy grouping. Acidic hydrolysis of the mixture was more revealing, showing the presence of both kaempferol and quercetin in the ratio of approximately 1:1. The sugar present was glucose, although there appeared to be more aglycone than sugar on a molar basis (ratio 1:0.8). With this result, and the information from the UV and TLC, the mixture appeared to contain kaempferol-3-monoglucoside plus a quercetin-3-glucoside which was not isoquercitrin nor a diglucoside (R_f values and hydrolysis). β-Glucosidase hydrolysis of the mixture yielded kaempferol, glucose plus an intact quercetin glucoside. Further hydrolysis with acid cleaved this glucoside to quercetin and glucose. These are shown schematically in Figure 2. Thus, the linkage of the glucose in kaempferol was β but that in quercetin was not. Partial hydrolysis of the mixture with acetic acid or TFA afforded only aglycone, glucose and starting material. Mild acid at room temperature left the mixture unchanged. Hydrolysis in alkaline media and the UV spectra pointed to the absence of cinnamic acids.

This unknown may be identical to a quercetin glucoside isolated but not characterized by Williams and Wender (1953a; b) from apricots and plums. However, the aglycone to sugar ratio of the quercetin-glucoside appears to be 2:1 from both acidic and enzymatic hydrolysis. Since this type of flavonol glycoside has not been reported, and as the experiment was performed on a mixture, this value must be taken with caution. Wrolstad et al. (1970a) reported the presence of an anthocyanin pigment, pelargonidin-3-glucoside, in strawberries which was different from the pelargonidin-3-monoglucoside usually found in this fruit. Their new pigment was not a chromatographic artifact, and could easily be converted to the known pelargonidin-3-β-monoglucoside by acid. In addition, it contained a 1:1 mole ratio of aglycone to sugar and did not possess acylating acids or solvents. It was suggested to be pelargonidin-3-monoglucoside where the glucose was either in the furanose form or the alpha anomer. Thus, there exists the real possibility that such a phenomenon could exist in the related flavonol glycosides. However, due to the paucity of material, this glucoside was not investigated further.

Thus, the -3-β-monoglucosides and -3-β-glucuronides of kaempferol and quercetin as well as a -7-monoglucoside, a -7-glucoside of kaempferol and a quercetin-3-glucoside have been isolated and characterized from strawberry. The first two compounds have already been identified from this fruit by Co and Markakis (1968); whereas, Creasy et al. (1964) and Duggan (1969) have indicated that this fruit contained more, but identification has only now been forthcoming.

Flavonol glucosides are quite common in plants but the glucuronides have been detected in only seven or eight species including the edible bean, *Phaseolus* (Endres et al., 1939), red grapes, *Vitis vinifera* (Ribereau-Gayon, 1964) and in the red raspberry, *Rubus idaeus* (Ryan and Coffin, 1971). This is the first report of these rarer glycosides in the strawberry.

Part of the oversight in identification of glucuronides lies with the compounds themselves. Many of the common techniques for isolation and characterization of glycosides result in the loss of glucuronides, because they contain a carboxylic acid group. There have been reports (Dumow, 1969) that glucuronides are irreversibly bound to polyamide columns

and cannot be isolated in this manner. However, their characterization has also been reported by just such a technique (Wagner et al., 1970). In any case, experimental techniques must be adjusted in the isolation and characterization of glucuronides to take into account their acidic nature.

Anatomical and geographic differences

Because of the high concentration of flavonol glycosides in the roots and leaves of plants, they can sometimes be obtained from these parts by direct crystallization. However, when the fruit or pulp of a species is the source of flavonoid compounds, this procedure is not possible, due to the low levels in these parts. As a comparison, the flavonols from the leaves of *Vaccinium myrtillus* (huckleberry) (Ice and Wender, 1953) were isolated at the concentration of about 1,500 mg/kg leaf. On the other hand, the flavonol content of whole grapes is about 10–15 mg/kg (Williams and Wender, 1952) and that of pears in the order of 10 mg/kg (Nortje and Koeppen, 1965). In this study the flavonol content of strawberries from various sources has been found to vary between 37 and 79 mg/kg fruit. Comparison of the flavonol glycoside content with the anthocyanins from the same source also shows a considerable difference. Wrolstad et al. (1970b) has found an anthocyanin value between 343 and 966 mg/kg with a mean of 573, values much larger than those of flavonols.

All of the samples of strawberries derived from Canadian, American and Polish sources contained glycosides 1 to 7 inclusive. Glycoside 8 was not detected in Polish fruit. In all instances glycosides 1, 2, 4 and 7 were the major components, whereas the relative amounts of 3, 5, 6 and 8 appeared to vary from source to source. Overall there were only minor differences between the fruit from different regions.

With these results in hand, a comparison can be made to the coloring pigments, the anthocyanins, which give the strawberry its attractive appearance. If attention is focused on the hydroxylation pattern in the B ring of these aglycones, the main anthocyanin found in strawberry is pelargonidin (monohydroxy), while cyanidin (dihydroxy) is a minor component. However, in this study it has been shown that kaempferol (monohydroxy) and quercetin (dihydroxy) occur in about equal amounts. The trihydroxy aglycones, myricetin and delphinidin, are both absent in this genus (Harborne, 1967). The glycosidic portion of anthocyanins in strawberry has been found to be only -3-glucosidic whereas the flavonols are combined as -3- and -7-glucosides and -3-glucuronides. Thus, there appears to be no direct correlation between these two classes of flavonoid in strawberry.

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PHENOLIC AND INDOLE CONSTITUENTS OF EDIBLE PEAS

SUMMARY—p-Hydroxybenzoic, ferulic and vanillic acids were isolated from hydrolyzed and non-hydrolyzed extracts of seeds of Pisum sativum *L. var. Feltham and var. Lincoln. In the Feltham variety syringic acid was only present as the free compound but p-coumaric acid occurred in both the free and bound forms. Indole-3-acetic and, tentatively, indole-3-carboxylic acids were identified in the Lincoln variety. It is very doubtful if phenols present in the minimal concentration found could play any role in the edible characteristics of the peas.*

INTRODUCTION

MANY CLASSES of compounds are found in peas (Karrer, 1958; Buch, 1960; Mentzer and Fatianoff, 1964; Mentzer, 1966; Kariyone, 1961–1967). Kaempferol and quercetin 3-(p-coumaroylglucosyl-sophoroside) have been found in pea tissue (Furuya et al., 1962) but very few simple phenols (caffeic, ferulic and chlorogenic acids) have been reported from this source (Elema, 1960), even though phenolic acids as their labile esters or glycosides are of widespread occurrence in plants.

Ferulic, caffeic and sinapic acids are present in many seeds and they have been referred to as germination inhibitors (Varga and Köves, 1959) although their role is still unclear (Harborne, 1964). The concentration of phenols in peas could be expected to make a substantial contribution to the taste (Horowitz, 1964) or toxicity (Singleton and Kratzer, 1969) of this food, which finds widespread use in the human diet. Accordingly, this study was carried out to ascertain whether phenols are present in significant amounts in immature pea seeds.

EXPERIMENTAL

Extraction and fractionation

Seeds of *Pisum sativum* L. var. Lincoln (1962-crop) or var. Feltham (1963-crop), dried at room temperature for several weeks, were soaked in methanol and homogenized in a blender.

The seeds were then extracted with methanol in the dark for 24 hr at room temperature with occasional shaking. The supernatant was decanted and the extraction repeated twice. The combined extracts were reduced in volume at 36–38° in vacuo, until only the extracted water remained. The aqueous solution was acidified with 1N HCl to pH 1–2, and extracted three times with ether in the ratio 1:1. The ethereal phase was then washed (3 times) with 7% $NaHCO_3$. The aqueous basic fraction was adjusted to pH 1–2 with 6N HCl and back-extracted with ether to yield the phenol-carboxylic fraction. The $NaHCO_3$ extracted ether phase contained the neutral and phenolic compounds.

Both fractions (carboxylic and phenol-neutral ones) were dried, in the dark, over anhydrous Na_2SO_4, filtered and evaporated to dryness in vacuo at 36–38°. They were redissolved in 5 ml methanol and chromatographed.

Hydrolysis

The original acidic extract was reduced to a small volume (50 ml), diluted with 150 ml methanol and hydrolyzed for 2 hr with 150 ml 10N H_2SO_4.

The methanol was evaporated and the hydrolysate filtered, extracted with ether and fractionated as previously described. The two fractions were dried, evaporated and chromatographed as above.

Chromatography

The phenol-carboxylic and phenolic fractions from both seed varieties were chromatographed on Whatman No. 1 paper using 2% acetic acid (2% HOAc) or isopropanol/ammonia/water (IAW) (10:1:1) as descending solvents. Different zones of the chromatograms were located by spraying marker strips with phenol or indole locating reagents. These regions were eluted from the chromatograms with methanol.

The most commonly used reagents for phenols were: (1) 2,6-dichloroquinonechlorimide oversprayed with saturated borax solution; (2) diazotized p-nitroaniline or (3) sulfanilic acid, both (2) and (3) with an overspray of 20% Na_2CO_3. Ehrlich reagent was used for indoles.

Table 1—R_f values of authentic samples and pea seeds eluates (descending paper chromatography)

	IAW		2% HOAc		EAW[a]	BA[a]
Acid	Lincoln	Feltham	Lincoln	Feltham	Feltham	Feltham
Eluate	0.19		0.29	0.24, 0.53	0.62	0.10
Ferulic	0.19		0.28	0.26, 0.52	0.58	0.09
Eluate	0.15	0.15	0.45	0.43	0.55	0.09
Vanillic	0.17	0.17	0.45	0.45	0.57	0.07
Eluate	0.21	0.20	0.51	0.49	0.59	0.11
p-Hydroxybenzoic	0.20	0.22	0.52	0.52	0.59	0.10
Eluate		0.42		0.37, 0.59		0.15
p-Coumaric		0.35		0.36, 0.59		0.16

[a]EAW = ethanol/ammonia/water (35:2:13); BA = butanol/1% ammonia.

Table 2—Ultraviolet data of authentic and unknown compounds in pea var. Feltham seeds

	λ_{max} nm	
Acid	Neutral pH	Alkaline pH
Unknown	256–7; 288–90	276–7; 296
Unknown • Hydrolysate	259	276
Vanillic	257; 290	282
Unknown	254	274; 330
Unknown • Hydrolysate	252–4; 325	274–6; 325–30
p-Hydroxybenzoic	251	274–6
Unknown	285–7; 305	305; 340–2
Ferulic	291; 314	306; 347
Unknown	290–3	332
p-Coumaric	291	334–5

Table 3–R_f values of methylated and reduced products of unknown and authentic ferulic acid (descending paper chromatography)

Compound	Ferulic acid	Unknown	System
Original	0.15	0.15	IAW
Reduced	0.18 0.69	0.18 0.69	IAW
Original	0.26 0.52	0.24 0.53	2% HOAc
Methylated	0.44 0.66	0.44 0.67	2%HOAc

Table 4–R_f values of unknown and authentic IAA (TLC on silica gel)

System	Eluate	IAA
Isobutanol/methanol/water (75:10:15)	0.49	0.48
Isopropanol/ammonia/water (10:1:1)	0.55	0.60
Chloroform/acetic acid (95:5)	0.68	0.68
2% NaCl	0.80	0.83

Spectroscopy and fluorimetry

The spectra of the eluates from the zones previously separated by chromatography were recorded in the range 220–325 nm at neutral and then at alkaline pH.

A Zeiss spectrophotofluorometer ZFM 4C with two monochromators and a Xenon arc-lamp was used to run the activation and fluorescence spectra.

Micro-scale reactions

In some cases, for a full characterization eluate aliquots containing approximately 20 μg of unknown were reduced with $LiAlH_4$ or methylated with diazomethane (Seoane and Carnicer, 1967) and their derivatives analyzed by chromatography.

RESULTS & DISCUSSION

THE ACIDIC fractions from the hydrolyzed and unhydrolyzed extracts were chromatographed with 2% HOAc. When a good resolution was not achieved, a second chromatographic separation was carried out.

The compounds were isolated and characterized by co-chromatography with authentic samples, chromogenic reactions and uv spectra in neutral and alkaline methanol (Tables 1 and 2).

Additionally, ferulic acid was detected by its green fluorescence under uv light on fuming with ammonia, and its identification confirmed by TLC with 2% NaCl as solvent (unknown R_f 0.87; authentic sample R_f 0.86). Its fluorescence spectra were indistinguishable from those of synthetic ferulic acid either at neutral or alkaline pH. Furthermore, unknown compound was methylated with diazomethane or reduced with $LiAlH_4$ on a microscale procedure (Seoane and Carnicer, 1967). The reaction products were chromatographed on paper with IAW or 2% HOAc (Table 3).

p-Hydroxybenzoic acid was identified by TLC in chloroform/acetic acid (95:5) (unknown and authentic sample R_f 0.96). Syringic and vanillic acids were characterized by the distinctive color with diazotized p-nitroaniline (dark blue and red violet, respectively). The reported presence of caffeic and chlorogenic acid (Elema, 1960) was not corroborated.

IAA was detected with Ehrlich reagent and identified by TLC (Table 4). Indole-3-carboxylic acid (ICA) was probably present as judged by chromatographic data.

The negligible concentrations of phenols were spectrally evaluated. Ferulic acid (50 μg/kg d.w. in both the hydrolysate and nonhydrolyzed extract of peas var. Lincoln and 3 and 1 μg/kg, respectively, in var. Feltham) and vanillic acid (6 and 2 μg/kg d.w. in both extracts of var. Feltham) were present in both varieties of pea, both in the hydrolysates and in the nonhydrolyzed extracts. Free and combined p-coumaric and syringic acids were detected in *P. sativum* var. Feltham, but their spectral evaluation showed technical difficulties.

In *P. sativum* var. Lincoln, IAA was characterized at R_f 0.49–0.56. ICA was possibly present in the same zone but in such small amounts that its identification requires further confirmation.

In peas, therefore, the concentrations of phenolic acids and their derivatives do not appear to be present in sufficient quantities to cause any harmful effects in human beings (Singleton and Kratzer, 1969) as other edible seeds are much richer in this class of compound without causing deleterious effects.

Similarly, it seems most unlikely that at so low concentrations they could make a substantial contribution to the taste of the peas.

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CHEMICAL COMPOSITION OF THE SEED OF SUNFLOWER HYBRIDS AND OPEN POLLINATED VARIETIES

SUMMARY—The seed of high oil hybrids and open pollinated sunflower varieties from experimental plantings at nine locations in six southern states in 1969 was analyzed for moisture, crude protein, total oil and fatty acid composition. Total oil content ranged from 28.8–44.7% with an average of 35.3% for hybrid varieties and 39.5% for open pollinated varieties. The crude protein ranged from 16.9–25.1%. All the introductions were relatively low in palmitic and stearic acids. Small amounts of palmitoleic, linolenic, arachidic, behenic and lignoceric acids were present in all samples. Oleic acid ranged from 33.4–62.7% and linoleic acid from 27.3–54.2%. The average oleic and linoleic acid content of the open pollinated varieties at the nine locations was 46.6 and 41.6%, respectively, as compared to 49.4 and 39.6% for the hybrids. The linoleic acid content of sunflower oil varied inversely with temperature during development of the seed. The oil of the sunflowers grown at the warmer locations and at the lower latitudes had a lower linoleic acid content than of those grown at somewhat cooler locations and higher latitudes.

INTRODUCTION

SUNFLOWER SEED is a high-quality vegetable oil which ranks second only to soybean oil in world edible vegetable oil production (USDA, 1971). Presently, there is considerable interest in sunflower oil in the United States for use in processed foods for human consumption.

The fatty acid composition of sunflower seed oil has been reported by a number of scientists to vary with planting location and with climatic conditions during the growing season (Cummins et al., 1967; Earle et al., 1968; Kinman and Earle, 1964; Putt et al., 1969). Using constant temperatures of 10, 16, 21 and 26.5°C for the period of seed development, Canvin (1965) found there was a decrease in linoleic acid and an increase in oleic acid with an increase in temperature. Putt et al. (1969) reported finding a significant variation in the stearic, oleic and linoleic acid compositions of sunflower oils of eight sunflower varieties which they believed were most likely under genetic control. Earle et al. (1968) found that sunflower oil from seed of varieties raised in northern United States or southern Canada typically contained about 70% linoleic acid. Cummins et al. (1967) found that the seed oil of 20 sunflower introductions grown at Experiment, Ga., had an average linoleic acid content of 45.9%. Kinman and Earle (1964) reported that the linoleic acid content of eight sunflower introductions grown at College Station, Tex., ranged from 31.6–52.8%.

Sunflower oil produced in the southern part of the United States with a lower content of linoleic acid and low iodine value may have better keeping qualities and stability characteristics than oil with a higher linoleic acid content. Information is therefore needed on the composition of the oil of new varieties or hybrids of sunflowers to aid in breeding for oil quality in the crop. Accordingly, a study of the chemical composition of selected sunflower varieties grown at nine locations in the Cotton Belt during 1969 was undertaken.

EXPERIMENTAL

THE SEED of high oil hybrid and open pollinated sunflower varieties was obtained from experimental plantings at nine locations in six southern states in 1969. These plantings were part of the 1969 U.S. Regional Sunflower Yield Tests conducted by Dr. Murray Kinman, Oilseed and Industrial Crops Research Branch, USDA, ARS, College Station, Tex.

Single 25-g samples of seed were analyzed for total oil content, crude protein, moisture and fatty acid composition of the oil. The whole seeds were finely ground in a high-speed micro-mill. Moisture was determined by heating 10-g samples of whole seed in a forced-draft oven at 135°C for 3 hr, total oil by extracting 2g of ground sample with petroleum ether in a Butt extractor for 6 hr and nitrogen by the semimicro-Kjeldahl procedure (Cocks and van Rede, 1966). The oils were converted to methyl esters by the BF_3-methanol procedure of Metcalfe et al. (1966). The methyl esters were analyzed by gas-liquid chromatography using a Varian 1740 chromatograph equipped with flame ionization detectors. An 8-ft by 1/4-in. stainless steel column packed with 10% stabilized diethylene glycol succinate (DEGS) on 60/70 mesh chromosorb W-AW-DMCS was used at 200°C. To quantitate linolenic acid, a 6-ft by 1/4-in. stainless steel column packed with 10% butane-1, 4-diol succinate (BDS) on 60/70

Table 1—Sunflower hybrids and introduced varieties tested in 1969

Entry No.	Description and identity	Type
1	P-21 mx × HA 60	High oil, hybrid
2	P-21 ms × HA 61	High oil, hybrid
3	Valley (CM 90RR × Peredovik)	High oil, hybrid
4	Peredovik	High oil, open pollinated
5	Peredovik (66)	High oil, open pollinated
6	VNIIMK 8931	High oil, open pollinated
7	VNIIMK 8931 (66)	High oil, open pollinated
8	Krasnodarets	High oil, open pollinated
9	NK HO 1	High oil, open pollinated
10	Majak	High oil, open pollinated
11	Mingren	Confectionery and birdfeed
12	Arrowhead	Confectionery and birdfeed
13	D-672	Confectionery and birdfeed
14	CM 90RR × Menn. RR-18-1	Confectionery and birdfeed
15	P-21 ms sel × Menn. RR-18-1	Confectionery and birdfeed
16	Manchurian	Confectionery and birdfeed
17	Greystripe	Confectionery and birdfeed
18	P-21-4-1#-4-18# × HA 60	High oil, hybrid
19	P-21-4-4-6# × HA 60	High oil, hybrid
20	P-21-4-4-12# × HA 60	High oil, hybrid
21	P-21-4-1#-2×3 × HA 60	High oil, hybrid
22	P-21-4-1#-4-6# × HA 60	High oil, hybrid
23	P-21-4-1#-4-18# × HA 61-1	High oil, hybrid
24	P-21-4-4-6# × HA 61-1	High oil, hybrid
25	P-21-4-1#-2×3 × HA 61-1	High oil, hybrid
26	P-21-4-1#-4-6# × HA 61-1	High oil, hybrid

Table 2–Average composition data of sunflower open pollinated varieties grown at different planting locations in the South in 1969[a]

Planting location	Planting date (1969)	No. of varieties	Moisture (%)	Protein[b] (%)	Oil[b] (%)	Fatty acid composition of oil (area, %)							
						16:0	16:1	18:0	18:1	18:2	20:0	22:0	Unknown[c]
Rocky Mount, N.C.	5/6	4	5.4	23.0	38.8	5.3	0.2	4.0	47.9	41.2	0.4	0.9	0.2
Clemson, S.C.	4/30	7	6.1	25.1	40.6	5.4	0.1	4.4	42.7	46.6	0.4	0.9	0.3
Experiment, Ga.	4/8	7	6.0	21.0	38.1	6.7	0.2	4.3	40.6	47.1	0.4	0.8	0.2
Tifton, Ga.	4/10	7	5.3	24.7	38.2	6.0	0.2	4.4	50.5	37.1	0.4	1.0	0.2
Crossville, Ala.	4/17	6	5.1	19.6	43.3	5.9	0.2	4.2	44.1	44.3	0.3	0.8	0.2
Crossville, Ala.	5/22	6	4.7	22.4	40.2	5.9	0.1	4.7	37.3	50.5	0.4	0.9	0.2
Camp Hill, Ala.	4/24	6	5.8	25.0	34.9	6.0	0.2	4.8	45.1	42.3	0.5	1.0	0.2
Brewton, Ala.	4/2	6	5.8	24.9	34.9	6.2	0.3	4.6	48.0	39.2	0.5	1.0	0.2
Baton Rouge, La.	4/14	6	5.2	22.4	40.8	5.6	0.2	3.7	54.0	33.9	0.3	0.7	0.2
College Station, Tex.	4/1	7	4.3	19.7	44.7	5.7	0.2	3.4	55.7	33.8	0.3	0.8	0.2
Average			5.4	22.8	39.5	5.9	0.2	4.3	46.6	41.6	0.4	0.9	0.2

[a]High oil type sunflower varieties.
[b]Percent dry basis.
[c]Less than 0.1% is linolenic acid; remainder is unidentified component.

mesh chromosorb W-AW-DMCS was used. The DEGS liquid phase did not separate linolenic acid and an unidentified fatty acid, whereas the BDS liquid phase separated them. The areas under the peaks of individual components were determined by an electronic integrator. Areas and percentages thus obtained were in good agreement with those of mixtures of known compositions. Analysis of fatty acid standards agreed with the stated composition, with a relative error of <2.0% for oleic and linoleic acids.

RESULTS & DISCUSSION

SEED COMPOSITION was studied on 26 high oil (open pollinated and hybrid), confectionery and birdfeed sunflower varieties grown at nine locations in the Cotton Belt in 1969 (Table 1). Six out of seven of the high oil open pollinated varieties were common at eight locations, two out of 12 high oil hybrids were common at nine locations, all of the 12 high oil hybrids were common at three locations, and from one to five confectionery or birdfeed varieties were common at four locations. On the basis of our data on individual varieties, we have concluded, as did Kinman and Earle (1964), that environmental conditions influence the fatty acid composition much more than the genotype of the variety. Therefore, since the chemical composition of the sunflower seed did not appear to be influenced by variety within a type, the average composition of the different varieties grown at each location is reported here.

The average chemical composition data of only the high oil open pollinated and hybrid sunflower varieties are shown in Tables 2 and 3, respectively. The open pollinated varieties were 11.3% higher in oil content than the domestic hybrid varieties (39.5 vs. 35.5%). Also, the oil of the open pollinated varieties contained more linoleic acid and less oleic acid than did the oil from the hybrids. The open pollinated varieties and hybrids had a mean protein content of 22.8 and 21.3%, respectively. The differences between these values are not considered significant, since protein variability among the samples within a variety was frequently as great as between varieties.

All the varieties were relatively low in the saturated fatty acids, palmitic and stearic. Small amounts of palmitoleic, linolenic, arachidic and behenic acids were present in all samples. Lignoceric acid was present at a concentration of 0.2–0.4%; however, we did not analyze all samples for this acid because of its long retention time. Linoleic acid and oleic acid together ranged from 86.8–91.3% of the fatty acid content of the oils. The oleic acid content of individual high oil varieties ranged as follows: open pollinated varieties–34.5% at Crossville, Ala., to 59.0% at College Station, Tex., and hybrids–37.1% at Crossville, Ala., to 62.7% at College Station, Tex. The linoleic acid content of individual high oil varieties ranged as follows: open pollinated varieties–30.4% at College Station, Tex., to 53.0% at Crossville, Ala., and the hybrids–27.3% at College Station, Tex., to 50.1% at Crossville, Ala.

Previous reports (Cummins et al.,

Table 3–Average composition data of sunflower hybrids grown at different planting locations in the South in 1969[a]

Planting location	Planting date (1969)	No. of hybrids	Moisture (%)	Protein[b] (%)	Oil[b] (%)	Fatty acid composition of oil (area, %)							
						16:0	16:1	18:0	18:1	18:2	20:0	22:0	Unknown[c]
Rocky Mount, N.C.	5/6	2	6.1	23.1	34.3	5.3	0.1	3.8	51.3	38.1	0.3	0.9	0.2
Clemson, S.C.	4/30	3	6.4	24.9	33.3	5.2	0.1	4.3	45.8	43.1	0.4	0.9	0.2
Experiment, Ga.	4/8	12	5.4	20.3	35.6	6.0	0.1	4.2	47.6	40.7	0.4	0.9	0.2
Tifton, Ga.	4/10	12	5.5	22.0	35.7	5.7	0.2	3.9	54.5	34.2	0.4	0.9	0.2
Crossville, Ala.	4/17	2	5.2	20.0	36.8	5.5	0.1	4.2	47.4	41.6	0.3	0.9	0.2
Crossville, Ala.	5/22	2	5.0	19.3	37.2	5.7	0.1	4.7	38.3	49.7	0.4	0.9	0.2
Camp Hill, Ala.	4/24	2	6.0	22.9	28.8	5.8	0.2	4.5	47.0	40.9	0.5	1.0	0.2
Brewton, Ala.	4/2	2	6.1	23.5	32.2	6.0	0.2	4.0	48.0	40.4	0.4	0.9	0.2
Baton Rouge, La.	4/14	2	5.4	20.2	36.7	5.5	0.1	3.2	54.8	35.9	0.2	0.7	0.2
College Station, Tex.	4/1	12	4.2	16.9	44.3	5.4	0.2	3.0	58.8	31.4	0.2	0.7	0.2
Average			5.5	21.3	35.5	5.6	0.1	4.0	49.4	39.6	0.4	0.9	0.2

[a]High oil type sunflowers.
[b]Percent dry basis.
[c]Less than 0.1% is linolenic acid; remainder is unidentified component.

1967; Earle et al., 1968) have indicated that sunflower seed oil contains 0.1–0.5% linolenic acid. On the 10% DEGS column, we obtained chromatograms which showed a peak with the proper retention time for methyl linolenate, and on subsequent analysis on the 10% BDS column, the peak was separated into linolenic acid and an unidentified component. A component similar to the latter was identified as eicosenoic acid by Cummins et al. (1967). However, using a fatty acid methyl ester standard containing methyl eicosenoate, we were unable to confirm the identity of this component as eicosenoate. Linolenic acid was found to be present in all of the sunflower seed samples at a concentration of 0.1% or less.

The effect of planting location on the fatty acid composition of sunflower cultivars can be seen by comparing the analyses of samples from six locations (Tables 2 and 3). The oil of both the open pollinated varieties and hybrids grown at the warmer locations (southern Georgia, Louisiana and southeast Texas) had a lower linoleic acid content than of those grown at the somewhat cooler locations (South Carolina, northern Alabama and central Georgia). In fact, the sunflower seed oil from the majority of the planting locations contained either about the same or a higher oleic than linoleic acid content. The only exception in both the open pollinated varieties and hybrids was the late planting in northern Alabama, where the linoleic acid content was substantially higher. The ratio of oleic acid to linoleic acid at the nine locations was 0.9:1 and 0.8:1 for the open pollinated varieties and hybrids, respectively. This can be compared with plantings in northern United States or southern Canada, where the ratio was approximately 4:1 (Earle et al., 1968).

The effect of date of planting and environmental temperature on the chemical composition of sunflower seed is shown in Table 4. The oil from the sunflower seed maturing at the higher temperatures and earlier planting date had a higher oleic acid and a lower linoleic acid content than seed from the later plant date, which matured during the cooler part of the growing season.

Table 4—Effect of planting date on total oil and fatty acid composition of sunflower varieties grown at Crossville, Ala., in 1969[a]

Planting date (1969)	Temperature (°F)[b] Maximum	Minimum	Total oil[c] (%)	Fatty acid (area, %) Saturates	Oleic	Linoleic
April 17	95.9	67.0	41.7	11.2	44.9	43.6
May 22	92.2	64.2	39.4	11.8	37.6	50.3

[a]Data are average of two hybrid and six open pollinated high oil varieties.
[b]Average daily temperature from fertilization to maturity.
[c]Percent dry basis.

The data in Table 4 also seem to indicate that the sunflower seed grown at the higher temperature has the higher oil content. The April 17 planting with a mean daily temperature of 81.5°F from fertilization to maturity had a 5.5% greater total oil content (41.7 vs. 39.4%) than the May 22 planting, which had an average daily temperature of 78.2°F during the same growth period. Canvin (1965) has reported that the total oil content of sunflower seed produced in temperature-controlled growth cabinets increased with increasing temperatures from 50–70°F but then decreased at a temperature of 80°F. However, the data in Tables 2 and 3 showed that the two plantings in Georgia had approximately the same oil content, although the mean temperatures were approximately 3°F higher at Tifton than at Experiment, Ga. Thus, other factors such as the availability of nitrogen and competition for carbon for protein synthesis may be affecting the oil content of the sunflower seed.

A number of explanations have been made to explain the effect of temperature on the biosynthesis of unsaturated fatty acids. Hilditch (1964) has suggested that the saturated fatty acids are synthesized by a different pathway from that of the unsaturated fatty acids. Since the more unsaturated fatty acids have been found to be most abundant in seed grown in cool conditions where the rate of synthesis was relatively slow, he postulated that the most unsaturated fatty acid (linoleic or linolenic) is formed before the monoethenoid acid (oleic). Canvin (1965) theorized that the enzymes responsible for converting oleic acid to linoleic acid were inactivated by the high temperature, and further conversion of oleic acid was prevented. The effect of temperature on the biosynthesis of fatty acids needs to be investigated further.

Plantings of confectionery or birdfeed sunflower varieties were made at only four locations in the Cotton Belt in 1969. Results from the analyses of samples from these plantings are shown in Table 5. The varieties had an average protein content of 19.5% and an average oil content of 26.7%. The percent fatty acid composition of the oil of the varieties was similar to that of both the high oil open pollinated and hybrid type cultivars.

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Table 5—Average composition data of sunflower confectionery and birdfeed varieties grown at different planting locations in the South in 1969

Planting location	Planting date (1969)	No. of varieties	Moisture (%)	Protein[b] (%)	Oil[b] (%)	Fatty acid composition of oil (area, %) 16:0	16:1	18:0	18:1	18:2	20:0	22:0	Unknown[b]
Clemson, S.C.	4/30	1	7.6	20.4	23.1	5.5	0.1	3.8	44.2	46.0	0.3	0.9	0.1
Tifton, Ga.	4/10	4	6.4	20.1	26.6	6.5	0.3	3.4	52.0	35.9	0.4	1.0	0.2
Baton Rouge, La.	4/14	2	6.1	18.4	23.8	6.2	0.3	3.0	49.9	39.5	0.3	0.7	0.2
College Station, Tex.	4/1	5	5.0	19.2	33.4	6.0	0.2	3.1	56.0	33.4	0.3	0.8	0.2
Average			6.3	19.5	26.7	6.1	0.2	3.3	50.5	38.7	0.3	0.9	0.2

[a]Percent dry basis.
[b]Less than 0.1% is linolenic acid; remainder is unidentified fatty acid.

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Seed samples were obtained from the following: W.T. Fike, North Carolina State University, Raleigh, N.C.; J.A. Martin, Clemson University, Clemson, S.C.; D.L. Thurlow, Auburn University, Auburn, Ala.; B.J. Johnson, Georgia Experiment Station, Experiment, Ga.; W.H. Marchant, Georgia Coastal Plain Experiment Station, Tifton, Ga.; B.E. Newman, Louisiana State University, Baton Rouge, La.; M.K. Kinman, Crops Research Division, USDA, ARS, College Station, Tex.

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IDENTIFICATION AND QUANTITATION OF SEVERAL NONVOLATILE ORGANIC ACIDS OF COCOA BEANS

SUMMARY—Organic acids precipitated as lead salts from water extracts of cocoa beans were converted to trimethylsilyl ethers. TMS ethers were then separated and identified using a combined gas chromatograph-mass spectrometer. Citric, phosphoric, lactic, oxalic, malic, tartaric, succinic and gluconic acids were present in every sample examined, irrespective of geographic origin. Five of the acids were quantitated using a GLC procedure which involved the preparation of methyl esters from freeze-dehydrated, water extracts. Concentration ranges found, g/100g beans, were: lactic, 0.11–0.71; oxalic, 0.24–0.43; succinic, 0.02–0.07; malic, 0.02–0.10; citric, 0.56–1.32. Concentrations of lactic acid were lowest and citric acid levels tended to be highest in commercial samples from countries where cocoa beans usually are not subjected to a planned fermentation. These trends were confirmed in an experiment involving beans from Trinidad which had undergone a carefully controlled fermentation. Only minor differences were found in organic acid concentrations between roasted and unroasted cocoa beans.

INTRODUCTION

ALTHOUGH PHOSPHORIC ACID and lactic, tartaric, malic, succinic, oxalic, and citric acids have been found in cocoa beans (Mosimann, 1947; Kaden, 1955; Wolf, 1958; Dietrich et al., 1964; Rohan and Stewart, 1964), only the latter two have been quantitated. Mosimann (1947) reported the shell-free mass to be 0.32–0.50% by weight oxalic acid and 0.45–0.75% citric acid. Diverse agronomic, harvesting, fermenting and drying practices for cocoa, a commodity of tropical agriculture, might be expected to affect the concentration of these acids which could ultimately influence quality characteristics in chocolate. Awareness of a need for more information about the organic acids of cocoa beans, especially quantitative aspects, prompted the investigation reported herein.

Initially, organic acids were isolated from cocoa beans as lead salts and separated by GLC of trimethylsilyl (TMS) ethers. This approach was useful for identification purposes, but it did not yield reliable quantitative data. Concentrations of five of the acids were established by GLC-methyl ester analyses of water extracts of the beans.

EXPERIMENTAL

Samples

Selected for analysis were beans from Ghana, Brazil (Bahia), Trinidad, Dominican Republic (Sanchez), Mexico (Tabasco), Ecuador (Arriba), Samoa and New Guinea. Most analyses were conducted on unroasted beans. The effect of the roasting variable was assessed by comparing cocoa beans before and after roasting 30 min in a laboratory forced-air oven at a circulating air temperature of 150°C.

Lead salt method

Procedures described by Fitelson and Bowden (1968) for vanilla and Brunelle et al. (1967) for wine were modified for the analysis of cocoa beans. 15g of beans were pulverized (in a Waring Blendor for 5 min) with 100 ml of water and 10 ml of a solution of internal standard (100 mg glutaric acid). The water dispersion was passed through a bed of Celite 545, and lead salts were precipitated, washed and dried according to the prescribed procedures.

A portion of the oven-dehydrated mass (20 mg) was dissolved in 2 ml pyridine and reacted with 1 ml hexamethyldisilazane and 0.6 ml trimethylchlorosilane. TMS ethers were separated on a 6-ft by 1/8-in. stainless steel column packed with 5% SE-30 on 80/100 mesh Hi-Performance AWDMCS-treated Chromosorb G (Supelco Co., Bellefonte, Pa.). The column was programmed 90–260°C at 6°C/min in a Hewlett-Packard Model 5750 gas chromatograph equipped with a hydrogen flame detector. Nitrogen was the carrier gas at 30 ml/min.

Methyl ester method

The procedure of Harvey et al. (1970) for tobacco leaf was adapted to cocoa beans. 100g of beans were pulverized with 475 ml water and 25 ml internal standard solution (5 mg glutaric acid/ml water) for 6 min in a Waring Blendor. The mixture was filtered through Whatman No. 1 paper and 40 ml of the extract was freeze dehydrated to constant weight. A relatively moisture-free sample is a requirement for a successful esterification reaction.

The freeze-dehydrated mass and 50 ml esterification solution (Harvey et al., 1970) were mechanically shaken for 30 min and then held 24 hr at room temperature. The reaction mixture was filtered through Whatman No. 1 paper into a separatory funnel, 50 ml water added, and the methyl esters recovered by extraction with 4 × 10 ml distilled chloroform. After drying over $MgSO_4$ the chloroform extract was filtered and concentrated to half volume.

Separation of methyl esters was obtained on an 8-ft by 1/8-in. stainless steel column packed with 2% H_3PO_4-treated diethyleneglycoladipate (10%) on 80–100 mesh Gas Chrom A (Applied Science Labs., State College, Pa.). The column was programmed 90–190°C at 6°C/min and then held isothermally for 9 min. Nitrogen was the carrier gas at 30 ml/min.

This procedure proved satisfactory for lactic, malic, succinic and citric acids. However, because oxalic acid sublimes, an alternative method was found necessary for quantitation of this acid. The modification consisted of making the extract alkaline with NaOH and freeze dehydrating a solution of sodium salts rather than free acids. Thereafter, the methyl ester method was the same as for the other acids.

Since the same amount of glutaric acid was added to all samples, peak area for each acid on a chromatogram divided by peak area of glutarate gave values that reflected quantitative differences among the bean samples. A standard curve for each acid was then used to convert these values to concentration expressed as g acid per 100g beans. The standard curve was constructed from data obtained in the analysis of solutions which contained varying levels of the acid in combination with the same amount of glutaric acid added to cocoa bean samples. These solutions were freeze dehydrated, methylated, extracted and analyzed by GLC exactly as described for cocoa beans. Plots of acid content relative to glutarate were linear over the range of concentrations found for each acid in cocoa beans.

Mass spectrometry

Peaks appearing on the chromatograms of organic acids recovered by both the lead salt and methyl ester methods were identified using an LKB Model 9000 combined gas chromatograph-mass spectrometer. Mass spectra and retention times were compared to data obtained for reference compounds and with published spectra for TMS and methyl ester derivatives. Glass columns, 6-ft by 1/4-in., containing the previously described packing materials were used in the GLC-MS phases of the investigation.

RESULTS & DISCUSSION

SEVEN ORGANIC ACIDS and phosphoric acid were identified in the lead salt precipitate of cocoa beans. Gluconic acid had not been reported previously. While the other acids, lactic, oxalic, succinic, malic, tartaric, citric and phosphoric, had been found in cocoa beans by others, the literature on the subject is quite meager and contradictory. The current investigation contributed a more positive identification for each acid.

Figure 1 is representative of the chromatograms obtained for extracts of cocoa beans using the lead salt-TMS method. The same acids were present in all samples, irrespective of geographic origin. In

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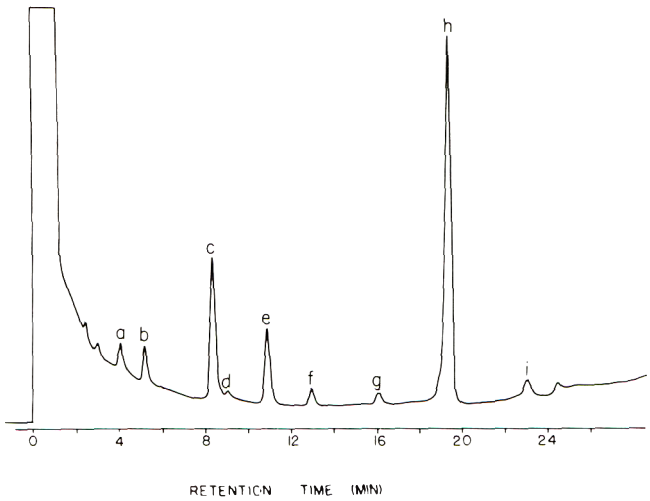

Fig. 1–Typical GLC chromatogram of organic acids extracted with water from cocoa beans, precipitated as lead salts and separated as TMS ethers. a) Lactic; b) oxalic; c) phosphoric; d) succinic; e) glutaric (internal standard); f) malic; g) tartaric; h) citric; i) gluconic.

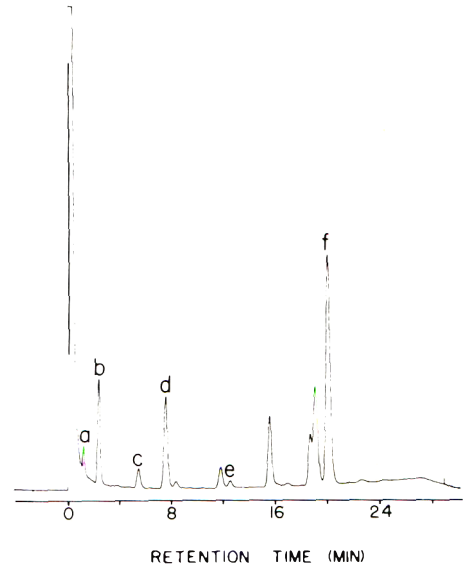

Fig. 2–Typical GLC chromatogram of organic acids extracted with water from cocoa beans and analyzed as methyl esters. a) Lactate; b) oxalate; c) succinate; d) glutarate (internal standard); e) malate; f) citrate.

every instance, phosphoric and citric acids were the dominant acids as judged by relative peak areas. This conforms with the observations of Rohan (1964), who found that the largest spots were caused by citric and phosphoric acids on paper chromatograms which also showed tartaric, lactic and two unidentified acids.

The lead salt method was found not applicable to quantitative measurements. Precipitation was not complete and losses occurred when the salts were washed to remove traces of sugars and other substances.

The methyl ester method for organic acids yielded chromatograms similar to that shown in Figure 2. Separations suitable for quantitative purposes were obtained for lactic, oxalic, succinic, malic and citric acids. Other peaks appearing on the chromatograms are the internal standard (glutarate) and the methyl esters of palmitic, stearic and oleic acids arising from traces of lipid extracted with the organic acids. All peaks, including the fatty acid esters, were identified according to retention times and mass spectra.

Cocoa fat contains 3% by weight linoleic acid which would elute near citrate under the GLC conditions employed in this investigation. However, mass spectral scanning did not reveal any linoleate under the citrate peak, and no allowance for this minor component was deemed necessary in calculating citric acid concentration.

Table 1–Concentrations of several organic acids in cocoa beans, extracted with water and measured as methyl esters

	Acid concentration, g/100g beans				
Type of bean	Lactic	Oxalic[a]	Succinic	Malic	Citric
Sanchez	0.14	–	0.03	0.10	1.32
Sanchez	0.12	0.24	0.02	0.05	0.67
Sanchez	0.14	–	0.03	0.10	1.04
Tabasco	0.11	0.28	0.03	0.05	0.68
Tabasco	0.11	–	0.04	0.07	0.58
Arriba	0.43	0.39	0.04	0.10	0.79
Trinidad	0.71	0.29	0.05	0.04	0.62
Trinidad	0.55	0.35	0.05	0.05	0.68
Ghana	0.31	0.36	0.04	0.06	0.59
Ghana	0.22	0.43	0.03	0.05	0.62
Bahia	0.42	0.38	0.04	0.04	0.56
Bahia	0.29	0.41	0.07	0.02	0.64
Bahia	0.13	–	0.03	0.03	0.63
Bahia	0.19	–	0.03	0.04	0.57
Samoa	0.66	–	0.05	0.10	1.05

[a]Not determined.

Concentrations of individual acids found in cocoa beans of different geographic origins are presented in Table 1. For some varieties, beans from several manufacturers are reported; for others only single samples were available for study. In most instances, values shown are averages of three or more complete analyses starting with the raw bean sample. Based on 14 trials using Sanchez beans, standard deviations, g/100g beans, were: lactate, ± 0.02; succinate, ± 0.007; malate, ± 0.017; citrate, ± 0.042. Standard deviation for oxalate, determined separately on extracts neutralized prior to freeze dehydration, was ± 0.028g/100g beans.

Among the five acids measured by the methyl ester method, citric acid was present in the highest concentration ranging from 0.56g/100g in a Bahia sample to 1.32g/100g in one lot of Sanchez beans. Concentration ranges for lactic and oxalic acids were 0.11–0.71 and 0.24–0.43g/-100g beans, respectively. Succinic acid and malic acid were present only in trace amounts. Values for oxalic and citric acids agree quite well with Mosimann (1947).

As reflected in Table 1, concentrations

Table 2—Concentrations of several organic acids in unfermented and fermented beans from Trinidad

Acid	Unfermented	Fermented
	(g/100g Beans)	
Lactic	0.15	2.34
Oxalic	0.25	0.28
Succinic	0.06	0.03
Malic	0.11	0.09
Citric	0.98	0.42

of individual acids varied as much among different lots of beans from the same locale as between samples chosen to represent the major geographic regions of their production. Most beans are subjected to a planned fermentation which might last as long as 8 days. Moisture content is then reduced to less than 8%, a storable level, most commonly by sun drying (Rohan, 1963). In the period between opening of the pods and final drying, many biochemical changes related to organic acid content would occur, the magnitude of which would be influenced by the processes employed, the degree to which these processes are standardized and by the variableness of climatic conditions in the tropics. Consequently, differences shown in Table 1 were not unexpected.

The only obvious correlation relating to bean type or origin involves the Sanchez and Tabasco beans which traditionally are not subjected to a fermentation, although they undoubtedly do receive a partial or quasifermentation during the early stages of sun drying. These beans were consistently low in lactic acid concentration and two of the three Sanchez samples were high in citric acid content.

Fortunately, through the cooperation of V.C. Quesnel, Cocoa Research Dept., University of West Indies, St. Augustine, Trinidad, we were able to examine beans which had undergone a rigidly controlled fermentation. A batch of beans, after opening of the pods, was divided and one portion, representing unfermented beans, was washed with water to remove the pulp and then rapidly dried. The other portion was fermented for 8 days in a sweat box and then dried as per the unfermented sample. The organic acid distribution in these unfermented and fully fermented beans is presented in Table 2.

This single, but controlled experiment, supports the interpretation given for the data in Table 1 concerning lactic and citric acids; namely, that the concentration of lactic acid increases and citric acid decreases during fermentation. Results also indicate that the acids measured in the study are normal components of ripe cocoa beans and, except for lactic acid, they remain relatively unchanged (oxalic acid) or decrease in concentration during fermentation (succinic, malic and citric acids). Rohan's (1963) review of the literature on cocoa processing states that citric acid in the pulp is consumed and decreases during fermentation.

The effect of roasting on the organic acids was assessed by comparing beans before and after roasting under conditions believed to simulate commercial conditions. A slight downward trend was observed, but the magnitude of change was within the range of experimental error. It was concluded that roasting does not materially affect the concentration of organic acids.

Concentrations of organic acids found in cocoa beans were relatively high even when compared to many acid type foods. However, chocolate usually is not distinguished as having a tart taste. This is attributable in part to the diluting effect of sugar and cocoa butter added during its manufacture. The high fat content and nonaqueous character of chocolate, its bitter and sweet tastes and the existence of the acids in bound and salt forms would further suppress tartness. Nevertheless, the variable and high concentrations found suggest that the organic acids, especially citric and lactic, could be considered as potentially important factors contributing to differences in flavor noted among some chocolate samples.

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STUDIES OF A METHOD FOR DETERMINING ORGANOCHLORINE PESTICIDE RESIDUES IN ANIMAL TISSUE

SUMMARY—Three different studies were conducted to evaluate the accuracy and reliability of a widely accepted method for determining organochlorine pesticide residues in animals' tissues. These investigations involved laboratories of both state and federal regulatory agencies. Various adipose tissues, heat-extracted and non-heat-extracted, spiked and unspiked samples were analyzed by these laboratories. Results of these studies demonstrated many inaccuracies and inconsistencies associated with using the current method. The laboratory performing the analysis, as well as the tissue sample used, was shown to significantly ($P < .05$) affect the results. Further, the heat-extraction phase, as well as the subsequent analytical phase of the method, was observed ($P < .05$) to present problems of repeatability.

INTRODUCTION

PUBLIC ATTENTION is currently focused on the widespread use of chemical pesticides in the environment (Train, 1970) and on their relationship to environmental health (Mrak, 1969; Kay, 1970). Man's food chain is probably the most important aspect of his environment that becomes contaminated from pesticide use (Westlake and Gunther, 1966). Consequently, there is always a pressing need for rapid and reliable analytical procedures to identify minute traces of unwanted chemicals in natural products (Lisk, 1970). With the advent of electron capture gas chromatography, Mills et al. (1963) of the Food and Drug Administration (FDA) revised an earlier method (Mills, 1959) for determining organochlorine pesticide residues in foods. Subsequent modifications of this revised procedure have been attempts at improving extraction (Kroger, 1966; Porter et al., 1967), clean-up (Langlois et al., 1964; Burke and Malone, 1966), concentration (Burke et al., 1966) and at reducing the sample size (Kroger, 1966; Furman and Fehringer, 1967). Currently, the FDA, the U.S. Department of Agriculture's Meat Inspection Division (MID) and the Pennsylvania Department of Agriculture (PDA) laboratories use a modified Mills procedure in their regulatory efforts. These agencies routinely sample man's food supply and subsequently condemn products in which the amount of pesticide residues exceeds the maximum legal tolerances established by the FDA (1969).

This paper reports results from several studies of the modified Mills procedure involving laboratories of both state (PDA) and federal (MID) regulatory agencies and the University (PSU). These studies were conducted to evaluate the accuracy and reliability of repeatability of the current procedure between laboratories.

EXPERIMENTAL

Experimental design

Three different studies were conducted in which all analyses were done in duplicate. All samples were coded to protect their identity. Solvents used were triple distilled.

In the first study, each of four cooperating laboratories received one heat-extracted fat sample from each of six bovine adipose tissue sites (2—internal sites: omental or intestinal, and kidney or perirenal; 4—external sites: the scrotal or cod area, chest or brisket, back or twelfth rib and caudal or tailhead). The experimental animal carcass was that of a castrated male (steer) Aberdeen Angus (*Bos taurus*) contaminated by ingesting DDT. Based on the pesticide residue determinations in our laboratory, a statistical analysis indicated no significant differences ($P > .05$) between sites, but all samples exceeded the maximum level of 5 ppm permitted by FDA regulations (1969). The purpose of this study was to evaluate the repeatability of the analytical procedure using many samples which did not differ in pesticide content.

In the second study, the repeatability of determining the amount of HEp, DDE, DDD, p,p′-DDT and total DDT (DDE + DDD + p,p′-DDT) in each of the six adipose tissue samples used in the first study was evaluated in our laboratory.

In the third study, each of the seven cooperating laboratories received 20 scrotal adipose tissue samples. Three heat-extracted and three raw tissue samples from each of three Angus steer carcasses were sent to each laboratory. In addition, from one of these carcasses each laboratory received one unspiked heat-extracted tissue sample, and one sample spiked with 2 ppm of p,p′-DDT. The p,p′-DDT was added to the liquid, heat-extracted sample and the sample thoroughly mixed before cooling. This study was designed to evaluate the repeatability of the extraction phase as well as the analytical phase of the procedure.

Sample preparation

Fresh adipose tissue samples were excised from the carcasses 48 hr post-mortem. They were then placed in glass jars, flushed with nitrogen gas and stored at 29°C. Subsequently, the heat-extracted samples were prepared by grinding 100g of the frozen tissue twice through a 0.65-cm plate in a 2°C cold room. This ground sample was then extracted at 105°C for 24 hr in a forced-air oven, the residue filtered away, the remaining liquid extract thoroughly mixed, 10-g liquid samples weighed out, cooled, reweighed and stored under nitrogen at 29°C. All samples (heat-extracted, raw tissue, spiked, unspiked) were shipped under dry ice to the cooperating laboratories. Detailed instructions for the handling of the samples, heat-extraction procedures and subsequent analytical determinations accompanied each shipment.

Pesticide analysis

The entire 10-g sample was extracted. All the laboratories involved in this study followed the modified Mills procedure (FDA, 1968), as detailed in the instructions. Electron capture

Table 1—Total DDT (in ppm) analysis of bovine adipose tissue samples by the four cooperating laboratories and our laboratory (ML)

Tissue sample	Laboratory A	B	ML	C	D	Deviation[b]
Internal						
Omental	16.00	7.99	7.39	3.14	1.63	65.0
Perirenal	15.10	12.82	7.61	2.88	0.83	79.5
External						
Scrotal	14.60	9.37	8.80	2.95	2.35	53.0
Caudal	15.20	8.91	7.93	2.94	0.41	65.4
Back	12.70	6.03	8.70	3.08	1.23	56.8
Brisket	16.00	7.11	8.35	3.10	1.06	64.2
All samples[a]	14.93	8.70	8.13	3.01	1.25	59.6
Deviation[b]	83.6	7.0	—	63.0	84.6	

[a]Any two means not connected by the same solid line are significantly different ($P < .05$) from each other.
[b]Average deviation (in percent) from the ML values.

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Table 2—Repeatability of procedure for determining the amount (in ppm) of various organochlorines in bovine adipose tissue samples[a]

Tissue sample	Organochlorine residue type				
	HEp	DDE	DDD	DDT	Total DDT
Scrotal	0.24	2.36	0.60	5.84	8.80
Back	0.25	2.66	0.57	5.47	8.70
Brisket	0.25	2.32	0.54	5.49	8.35
Caudal	0.27	2.15	0.70	5.08	7.93
Perirenal	0.22	2.00	0.56	5.05	7.61
Omental	0.22	1.93	0.61	4.85	7.39
Mean (ppm)	0.24	2.24	0.60	5.30	8.13
Deviation (%)	8.3	12.5	10.0	2.5	7.1

[a]Any two means not connected by the same solid line are significantly different ($P < .05$) from each other.

Table 3—Repeatability of extraction and detection procedures for determining the amount (in ppm) of DDT in bovine adipose tissue samples

Tissue sample	Cooperating laboratory						
	A	B	C	D	E	F	G
Extraction samples[a]							
MLEX[b]	14.8	17.5	44.1	19.4	14.4	8.5	8.6
RTSSE[c]	8.8	20.9	42.8	20.1	14.9	8.1	8.4
	6.0	3.4	1.3	0.6	0.5	0.4	0.2
Detection samples							
SPIKE[d]	11.4	11.6	30.9	13.7	9.8	6.6	6.7
UNSPK[e]	9.6	10.7	27.3	11.5	7.9	6.4	6.5
Observed	1.8	0.9	3.6	2.2	1.9	0.2	0.2
Actual	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	0.2	1.1	1.6	0.2	0.1	1.8	1.8

[a]Any two means not connected by the same solid line are significantly different ($P < .05$) from each other.
[b]ML heat-extracted (105°C) fat samples.
[c]Raw tissue samples subsequently heat extracted (105°C) by each laboratory.
[d]ML heat-extracted fat sample, spiked with 2 ppm of p,p'-DDT.
[e]ML heat-extracted fat sample, unspiked.

(EC) gas chromatography was employed in the determinative step. However, not every laboratory used the same make or model instrument. Nevertheless, operating conditions were similar. Separation was performed on 6- by 1.8-mm columns coated with 10% DC-200 (Dow Corning Silicone Fluid 12,500 cst) on 60–100 mesh support (Anakrom ABS or Gas Chrom Q). The carrier gas was either nitrogen or helium, depending on the laboratory. Column temperatures were 225°C and injection temperatures 250°C. Quantitative and qualitative standards, thin-layer chromatography and other GLC columns were used to verify identification of the organochlorine compounds (FDA, 1968).

Data analysis

Quantification of all results involved peak area determinations using the triangulation method. The ppm concentration found in different tissue sites was obtained using the following formula:

$$\text{ppm} = \frac{1.67 \times P_W \times C_S \times A_u}{A_k \times F \times W}$$

where 1.67 = a correction factor based on the 3-g weight of 5 ml of ether; P_W = weight in grams of petroleum ether fraction containing pesticide residues used in injection of GLC column; C_S = concentration of known standard in nanograms; A_u = area of unknown peak in square centimeters; A_k = area of standard peak in square centimeters; F = fraction of P_W weight injected in μl; W = tissue sample weight in grams. The data collected were analyzed by the analysis of variance (Snedecor, 1956) using an IBM 360/67 Computing System. Between mean differences from group to group were compared by Duncan's multiple range test (Duncan, 1955).

RESULTS

First study

The total DDT analyses of bovine adipose tissue samples by the four cooperating laboratories (representing both state and federal agencies) as well as our own laboratory are summarized and presented in Table 1. Statistically significant ($P < .05$) differences between laboratories for the analyses were observed. The amount of deviation from the values obtained in our laboratory were calculated and are recorded in this table.

Second study

The repeatability or difficulty in determining the individual type of organochlorine pesticide residue in bovine adipose tissue samples can be observed by examining the data tabulated in Table 2. No statistically significant ($P > .05$) differences within residue type were noted. The error percent associated with the determination of each type ranged from 2.5% for DDT to 12.5% for DDE. Most of the values were 10% or less.

Third study

Table 3 summarized the data compiled from seven state and federal cooperating laboratories. The repeatability of the heat-extraction phase was noted to differ statistically ($P < .05$) between the various laboratories. It can also be observed that the laboratories which showed some trouble in adequately heat extracting the samples were not necessarily those that had trouble with the analytical phase.

DISCUSSION

RESULTS OF THE STUDY tabulated in Table 1 indicate the method itself is not consistently accurate and reliable when the analyses of various laboratories are compared. Although the statistical analyses could not demonstrate ($P > .05$) any difference between the mean DDT values of all samples analyzed by Laboratory B and our laboratory (ML), results of the analyses from the other cooperating laboratories did differ significantly ($P < .05$) from ours. Values reported by Laboratory A were higher, whereas those from C and D were lower than ML values. Analyses from both Laboratories A and D deviated approximately 84% from our own. A 63% deviation was noted for those from Laboratory C. Based on the composite analyses of all samples by ML, the total DDT value of 8.13 ppm indicates this carcass should be condemned for human food, since it exceeds the maximum permissible legal (FDA, 1969) limit of 5 ppm. However, the data show that only Laboratories A and B would recommend condemnation, whereas C and D would pass it as acceptable for human consumption. Such results appear to indicate that the data from food surveillance programs are questionable and certainly suggest law enforcement and compliance efforts will be difficult.

Another observation (see Table 1) is that the location or type of sample may affect the results obtained. More deviation from ML DDT values was noted for

the internal (72.3% average) as compared to the external (59.9% average) tissue samples. This is quite interesting, since it is the usual practice of state and federal regulatory agencies to use the analyses of internal carcass tissue samples as the basis for either acceptance or condemnation of meat for food. Our results would suggest external samples should be used and preferably scrotal samples, since these showed the least variation (53.0%). Perhaps an explanation of this sample type effect is related to the chemical and morphological differences between tissues. We have noted that the more saturated lipids are found in internal rather than external sites (Huston et al., 1965). Lisk (1970) comments that the solubility of DDT in the fat is affected by the degree of hardness (saturation) of the fat. We have also observed, although not reported, that more nonlipid-extractable tissue residues are associated with the internal adipose tissue.

It is rather obvious from the data presented in Table 2 that the procedure was quite repeatable for determining the individual amount of each pesticide residue species. With the exception of DDE, all samples were within 10% of the mean value. It also appears that DDE is the primary metabolite of DDT and this supports the degradation pathway of DDT metabolism suggested by Datta (1970).

A statistical analysis of that portion of the third study related to examining the heat-extraction phase (see Table 3) demonstrated significant ($P < .05$) differences between laboratories. Results reported by Laboratory A showed a 6 ppm discrepancy in their ability to satisfactorily heat extract the tissue sample. The best correspondence between the values of MLEX (ML heat-extracted samples) and RTSSE (raw tissue samples subsequently heat extracted by each laboratory) was recorded for Laboratories F and G. Although the results of Laboratory A as compared to F and G were significantly different ($P < .05$), those between other laboratories were not ($P > .05$). The spiked-unspiked comparisons indicated that those laboratories which had trouble with the heat-extracting phase did not necessarily have problems in performing the subsequent analysis. However, these results do suggest inconsistencies in this portion of the procedure too. Three laboratories (A,D,E) were accurate, three (C,F,G) inaccurate and one (B) intermediate in determining the amount of DDT in the spiked sample.

Although detailed instructions were sent to every laboratory, human errors in interpreting and executing these instructions cannot be discounted as a source of variation in the results obtained.

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Abbreviations: HEp, 1,4,5,6,7,8,8-heptachloro-2,3-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindan; DDE, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene; DDD, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane; DDT, 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane + 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane; total DDT = DDE + DDD + DDT; ppm, parts per million.

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ON THE NATURE OF COOKED MEAT HEMOPROTEIN

SUMMARY—Reflectance spectra of cooked meat and the precipitates obtained on heating aqueous muscle extracts, mixtures of bovine serum albumin (BSA) and horse heart metmyoglobin (Mb) at 60°C, and Mb alone at > 80°C were all similar. BSA-Mb mixtures in 5.5 M urea at pH 6.0 yielded soluble complexes that were spectrally the same as those present in cooked meat. Sephadex chromatography showed these complexes to be denatured hemoproteins where the protein could be denatured BSA or dimers or higher aggregates of denatured apomyoglobin (ApoMb). The affinity of the hematin for denatured BSA was much greater than for the ApoMb aggregates. No evidence was obtained for complexes of a type where hematin was coupled with both denatured ApoMb and denatured BSA. It is suggested that the complexes present in cooked meat are also denatured hemoproteins, where the protein may be any of several of the denatured proteins present in cooked meat. An electron-spin resonance (ESR) spectra of cooked meat indicated that at −196°C the ferric ion in the hematin was essentially low-spin. The mechanism of formation and the nature of the possible bonding between hematin and denatured protein are discussed.

INTRODUCTION

WHEN MEAT is heated, the hemoproteins (mainly myoglobin plus some hemoglobin) react to form the brown insoluble pigments typical of cooked meat. It is well established that the iron in the porphyrin is in the ferric state (Tappel, 1957). Only tentative suggestions, using analyses of the reflectance spectra of cooked meat (Tappel, 1957; Tarladgis, 1962), have been made about the nature of the two ligands on the ferric ion at the positions not bonded to the four nitrogen atoms of the porphyrin.

With the increasing tendency to market precooked, partially cooked and pasteurized meats, it is desirable to know the nature of these cooked meat hemoproteins, expecially since they allegedly catalyze lipid oxidation and so decrease storage life (Tarladgis, 1962).

Tappel (1957) suggested that the hemoproteins in cooked meat were mixed denatured globin nicotinamide hemichromes. Tarladgis (1962) argued that such compounds would yield low-spin ferric ion (Brill and Williams, 1961), i.e., one unpaired *d* electron. Since the reflectance spectrum of cooked meat was typical of high-spin ferric ion, i.e., five unpaired *d* electrons, Tarladgis suggested that the ligands at the fifth and sixth positions were a carboxylate group from denatured globin and water.

Thus, both workers postulated denatured globin as one of the ligands in the complex. However, myoglobin is precipitated in meat at temperatures of 60°C and above, although in pure solution, at the same pH, marked denaturation and precipitation occur only at temperatures above 75°C (Bernofsky et al., 1959). Draudt (1969) found that the precipitation of myoglobin in meat was dependent on the nature of the ligand at the sixth position, and on the oxidation state of the iron. The stabilities of the various myoglobin derivatives were in the same order as in pure solution, although precipitation in meat occurred at temperatures about 15–30°C below that required in the pure system at the same pH. He concluded that myoglobin apparently co-precipitates with other proteins and part of the thermal denaturation behavior of myoglobin has to be attributed to the denaturation of the other proteins in meat.

The thermal denaturation of myoglobin, in aqueous solution, has been extensively studied (Acampora and Hermans, 1967; Awad and Deranleau, 1968), as has the stability of myoglobin in the presence of inorganic ions and organic solvents (e.g., Cann, 1964; Herskovits and Jaillet, 1969). On the other hand, little work appears to have been done on the stability of myoglobin in meat, viz., in the presence of an excess of other proteins. It is known, however, that the hematin of hemoglobin is transferred to gelatin if incubated with a large excess of gelatin at 37°C (Blyumenfel'd and Charnyi, 1950) and that albumin can combine with hematin to yield methemalbumin, the albumin being attached by means of the carboxyl groups of the protoporphyrin (O'Hagan, 1959).

The present study was undertaken to explain the nature of the hematin complex(es) present in cooked meat and the role of the other water-soluble proteins in its (their) formation.

EXPERIMENTAL

Materials & Methods

Bovine serum albumin (BSA) (Cohn fraction V, Sigma Chemical Co.) and salt-free horse heart metmyoglobin (Mb) (Mann Research Labs.) were used without further purification. Any insoluble Mb was removed by filtration after preparation of the solution. The urea solutions used in the chromatography experiments were frozen after 120 hr, stored at −20°C and thawed when required.

Aqueous muscle extracts were prepared by vigorously shaking minced, lean beef muscle with an approximately equal volume of either distilled water or 0.05 M phosphate (pH 6.0) at 4°C and filtering after standing overnight. The extract usually had a total hemoprotein concentration of 3–7 mg ml^{-1}. The distilled water extracts were adjusted to the required pH with 0.1 N HCl or 0.1 N NaOH. The phosphate extracts were used in the chromatography experiments, as at these dilutions a marked change in pH occurred on heating. This was not observed in the concentrated, unfractionated samples.

10-ml aliquots of the distilled water extracts were heated in preheated, stoppered thin-walled glass test-tubes at the required temperature (± 0.05°C). After the appropriate time (usually 2 hr) the test-tubes were cooled in ice-water and the contents filtered. When necessary, the total weight of protein precipitated was determined by weighing the ether-washed filtrate, dried at 37°C. The total hemoprotein concentration in the solutions was determined from the absorption at 525 nm, an isobestic point for the oxy, reduced and oxidized forms of both hemoglobin and myoglobin (Bowen, 1949; Lemberg and Legge, 1949) ($\sum_{525}^{1mM} = 7.5$).

Sephadex chromatography

All solutions were fractionated at 22 ± 1°C, on Sephadex G-100 (superfine) (bed volume 360 ml, height 43 cm and flow rate about 12 ml hr^{-1} in urea/phosphate; bed volume 350 ml, height 42 cm and flow rate about 15 ml hr^{-1} in phosphate).

To determine which species were responsible for the increased myoglobin precipitation in aqueous muscle extracts, the following experiments were performed. 5-ml aliquots of a concentrated muscle extract, in 0.05 M phosphate buffer (pH 6.0) were fractionated, and the absorbances of the 5-ml eluted fractions determined at 410 nm (for the hemoprotein) and 280 nm (for all proteins). From each separation the fractions containing myoglobin (Mb) were combined and 4-ml aliquots of this Mb solution were mixed with 4 ml of the fractions corresponding to the other protein-rich peaks in the elution profile. These mixtures, plus solutions containing only the separated hemoglobin (Hb) and separated Mb, were heated and the amount of Hb and Mb precipitated determined.

To determine the complexes present in the Mb-BSA solutions, in 5.5 M urea (> 99.5% pure) at pH 6.0, 5-ml aliquots of these solutions were fractionated and the hematin and protein contents of the eluted fractions determined as the absorbances at 410 nm (hematin) and 280 nm (protein). In all cases the eluted fractions were collected by counting 128 drops.

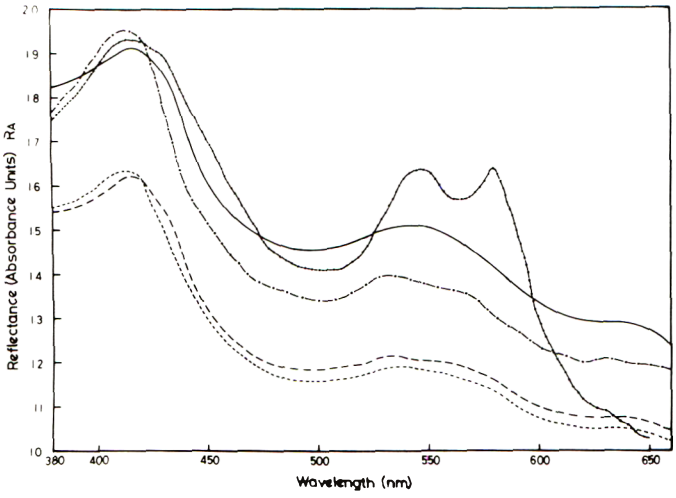

Fig. 1–Reflectance spectra of ——cooked meat; – – – precipitate from aqueous muscle extract heated to 60°C; · · · · · precipitate from a solution of Mb-BSA heated to 60°C; – • – • precipitate after heating Mb to 90°C; · ·· ·· ·· uncooked meat (mainly MbO_2).

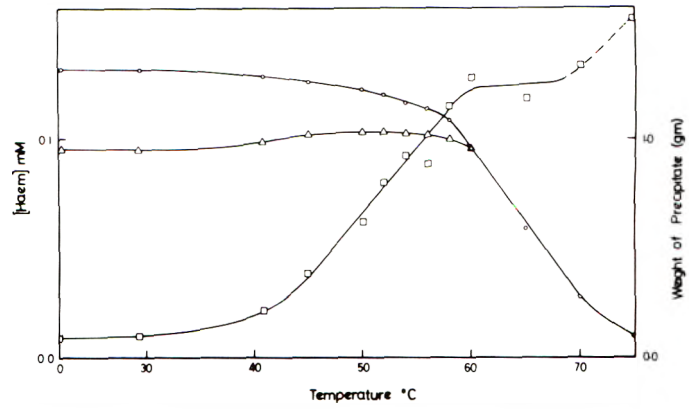

Fig. 2–The effect of heating an aqueous muscle extract for 2 hr at pH 6.0 on the amount of hemoprotein remaining in solution (○–○) (measured as the absorption of the solution) and the precipitation of the other water-soluble proteins (□–□) (determined by the weight of precipitate). △–△ is the amount of hemoprotein remaining in solution after 2 hr at 60°C after preheating at the temperature shown for 2 hr. In all cases the change in pH after heating was less than 0.05 pH unit.

Dialysis

20 ml of a 4-day-old mixture of 10 mg ml^{-1} BSA and 1.46 mg ml^{-1} Mb in 5.5 M urea at pH 6.0 was dialyzed against 0.05 M phosphate (pH 6.0). The dialysis was performed over 4 days at 0°C using four changes of 8 liters of phosphate.

Spectral analysis

All absorbances, at single wavelengths, were determined, in 1-cm cells, with a Perkin-Elmer Model 139 Spectrophotometer.

Reflectance spectra, on meat cooked to the required temperature in polythene pouches immersed in a water bath, or on the precipitate obtained on heating aqueous muscle extract or Mb-BSA solutions to 60–65°C, were determined with a Shimadzu Seisakusho Model MPS-50L Spectrophotometer with reflectance attachment. Absorption spectra on the solutions on BSA and Mb were determined with the same instrument.

RESULTS

Reflectance spectra of cooked beef and precipitate from heated muscle extract

Typical reflectance spectra of cooked beef and the precipitate obtained on heating an aqueous muscle extract are shown in Figure 1.

In the range 60–90°C the spectra of the precipitates changed little with temperature, but at temperatures below about 75°C the spectrum of the interior, but not exterior, of a cooked beef sample indicated the presence of unreacted oxy- and met-hemoproteins. Upon reduction with dithionite, both the precipitates and cooked meats yielded spectra identical with those described by Tappel (1957) for dithionite-treated cooked meat. Treatment with ferricyanide had negligible effect on the spectra of both the precipitates and cooked meats. Thus, the hemoproteins formed in both cooked meat and in the extracts appear to be the same, and it is reasonable to assume that muscle extracts may be used to study the reactions of myoglobin during the cooking of meat.

Spectral analysis of the filtered solution, before and after oxidation with ferricyanide, indicated the presence of only the oxy- and met-hemoproteins. Thus, for all practical purposes, all the cooked meat hemoproteins formed are precipitated from the solution.

Slight spectral differences among samples were observed for both the meat and the precipitate. For example, a slight shoulder at about 570 nm was apparent in a few of the meat samples and precipitates analyzed. The position of the Soret absorption maximum also varied from 409–417 nm.

Precipitation of cooked meat hemoprotein from aqueous muscle extracts

Effect of temperature. The results of a typical experiment demonstrating the effect of temperature on the precipitation of hemoprotein from solution are summarized in Figure 2. The solutions were heated for 2 hr, after which no further precipitation occurred.

It is apparent that at pH 6.0 most of the water-soluble non-heme proteins precipitate at temperatures below 60°C, whereas the hemoproteins (mainly myoglobin) precipitate only at higher temperatures. These two phenomena are closely related, as preheating a sample in the range 50–58°C, where most of the water-soluble proteins other than the hemoproteins precipitate, resulted in less hemoprotein precipitation on subsquent heating at 60°C (Fig. 2). This was verified on further extracts (Table 1) at other pH values and with other temperature regimes.

Effect of different protein fractions. The chromatogram of an aqueous muscle extract on Sephadex G-100 is shown in Figure 3. The molecular weight fractions responsible for the precipitation of the hemoproteins separated from this extract,

Table 1–Effect of temperature changes on the precipitation of hemoprotein from aqueous muscle extracts

Solution	Initial pH	Hemoprotein mM	Temperature history (°C)[a]	Precipitated (%)
A	5.55	0.227	52 then 60	41
A	5.55	0.227	60	61
B	5.8	0.101	60 then 70	69
B	5.8	0.101	70	96
B	5.8	0.101	60 then 70 then 75	90
B	5.8	0.101	75	98

[a]The solutions were held only for 20 min at 75°C and for 2 hr at each of the other temperatures.

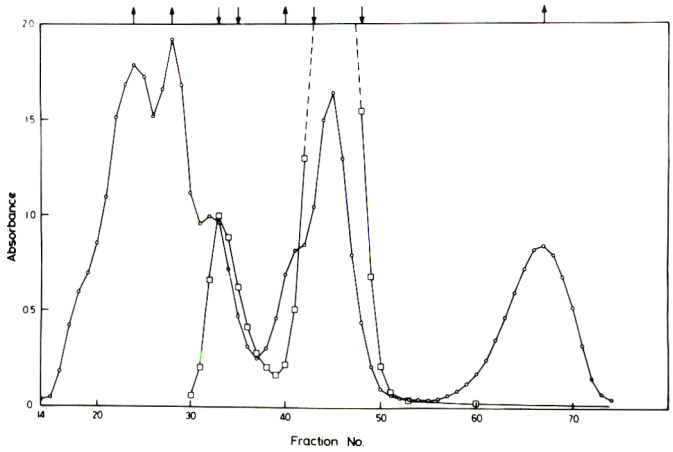

Fig. 3—Elution profile of 5.0 ml of an aqueous muscle extract (pH 6.0) after fractionation on Sephadex G-100 (bed volume approx 350 ml, height approx 42 cm and flow rate approx 15 ml hr^{-1}). The absorbances of the eluted fractions (5 ml) were recorded at 280 nm (○) and 410 nm (□). The fractions marked ↑ are the ones described in Table 2 and the fractions between ↓ and ↓ in the two hematin-positive peaks were combined to yield the hemoglobin and myoglobin solutions used.

Table 2—The amount of myoglobin (Mb) precipitated by the fractions obtained by chromatography of a muscle extract (2 × 5 ml) on Sephadex G-100 after heating for 2 hr at 60 or 65°C. The concentration of Mb in 0.05 M phosphate (pH 6.0) after mixing with an equal volume of the fraction was 0.0157 mM at 60°C and 0.0151 mM at 65°C

Elution vol, Ve,[a] of fraction (ml)	Absorbances at 280 nm of the added fraction	Temp (°C)	Mb lost (%)
130	Approx 2.2	60	10.2
129	1.78	65	61.0
150	Approx 2.4	60	20.5
150	1.93	65	38.0
176(Hb)	0.88	60	4.0[b]
178 (Hb)	0.70	65	14.0[b]
217	0.82	60	6.0
214	0.70	65	17.5
358	0.80	60	6.8
358	0.85	65	23.0
Eluant	0.00	60	3.0
Eluant	0.00	65	16.0

[a]Ve for Mb = 237 ± 2 ml. All the Mb was collected in a volume of approx 30 ml in both experiments. Spectral analysis showed it to be > 80% metmyoglobin in all samples.
[b]Corrected for hemoglobin precipitation (see text).

at 60 and 65°C, can be seen in Table 2. The samples containing the fractionated hemoglobin (Hb) had total hematin concentrations of 0.0195 and 0.0177 mM at 60 and 65°C. Solutions of the separated Hb gave 75% hemoprotein precipitation when heated to 60°C and 96% when heated to 65°C. If this amount of Hb was lost in the Hb-Mb solutions, then the % Mb lost would be about 4% at 60°C and 14% at 65°C, i.e., a negligible increase in the amount of Mb precipitated compared to the amount lost in solutions containing only Mb (Table 2). Thus, Hb has no marked effect on the precipitation of Mb.

These results indicate that, in muscle extracts, most of the myoglobin precipitation, at 60–65°C, is caused by the higher molecular weight proteins, although slightly increased precipitation also occurs with some of the smaller molecular weight proteins. At 60°C most of the myoglobin is precipitated by the protein(s) eluting at about 150 ml, whereas at 65°C the major precipitation is caused by the protein(s) eluting in the peak at about 130 ml.

Model systems

It is difficult to determine the role of the other proteins in the formation of cooked meat hemoprotein from meat or muscle extracts, due to the complexity of reactants and insolubility of the complex. Thus, attempts were made to prepare a similar, but soluble, complex from known reactants to gain further insight into the formation and nature of cooked meat hemoprotein.

Mixtures of BSA and Mb, at pH 6.0 and 60–65°C, yielded precipitates that were similar, as judged by reflectance spectrophotometry, to those obtained on heating meat (Fig. 1) but with the Soret peak always at 410–412 nm. The spectrum behaved similarly to that of meat upon reaction with dithionite or ferricyanide. Mb (> 99% of which eluted as one peak on Sephadex G-100) heated to > 80°C yielded a spectrally similar precipitate (Fig. 1).

Soluble complex. Both acid (0.1 N HCl) and alkali (5 N NaOH) solubilized the original hemoprotein in the precipitate from heated aqueous meat extracts, yielding absorption spectra typical of acid hematin and alkali-denatured metmyoglobin, respectively (Lemberg and Legge, 1949). Pyridine (40% v/v), when blended with the precipitate at neutral pH, solubilized only a small amount of the hematin, yielding on reduction the typical pyridine hemochrome.

The precipitate was solubilized to an appreciable extent by 8 M urea, yielding an aqueous solution whose absorption spectra were similar to the reflectance spectra of the insoluble cooked meat hemoprotein.

The ratio of E_{538} (absorption maximum for cooked meat hemoprotein) to E_{505} (absorption maximum for Mb) showed that maximum formation of cooked meat hemoprotein in BSA (6 mg ml^{-1}) –Mb (1.7 mg ml^{-1}) occurred at urea concentrations of 5.0–5.5 M buffered to pH 6.0 by 0.05 M phosphate. After 20 hr at 22°C the ratios were 0.750, 0.990, 1.016 and 0.986 at 4, 5, 5.5 and 6 M urea, respectively. All the ratios increased by 2–5% over a further 24 hr. The lower concentrations of urea yielded spectra rich in unreacted Mb, whereas the higher concentrations yielded

Table 3—Influence of BSA concentration on formation of soluble cooked meat hemoprotein at 22°C in 5.5 M urea (pH 6.0) at an Mb concentration of 1.46 mg ml^{-1}. Spectra recorded after 23 and 120 hr

(BSA) mg ml^{-1}	23 hr E_{538}/E_{505}	120 hr E_{538}/E_{505}	Soret λ max ± 0.5[a]	Eλ max
0	0.955	0.940	399	0.392
1	0.964	0.956	398	0.390
2	0.981	0.978	397	0.387
5	1.008	1.004	398	0.386
10	1.038	1.039	403	0.406
15	1.042	1.057	407	0.419
20	1.051	1.069	408	0.442
50	1.082	1.113	411	0.525

[a]Solution diluted 1:10 to record this region of the spectra after 120 hr.
E = Absorbance, and λ = wavelength (nm).

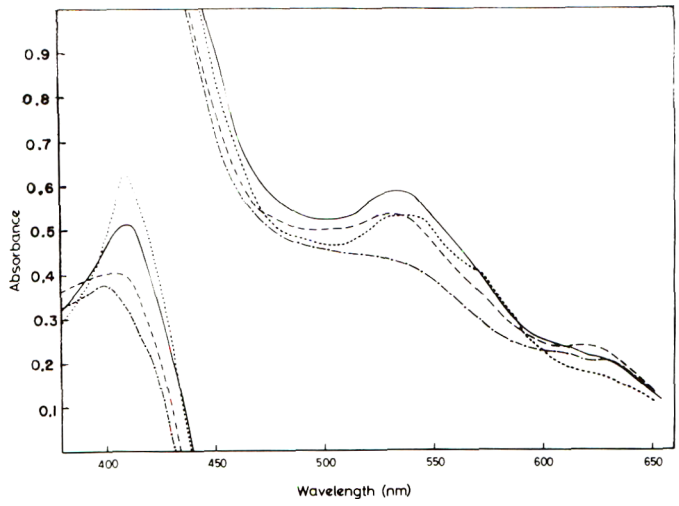

Fig. 4–Absorption spectra of Mb-BSA mixutres in 5.5 M urea and 0.05 M phosphate (pH 6.0) after standing for 120 hr. The Mb concentration was 1.46 mg ml^{-1} and BSA concentrations were 0 mg ml^{-1} – • – •; 10 mg ml^{-1} – – ; 50 mg ml^{-1} – and • • • • 10 mg ml^{-1} after dialysis against 0.05 M phosphate. To record the spectra from 380–440 nm the solutions were diluted 1:10.

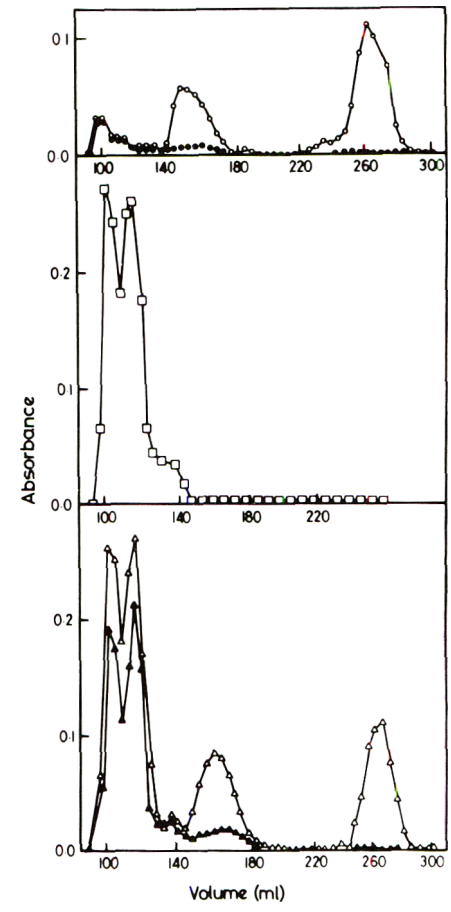

Fig. 5–Elution profile of BSA-Mb mixtures in 5.5 M urea and 0.05 M phosphate (pH 6.0) after fractionation on Sephadex G-100 (superfine) (bed volume approx 360 ml, height approx 43 cm and flow rate approx 12 ml hr^{-1}). The absorbances of the eluted fractions (128 drops) were recorded at 280 nm (open symbols) and 410 nm (closed symbols). ○, ● 1.46 mg ml^{-1} Mb, no BSA; □, ■ no Mb. 2 mg ml^{-1} BSA; and △, ▲ 1.46 mg ml^{-1} Mb and 2 mg ml^{-1} BSA. Volume, rather than fraction number, is plotted as the decreased surface tension of the fractions of high protein content caused the volume of 128 drops to be less.

spectra with decreased Soret absorption.

At fixed Mb (1.46 mg ml^{-1}) and urea (5.5 M) concentrations, markedly increased formation of cooked meat hemoprotein occurred on increasing the BSA concentration (Fig. 4 and Table 3).

Dialysis of the urea solution against 0.05 M phosphate yielded, after filtration, a solution whose spectrum was even more characteristic of the insoluble cooked meat hemoprotein (cf. Fig. 1 and 4).

Chromatography on Sephadex G-100. The protein concentrations of the fractions eluted from the column were measured as the absorbance at 280 nm (Fig. 5). Due to conformational differences, changes in the UV spectra of proteins are known to occur upon denaturation (e.g., Schechter and Epstein, 1968). It was found that, at pH 6.0, the changes of 280 nm for urea solutions of BSA were completed well below 4 M urea, no further changes occurring up to at least 6 M urea. Thus, the 280 nm absorbance could be used to measure BSA concentration in the 5.5 M urea employed here.

It has been shown that the hematin contributes significantly to the 280 nm absorption of hemoprotein (Drabkin, 1959) (E_{280} for Mb is about $2 \times E_{280}$ for ApoMb). The ApoMb fractions separated after chromatography of urea solutions of BSA-Mb all had similar, and insignificant, hematin contents as judged by the negligible absorption at 410 nm (Table 4). Thus, quantitative comparisons at 280 nm for these hematin-free ApoMb fractions, which are completely denatured below 5 M urea (Schechter and Epstein, 1968), are valid.

Figure 5 shows the chromatograms of some of the solutions described in the previous section.

The central elution profile of Figure 5 shows that the BSA elutes as two peaks, the dimer and higher aggregates eluting in the void volume, followed closely by the monomeric BSA. The other elution profiles in Figure 5 show that the ApoMb elutes as two main peaks, the behavior being independent of BSA concentration, although in the mixtures hematin is strongly associated with the BSA. This hematin-BSA association does not affect the elution behavior of the BSA. Results for all the mixtures are summarized in Table 4. Spectra of the more concentrated hematin-BSA fractions displayed small absorption maxima at 530–545 nm and about 630 nm and strong absorption in the 390–410 nm range, cf. cooked meat, the Soret peak becoming more intense and shifting to longer wavelengths with increasing protein concentration. "Free" hematin was apparently absorbed in the column during the fractionation of the solutions containing little or no BSA, since on subsequently eluting with a BSA solution the first BSA peak contained some typical hematin complex. All the free hematin was scavenged in this manner, as no complex was observed in the subsequent protein fractions. These experiments indicated that in 0 mg ml^{-1} BSA > 90% of the hematin was free, about 60% at 2 mg ml^{-1} BSA, about 10% at 10 mg ml^{-1} and $\leqslant$ 5% at the higher BSA concentrations.

The main hematin-denatured ApoMb peak elutes after the monomeric BSA (Ve for BSA = 133–142 ml and Ve for globin = 155–164 ml) and probably consists of dimeric ApoMb, since small peaks are present which could correspond to the trimer and tetramer, higher aggregates eluting in the void volume (97–105 ml) (Fig. 5). The amount of "dimeric" denatured ApoMb and hematin-free denatured ApoMb (as judged by the absorption at 280 nm) is, within experimental error, independent of the concentration of BSA in the unfractionated solution (Table 4). As these two fractions account for over 85% of the total denatured ApoMb, and since the scavenging experiments showed that most of the hematin was bound to protein, at least in the most concentrated BSA solutions, it would appear that the hematin-BSA complex(es) do not involve globin.

Table 4–Total absorbances of the heme-free and hemoproteins fractionated on Sephadex G-100 from Mb-BSA in 5.5 M urea at pH 6.0. The initial Mb concentration was 1.46 mg ml^{-1} in all samples, temperature 22 ± 1°C

(BSA) mg ml^{-1}	BSA[a]		Dimeric ApoMb[b]		Heme-free ApoMb[c]
	E_{280}	E_{410}	E_{280}	E_{410}	E_{280}
0	0.12 ± 0.01	0.14 ± 0.01	0.32 ± 0.02	0.05 ± 0.01	0.37 ± 0.05
1	0.86 ± 0.06	0.63 ± 0.05	0.35 ± 0.04	0.06 ± 0.01	0.39 ± 0.04
2	1.48 ± 0.12	1.20 ± 0.10	0.37 ± 0.06	0.08 ± 0.01	0.39 ± 0.08
5	4.05 ± 0.15	1.94 ± 0.10	0.32 ± 0.08	0.08 ± 0.01	0.37 ± 0.06
10	6.80 ± 0.15	3.63 ± 0.10	0.38 ± 0.06	0.05 ± 0.01	0.40 ± 0.08
15	8.20 ± 0.30	5.51 ± 0.10	0.36 ± 0.06	0.07 ± 0.01	0.39 ± 0.05
20	8.50 ± 0.40	6.24 ± 0.15	0.37 ± 0.06	0.07 ± 0.01	0.38 ± 0.06
50	> 25	> 6.8	Not recorded		
10[d]	6.70 ± 0.10				
2[d]	1.60 ± 0.10				

E = Absorbance of each fraction cm^{-1}.
[a]This is the total E of the fractions in the first two peaks of the chromatograms, i.e., the monomer and higher aggregates of BSA and trace amount of high molecular weight denatured Mb.
[b]This is the total E of the fractions in the third major peak in the chromatograms, i.e., the first main Mb fraction.
[c]This is the total E of the fractions in the fourth major peak in the chromatograms, i.e., the other main Mb fraction (hematin-free).
[d]These solutions contained no Mb.
Where necessary, corrections were applied for any slight differences in volume between the fractions from different runs; these corrections were always less than 10% of the total E.

The percentage of monomeric BSA in the mixtures, which decreased from about 54% at a concentration of 1 mg ml^{-1} to about 6% at 20 mg ml^{-1} was the same, within experimental error, whether determined from the absorbances at 280 nm or 410 nm. Also, the percentage monomeric BSA was the same in both the presence and absence of Mb at concentrations of 2 mg ml^{-1} (Fig. 5) and 10 mg ml^{-1}. These results indicate that the monomer and higher polymers of BSA have equal affinity for hematin and that complexes of the type BSA-hematin-BSA are not present in the mixtures to any appreciable extent.

Sephadex chromatography, in phosphate buffer, of the dialyzed BSA (10 mg ml^{-1}) –Mb (1.46 mg ml^{-1}) solution, yielded an elution profile similar to that obtained in urea (Fig. 5), except that the hematin-BSA complexes eluted just before the hematin-free "renatured" BSA. Some renatured monomeric ApoMb was bound to hematin after dialysis, the binding between the dimeric ApoMb and hematin being unchanged.

DISCUSSION

THE SPECTRAL similarity between the complexes of hematin and BSA in urea solution at pH 6.0 and the insoluble cooked meat hemoproteins suggests that the hematin environment is very similar in both cases, although the behavior of the Soret band indicates decreased hematin-protein interaction in urea (Fig. 4).

The position and intensity of the Soret band in methemoproteins is dependent on the nature of the ligands at the fifth and sixth positions (Brill and Williams, 1961). Also, the intensity of the band is markedly decreased and its position shifted to shorter wavelengths if the hematin-protein interactions are decreased by urea, guanidine hydrochloride (Schechter and Epstein, 1968), amides, alcohols (Herskovits and Jaillet, 1969), inorganic ions (Cann, 1964) and acid or heat (Acampora and Hermans, 1967; Awad and Deranleau, 1968). With heat, the intensity increases again on denaturation at pH 6 (Awad and Deranleau, 1968). No such increase occurs on denaturation by the other means.

The chromatographic results showed the complexes in urea to be hematin-denatured proteins, no evidence being found for complexes of the type ApoMb-hematin-BSA. It is well established that in alkaline solution hematin can combine with ϵ-amino groups of denatured ApoMb or albumin (Kaziro and Tsushima, 1959). The present results indicate that combination can also occur at pH 6.0, although the attachment is presumably not by the ϵ-amino groups, which combine only with hematin at pH > 10.

Increasing urea concentration weakens the interactions between the hematin and ApoMb (Schechter and Epstein, 1968), and displacement by denatured BSA can presumably occur. Thus, results in Table 3 are explained as a breakdown of Mb with time and the formation, increasing with time, of hematin-BSA. The variation in the Soret absorption maxima in Table 3 and Figure 4 are indicative of increased hematin-protein interaction on increasing BSA concentration, or removal of urea (Schechter and Epstein, 1968).

In aqueous Mb solutions, increasing temperature leads to conformational changes in the hematin environment, these changes occurring well below the denaturation temperature (Awad and Deranleau, 1968; Atanasov et al., 1968). These conformational changes may allow attack by the denatured protein on the hematin, the ApoMb being displaced by the other protein and subsequently, or simultaneously, denatured. Alternatively the denatured proteins may decrease the stability of Mb, allowing "normal" denaturation to occur at lower temperatures. Recent evidence (Atanasov and Mitova, 1970) suggests that in thermally denatured myoglobin the hematin remains firmly bound to the ApoMb. Thus, if the effect of the other proteins is merely to destabilize the globin, the only hemoprotein in cooked meat will be denatured ApoMb hemichrome which, at constant pH, would be expected to yield a characteristic spectrum with unique absorption (reflectance) bands. This is not so, as differences are observed in the position of the Soret absorption maximum for different cooked meats (Tarladgis, 1962) and precipitates from different heated muscle extracts. Also, the reflectance spectra of some of the precipitates from muscle extracts had a shoulder at approximately 570 nm. These spectral differences suggest that the hematin environment in cooked meat hemoprotein is not unique and on present evidence it would appear that attack on the hematin by the denatured proteins causes displacement and denaturation of the globin. The great affinity of urea-denatured BSA for the hematin of urea-denatured myoglobin would support this type of mechanism.

Therefore, when meat is heated a range of denatured hemoproteins will form, which aggregate and precipitate with the other unreacted denatured proteins to yield the brown cooked meat pigments.

Tarladgis (1962) suggested that denatured ApoMb was attached to the ferric ion of the hematin by a carboxylate group with a water molecule at the sixth position on the ferric ion, resulting in a high-spin ferric hematin complex. Hollocher (1966) found that the ESR spectrum of heat-denatured Mb, which has a reflectance spectrum similar to that of cooked meat (Fig. 1), had no high-spin ferric hematin signal at g ≑ 6. The spectrum was similar to that of a hematin-imidazole complex. During the course of the present work an ESR spectrum, at −196°C, of meat cooked to 70°C was recorded by Dr. M.E. Winfield (CSIRO Division of Applied Chemistry, Fishermen's Bend, Victoria, Australia). As with denatured Mb, this had no signal at g ≑ 6. This signal was present, however, in uncooked meat due to the high-spin ferric

ion of metmyoglobin (Hollocher, 1966). As the ferric ion in cooked meat would appear to be predominantly low-spin at −196°C, it is unlikely that the ligands at the fifth and sixth positions are carboxylate and water (Brill and Williams, 1961). If the denatured protein is attached directly to the ferric ion, more probable ligands that would account for the low-spin character of the cooked meat complexes are an N atom of histidine or an S atom of a methionyl or cysteine residue, or both. The reflectance spectra of cooked meat at 20°C possess some apparently high-spin character (Tarladgis, 1962), including the absorption band at about 630 nm which is characteristic of liganded water (Brill and Williams, 1961). If water and a low-spin-inducing ligand are bonded to the ferric ion, this could give rise to a mixed high- and low-spin equilibrium, being about 100% low spin at −196°C and the proportion of high-spin complex increasing with temperature. This type of equilibrium is well established for other ferric hematin compounds (e.g., George et al., 1959).

It is also possible that the hematin-protein linkage in the cooked meat hemoprotein does not involve the ferric ion but, as with methemalbumin (O'Hagan, 1959), is formed between the protein and the side chains on the hematin, e.g., the carboxylate groups and suitable sites on the denatured protein. Even if an iron protein bond is involved these, or other linkages, e.g., hydrophobic hematin-protein interactions which would be absent in urea, may confer additional stability on the complexes. This would explain the limited reactivity towards pyridine of the hematin in the precipitate from the muscle extracts. The hematin environment in such structures would probably be similar to that obtained upon dialysis of the urea from the BSA-Mb system, although further work to relate this model system to cooked meat would be desirable.

Further study will also be necessary to determine what are the actual ligands attached to the fifth and sixth positions of the ferric hematin in cooked meat, but it is likely that these will be any of the possible side chains along the different denatured proteins or water, or both.

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A COMPARISON OF SALMONELLA EXCRETION BY STRESS-SUSCEPTIBLE AND STRESS-RESISTANT PIGS

SUMMARY—A relatively high percentage of apparently healthy pigs were Salmonella carriers and excreted salmonellae in the feces. Stress imposed by trucking did not significantly alter the excretion of salmonellae by the pigs. The incidence of salmonellae in fecal samples from 32 stress-resistant and stress-susceptible animals was investigated. There was a high incidence of salmonellae in cecum and colon contents, but salmonellae were not isolated from various muscles or from spleen, liver and kidney samples. Three of eight pigs were positive for Salmonella *in the mesenteric lymph nodes. There was no apparent pattern of salmonella detection in regard to the stress-susceptible condition of the animal.*

INTRODUCTION

DOMESTIC LIVESTOCK constitute one of the chief reservoirs of salmonellae, and the presence of salmonellae in the gut contents of food animals is a serious problem in the meat-packing industry. Several groups have examined the incidence of salmonellae in poultry, cattle and swine. One of the more interesting aspects of these studies has been the observation that the incidence of salmonellae in the excreta of animals, particularly swine at the farm, is much lower than in the same animals after transport to market. For example, Galton et al. (1954), McDonagh and Smith (1958), Leistner et al. (1961), Hansen et al. (1964) and Shotts et al. (1962) demonstrated that the incidence of salmonellae in rectal swabs of pigs on farms was quite low, but a sudden increase occurred when the pigs were transported to market. The observed incidence in transported pigs ranged from 30–80% by rectal swab examination. Williams and Newell (1970) offered two alternate explanations for these findings: 1) the sudden increase in salmonella excretion is due to animals infecting each other in the trucks and in the holding pens or 2) the true incidence of salmonellae in pigs on the farm cannot be assessed by means of rectal swabs because salmonellae are either absent in the rectum or if present they are in extremely low number. When swine are disturbed by transport, overcrowding in holding pens or by rough handling before slaughter, cecal contents reach the rectum and the previously hidden or masked salmonellae become more readily detectable. This increase could be considered the response of the animals to imposed stress.

Stress also influences body weight and pituitary and adrenal gland characteristics as described by Juszkiewicz and Jones (1961) and King et al. (1958). These changes are evident in postmortem examination and the condition is termed pale, soft, exudative (PSE) muscle (Briskey, 1964). Reddy et al. (1970) have shown that leakage into the blood of sarcoplasmic enzymes from muscle after stress is characteristic of the stress-susceptible syndrome in swine. Sayre et al. (1963) noted a higher incidence of PSE muscle in the Poland China breed of pigs than in the Chester White breed.

The purpose of this study was to determine whether there was a difference in the incidence of salmonellae in the intestinal contents of stress-resistant and stress-susceptible pigs and to ascertain whether exposure to stress resulted in an increased excretion rate and altered distribution of salmonellae in the muscle and organs of these pigs.

MATERIAL & METHODS

Animal handling

Thirty-two market-age pigs (16 Chester White and 16 Poland China) were used in this experiment, conducted during June and July, 1970. The animals were delivered from farm sources and held in group pens for 1 wk and fed an antibiotic-free ration. The ration constitutents were ground corn, soybean oil meal, alfalfa leaf meal, brewer's yeast, bonemeal, trace mineral salt and zinc. Following this 1 wk equilibration period, each pig was placed in an individual pen and fed ad libitum during the experimental period. Three fresh fecal samples were taken consecutively at 24-hr intervals from the piggery floor. On the fourth day of experiment, four Chester White and 10 Poland China pigs were loaded on a truck and separated in individual pens by moveable gates. The truck and gates were steam-cleaned before use. The truck was then driven for 60 miles for the purpose of stressing the pigs. Fecal samples were obtained from each pig upon completion of the transportation. After the fecal samples were examined for salmonellae, eight pigs were selected for further investigation. This group included four pigs that had excreted salmonellae (two Chester White and two Poland China) and four pigs that had not excreted salmonellae (two Chester White and two Poland China) after stress. A 1-wk recovery period was allowed before the animals were slaughtered. Stress susceptibility of these eight animals was estimated by following pH and rigor patterns of the muscle postmortem (Schmidt et al., 1968).

Muscle and organ sampling

Longissimus, trapezius and biceps femoris muscles, liver, spleen and mesenteric lymph nodes and cecum and colon contents were obtained from the slaughtered animals (Kampelmacher et al., 1961). The viscera were placed on a tray which had been washed and steamed before use. Forceps and knives were cleaned and immersed in boiling water 2 min before sampling. Mesenteric lymph nodes were collected by removing several lymph nodes together with surrounding tissue from the viscera. Cecum and colon contents were obtained by tying off a segment of cecum or colon prior to incising with a sterile knife. A portion of the contents was removed with a sterile spatula without touching the sides of the intestinal wall. Muscle, liver, spleen, kidney samples and lymph nodes were immersed in boiling water for 10 sec to eliminate surface contamination (Chung and Frost, 1969).

Testing for salmonellae

Muscle, liver, spleen and kidney were cut into small pieces with sterile scissors. The connective tissue surrounding the mesenteric lymph nodes was removed using sterile scissors and the lymph nodes were homogenized in a precooled sterile blender with M-tetrathionate broth for 2 min. 10g of fecal samples, cecum and colon contents and 20g from muscle, liver, kidney and lymph nodes were inoculated into 200 ml of modified M-tetrathionate broth (Difco). The medium was modified by addition of one-half the recommended quantity of iodine solution. This modification was made because preliminary trials showed that when the recommended level of iodine solution was used, more than 24 hr of incubation was necessary for recovery of salmonellae. The enrichments were incubated at 37°C for 20 ± 2 hr. Subsequently, a loop from the enrichment medium was streaked on to Brilliant Green (BG) agar plates, which were then incubated at 41°C for 20 ± 2 hr (Spino, 1966). Two suspect colonies were picked from the BG agar plates and inoculated into 9 ml of brain heart infusion broth (BHI) and lysine iron agar. Each medium was incubated at 37°C for 24 hr. A polyvalant flagellar antigen test (Silliker et al., 1965) was performed on the cultures that gave a typical salmonella reaction in lysine iron agar. Serotyping of the isolates was carried out by the Wisconsin State Laboratory of Hygiene.

RESULTS & DISCUSSION

Table 1 shows that on individual days the number of pigs detected as shedding salmonellae was high. 75% of the Chester White pigs and 62.5% of the Poland China pigs shed salmonellae in the feces in at least one of the three fecal samples taken before stressing. Three Chester White and four Poland China pigs excreted salmonel-

Table 1–Salmonella excretion pattern of healthy pigs[a]

Breeds		Chester White																Poland China															
Pig No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Treatments																																	
Prior	Day 1	+	–	–	–	+	–	–	+	–	+	+	+	+	+	+	–	–	+	–	–	+	–	–	–	–	–	+	+	+	–	–	+
to	Day 2	+	–	–	–	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	+	–	+	–	–	–	–	–	+	+	–	+	+
stress	Day 3	–	–	–	–	+	+	–	+	–	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	+	+	–	+	+
After	Day 1	+	–	–	–													–	b	–	–	+	–	–	–	–	–						
stress	Day 2	–	–	–	–													–		–	–												
	Day 3	–	–	–	–													–		+	–												

[a]Nine serotypes of *Salmonella* were isolated from various positive samples. These included *S. newington, S. heidelberg, S. thompson, S. saint paul, S. london, S. anatum, S. senftenberg, S. typhimurium* and *S. siegburg.*
[b]Animal died due to stress.

lae in all three pre-stress samplings, whereas the remaining pigs shed intermittently; i.e., salmonellae were present in only one or two of the three fecal samples. These results establish, therefore, that a relatively high percentage of the apparently healthy pigs were salmonella carriers and were excreting salmonellae in the feces.

The high incidence of salmonella excreters in this study is in contrast with data previously reported by others. One possible explanation is the difference in the method of obtaining samples; i.e., the examination of 10g of fecal material rather than rectal swabs. Haddock (1970) demonstrated six salmonella carriers in 16 market-age pigs after three rectal swabs from each pig. Six more carriers were detected after 16 rectal swabs from each pig had been examined. Cecal swabs of 15 of the 16 pigs were positive for *Salmonella*. McCall et al. (1964; 1966) pointed up the rather poor efficiency of rectal swabs compared to fecal samples in detecting carriers of salmonellae. Our results indicate that a more significant problem than previously indicated may exist. The incidence of shedding salmonellae on the farm by the market-age pigs used in this study was not ascertained. This is unfortunate, because the stress imposed on the pigs by trucking from the farms to the University may have altered the salmonella excretion pattern. It is conceivable that transport from the farms may have enhanced the shedding of salmonellae, thereby accounting for the high incidence of salmonellae when the pigs were examined. However, Williams et al. (1967) showed that a holding period may negate this effect.

Another factor that may have influenced the shedding pattern was the feed. When the animals were received at the University, they were fed a ration free of antibiotics. This antibiotic-free feed was used throughout the experimental period. Because the ration contained some animal by-product material, it quite likely contributed to the salmonella population of the pigs. Previous tests on the feed material had revealed that it was contaminated with salmonellae (Goepfert, unpublished data).

Table 1 shows the effect of stress-by-trucking on the salmonella excretion pattern of the healthy pigs. Only one of nine pigs that had not previously excreted salmonellae did excrete these organisms after stress. Therefore, it was concluded that stress as imposed by trucking did not significantly alter the excretion of salmonellae by these pigs. These results contrast with earlier studies (Williams et al., 1970), which found that the stress imposed by transportation did significantly increase the incidence of salmonellae in the feces of the pigs examined. Results of the present experiment may be due to the high percentage of pigs (68.75%) that were already excreting salmonellae before the application of stress.

One Poland China pig died during the process of transportation (temperature was 85°F). This illustrates the extreme sensitivity of some pigs to the stress of transportation; it is not unusual to have stress-susceptible pigs die during handling or transport.

Eight pigs, randomly selected, representing equal numbers of the two breeds, were selected for study of the association of stress-susceptibility with salmonellae content of muscle, various organs and cecum and colon contents. The pigs were rested 1 wk before slaughter. Four had been shown previously to be excreting salmonellae and four were negative. Post-mortem muscle characteristics of pH decline and rigor mortis pattern (Briskey, 1964) of eight slaughtered pigs showed three stress-susceptible and five stress-resistant (Table 2). A very high incidence of salmonellae was found in the cecum and colon contents of the slaughtered pigs. Two Chester White and two Poland China pigs that were not shedding salmonellae in the feces had salmonella-positive cecal and colon contents. Three of the four pigs that were shedding salmonellae also had salmonella containing cecum and colon contents. Salmonellae were not isolated from longissimus, trapezius or

Table 2–Salmonella detection in muscle and organs of healthy pigs

Samples	Feces salmonella-negative				Feces salmonella-positive			
	Chester White[a]		Poland China[b]		Chester White[c]		Poland China[d]	
L.D.	–	–	–	–	–	–	–	–
Trapezius	–	–	–	–	–	–	–	–
Biceps	–	NT[e]	–	NT	NT	NT	NT	NT
Spleen	–	–	–	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–
Kidney	–	NT	–	NT	NT	NT	NT	NT
Mesentric lymph nodes	–	–	–	+	+	+	–	–
Cecum	NT	–	NT	+	NT	+	NT	+
Colon	+	–	+	–	+	+	+	+
Muscle pH[f]	5.8	6.5	5.4	6.2	5.8	6.2	5.4	6.1
Classification[g]	SS	SR	SS	SR	SR	SR	SS	SR

[a]Numbers 1 and 5 from Table 1.
[b]Numbers 4 and 5 from Table 1.
[c]Numbers 4 and 3 from Table 1.
[d]Numbers 10 and 9 from Table 1.
[e]Not tested.
[f]3 hr post-mortem.
[g]Stress-susceptible (SS) or stress-resistant (SR) based on muscle pH and rigor pattern post-mortem.

biceps femoris muscles or from the spleen, liver and kidney samples of any of the slaughtered pigs. Three of the eight pigs were positive for salmonellae in the mesenteric lymph nodes (Table 2). There was no apparent pattern of salmonella detection in regard to the stress-susceptible condition of the animal. The hypothesis for this experiment was that the stress-susceptible animals might undergo physiological changes (such as membrane permeability) that would allow infection by salmonellae of certain organs of the body. This did not appear to be the case, at least within the limitations of the experiment.

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MODE OF ACTION OF ETHYLENE OXIDE ON SPORES OF Clostridium botulinum 62A

SUMMARY—Death of spores of Clostridium botulinum *when exposed to gaseous ethylene oxide followed first order kinetics. Supplementation of a synthetic medium with the purine and pyrimidine bases of DNA and RNA indicated, as judged by outgrowth from spores which had received sublethal ETO treatments, that the lethal action of ETO on the spores was through alkylation of the guanine and adenine components of DNA. Observed impairment of RNA and protein synthesis was considered an indirect effect resulting from alkylation of DNA components; however, additional evidence bearing on this point is needed to support a more definite conclusion.*

INTRODUCTION

ETHYLENE OXIDE (ETO) in the vapor phase has been shown to be an effective sterilant by many workers during the past four decades. Death kinetics of a wide variety of microbial species, as well as environmental factors affecting these kinetics, have been quite thoroughly investigated (Kaye and Phillips, 1949; Phillips, 1949; Opfell et al., 1959; Ernst and Shull, 1962; Gilbert et al., 1964; LIU et al., 1968; Kuzminski et al., 1969; Kereluk et al., 1970a, b; Blake and Stumbo, 1970; Marletta and Stumbo, 1970; Michael and Stumbo, 1970; and Savage and Stumbo, 1971). Notwithstanding the thoroughness of these kinetic studies, only limited studies regarding the mode of lethal action of ETO have been reported.

There is little doubt that the mode of action is alkylation (Ross, 1962; Wheeler, 1962; Alexander et al., 1957, 1961). However, there has been only one report dealing specifically with what is alkylated to cause the microbial cell to lose reproductive ability. Michael and Stumbo (1970) studying the mode of action of ETO on *Salmonella senftenberg* and *Escherichia coli* concluded that it was alkylation of the guanosine triphosphate of DNA that caused the bacterial cells to lose their power of reproduction.

Kuzminski et al. (1969) reported on the death kinetics of spores of *C. botulinum* Type A, strain 62, when exposed to ETO under different conditions of temperature and relative humidity; however, their studies did not consider the mode of action which inactivated the spores. Therefore, the present study was undertaken with the hope of elucidating this point.

MATERIALS & METHODS

Test organism

Clostridium botulinum Type A, strain 62. That the organism was Type A was verified by mouse protection tests employing specific antitoxin.

Sterilant gas

A mixture of ethylene oxide (12%) dichlorodifluoromethane (88%), by weight, was employed throughout.

Growth and sporulation media

A trypticase medium developed by Day (1960), with some modifications, was employed. A growth medium was prepared as follows: 40.0g trypticase and 0.5g/glucose were added to 1 liter of 0.067M phosphate buffer. After solution of these ingredients, the pH was adjusted to 7.2, the medium distributed in bottles or tubes and sterilized by autoclaving for 20 min at 121°C. In any study, just prior to spore inoculation, sufficient sterile thiamine solution, in phosphate buffer (pH 7.2) was added to give a thiamine concentration of 1 ppm. The thiamine solution was sterilized by filtration using a 0.2 μm filter membrane (Millipore Corp., Bedford, Mass.).

For sporulation, this medium was further supplemented with $(NH_4)_2SO_4$ to a concentration of 0.5%. It has been shown that the joint effect of $(NH_4)_2SO_4$ and thiamine is to increase spore yield (Schmidt, 1964).

Subculture medium for colony development

This was prepared by adding 0.05% sodium thioglycollate and 1.5% agar to the growth medium. Prior to sterilization the pH was adjusted to 7.2 and the medium distributed in Prickett tubes (20 ml per tube).

Medium for studying repair process

A synthetic medium supplemented with purine and pyrimidine bases or their nucleosides was used to follow growth patterns of progeny of ETO treated spores. The medium was composed of Bacto Synthetic Broth AOAC supplemented with biotin, p-aminobenzoic acid, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $MnSO_4 \cdot H_2O$ at concentrations per 10 ml of medium of 0.005 μg, 0.10 μg, 0.02 μg, 0.02 μg and 0.10 μg, respectively. The chemical composition of the dehydrated Bacto Synthetic Broth AOAC was as given by AOAC (1964). Seventeen grams of this dehydrated broth were dissolved in 1 liter of distilled water and the solution heated to boiling. Quantities (0.10 ml each) of stock solutions of biotin, p-aminobenzoic acid, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$, and $MnSO_4 \cdot H_2O$ were added to give the desired concentrations. The broth was then tubed and sterilized. Just prior to inoculation, 0.1 ml of sterile 10% dextrose solution was added aseptically to each tube (containing 20 ml broth).

Spore production

Tubes of trypticase medium were inoculated with spores from a stock suspension, heated at 80°C for 15 min, and incubated overnight at 30°C. The resulting cultures were used for the first of three cell transfers, 6–7 hr apart. The first transfer was to 100 ml of growth medium, the second to 2.5 liters, and the third to 7.5 liters. Each of the successive inoculations was approximately 10% by volume. Incubation was at 30°C and anaerobiosis was maintained by continual slow passage of nitrogen gas through the incubating cultures.

The third culture was incubated until sporulation was virtually complete as judged by periodic microscopic examinations (bright field, phase contrast and malachite green spore stains). This required about 60 hr, but the culture was held an additional 3 days at 30°C to permit lysis of any remaining vegetative cells and/or sporangia containing forespores. Microscopic examination revealed no regermination during the 3 day holding period.

This culture containing mature spores was dispensed in centrifuge bottles and centrifuged (5000G) for 20 min at 2°C. The supernatants were then discarded, the spores resuspended in cold sterile distilled water and again centrifuged. This washing procedure was repeated three times. (After each centrifugation, the dark extraneous material was removed from the surface of the sediments.) These washed spores were then further cleaned by the method of Powers (1968) employing a 300 μg/ml filter-sterilized solution of lysozyme in 0.067M phosphate buffer (pH 7). Finally, the cleaned spores were suspended in 0.05M hydroxymethylaminomethane (pH 7.2 Tris buffer) and stored at 2°C until needed.

Production of germinated spores

The procedure followed was essentially that described by Simmons and Costilow (1962). About 10g (wet weight) of the cleaned spores were suspended in 15 ml of a sterile solution of 4% trypticase and 0.1% sodium bicarbonate in 0.067M phosphate buffer (pH 7.0) and heated at 80°C for 15 min. This suspension was then added to 4 liters of the same medium and incubated at 30°C. During incubation, nitrogen gas was passed slowly through the culture to maintain anaerobiosis and provide mild agitation. After about 3 hr incubation more than 95% of the spores had germinated as judged by microscopic examination. These germinated spores were harvested by centrifugation at 5000G and washed three times with cold sterile distilled water. After the final washing, the germinated spores were resuspended in 0.05M Tris buffer and stored at 2°C until needed.

Preparation of test samples for ETO exposure and exposure procedure

The procedures employed were essentially as described by Kuzminski et al. (1969). However, in this study, relative humidities of 11%,

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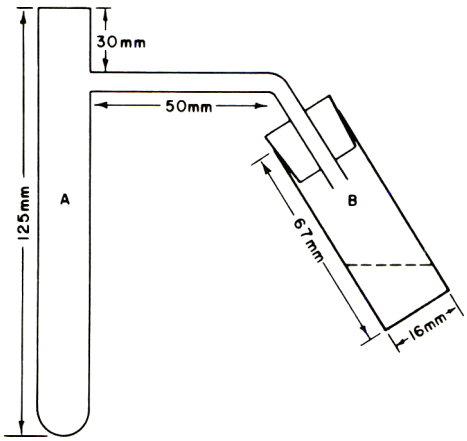

Fig. 1—Schematic diagram of growth system.

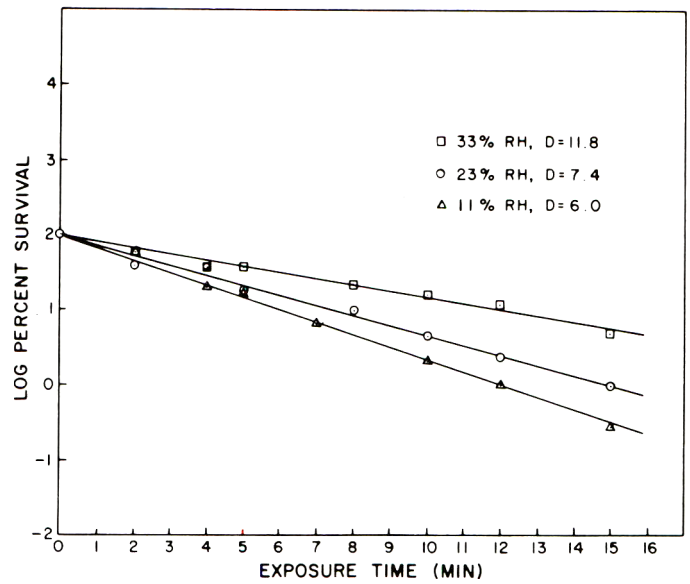

Fig. 2—Survivor curves for dormant spores of C. botulinum 62A, when exposed (40°C) to ETO (700 mg/L).

23% and 33% only were employed and all exposures were at 40°C. Ethylene oxide concentration in all exposure atmospheres was maintained at 700 ± 20 mg per liter by keeping the exposure atmosphere at 5 psig (within limits of control).

Death kinetic studies

Absorbent paper discs were inoculated with ungerminated or germinated spores and exposed for varying lengths of time. The exposed discs were placed in tubes of sterile distilled water and pulverized on a Vortex Genie Mixer. The slurries were then appropriately diluted and subcultured in trypticase agar for incubation at 30°C. Colony counts were made after 4–6 days incubation. Survivor curves were constructed by plotting logarithms of survivors against corresponding times of exposure. Lines of best fit were located by the method of least squares and D values (times for inactivation of 90% of the spores) obtained from the resulting regression coefficients.

Technique for studying growth patterns

A system was devised whereby optical density measurements could be made periodically during incubation with minimum disturbance of growing cultures. A schematic diagram of the simple apparatus is shown in Figure 1. Basically the system consisted of a growth tube (A) connected with glass tubing to another tube (B) containing an oxygen absorbing reagent. Before use, the empty tubes (A and B) were cotton plugged and sterilized by autoclaving. Sterile medium (7.5 ml) was placed in tube A and the cotton stopper replaced with a sterile rubber diaphram stopper. Prior to inoculation the tube was placed at 30°C for 2 hr to attain temperature equilibrium. Just before inoculation of the medium in tube A, 2g of pyrogallic acid and 2.0 ml of 20% Na_2CO_3 were placed in tube B. Tube B was then connected to tube A as shown in the diagram. The medium in tube A was inoculated via syringe through the rubber diaphram. Periodically during incubation at 30°C, growth was followed by making optical density (O.D.) measurements with a Bausch & Lomb spectronic 20 colorimeter set at 600 nm.

Repair process studies

The synthetic medium described above, unsupplemented and supplemented with one purine or pyrimidine base, or a combination of two, was employed for studying growth patterns of progeny of ETO-treated spores. Exposure was in an atmosphere containing 700 ± 20 mg per liter of ETO maintained at a relative humidity of 23% and a temperature of 40°C. Time of exposure was 7.4 min, the time according to kinetic studies, which was required to inactivate 90% of the spores in the population (1 D value). Growth at 30°C was followed by O.D. measurements as explained above.

Effect of ETO exposure of spores on protein, RNA and DNA synthesis of progeny

The synthetic medium was inoculated with exposed (1 D value) and unexposed spores. The same system used in growth pattern experiments was employed for culturing. During incubation at 30°C, protein (Lowry et al., 1951), RNA (Ceriotti, 1955) and DNA (Burton, 1956) determinations were made at selected time intervals (2–3 hr) to ascertain the effects of ETO treatment on synthesis.

RESULTS & DISCUSSION

KINETICS of death of mature (dormant) spores of *C. botulinum* Type A, strain 62 exposed at 40°C to an atmosphere containing 700 mg of ETO per liter are depicted in Figure 2. The striking effect of relative humidity on death rates observed in this study was consistent with that observed by Kuzminski et al. (1969). For example, in this study, for relative humidities of 33 and 23%, D values of 11.8 and 7.4 min respectively, were observed; whereas Kuzminski et al. (1969) under the same conditions of exposure observed values of 13.75 and 8.18 min. The somewhat lower D values observed here might well be due to the fact that spores for this study were produced in a trypticase broth; spores used by Kuzminski et al. (1969) were produced in a beef heart casein medium which had a much higher fat content than the trypticase broth. That cell resistance to ETO increases with increases in concentration of fat and fat-like materials has been reported (Church et al., 1956).

Further kinetic studies with germinated spores showed that germination reduced resistance to ETO somewhat, but nothing like it reduces resistance to heat. In this study, death of germinated spores, exposed under the same conditions as was the dormant spores, was characterized by D values of 9.8, 4.5 and 4.3 min at relative humidities of 33, 23 and 11%, respectively, as compared with the D values of 11.3, 7.4 and 6.0 min for dormant spores.

Michael and Stumbo (1970), from their studies with *S. senftenberg* and *E. coli*, concluded that alkylation of the guanosine triphosphate component of DNA was the probable action of ETO that caused the bacterial cells to lose their power of reproduction. Therefore, a study was designed to determine if the lethal action of ETO on spores of *C. botulinum* might be the same or similar. The synthetic medium described above was supplemented with one purine or pyrimidine base (or a combination of two) and inoculated with spores of *C. botulinum* which had been untreated or treated with ETO for a time equivalent to 1 D value (according to kinetic studies using trypticase agar as the subculture medium). The purine and pyrimidine bases employed (in 0.1 mM concentration) were thymine, cytosine, uracyl, adenine and guanine (or their nucleosides). The outgrowth pattern from exposed spores was not improved by supplementing the medium singly with the bases, thymine, cytosine, uracyl or ade-

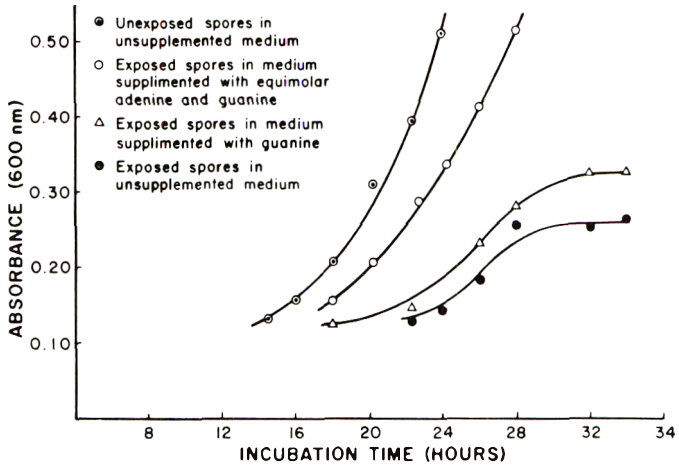

Fig. 3–Growth patterns of progeny of ETO exposed and unexposed spores of C. botulinum 62A in synthetic medium.

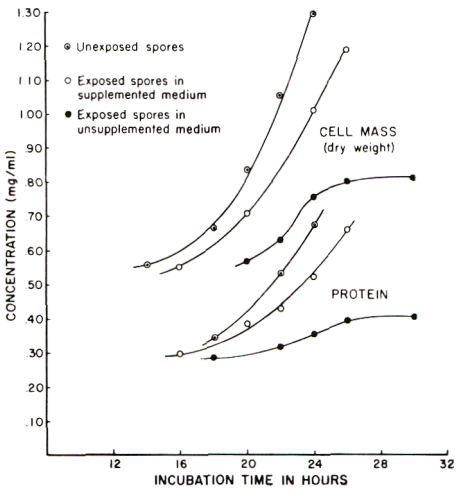

Fig. 4–Effect of ETO exposure of spores of C. botulinum 62A on cell mass and protein synthesis.

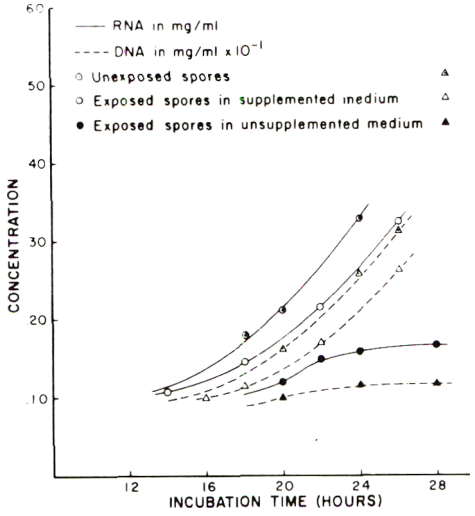

Fig. 5–Effect of ETO exposure of spores of C. botulinum 62A on RNA and DNA synthesis.

nine. Guanine supplementation improved outgrowth only slightly.

In an experiment using different combinations of two bases, only supplementation with the combination of guanine and adenine resulted in good outgrowth from the ETO "damaged" spores. Supplementation with equimolar concentrations of guanine and adenine resulted in best recovery. Results of this study are depicted in Figure 3. It should be noted, in comparing results in Figures 3, 4 and 5 that the concentration of potentially viable spores in the treated inoculum was only aobut 0.1 that of the untreated, ETO treatment having destroyed viability of about 90% of the spores initially contributing to optical density.

These results show that, even though adenine alone could not improve outgrowth from treated spores, it was essential in combination with guanine for good recovery. Further study demonstrated that supplementation with adenosine triphosphate and guanosine triphosphate gave the same results.

From these results it appears most probable that the lethal action of ETO on spores of *C. botulinum* is through alkylation of both guanosine triphosphate and adenosine triphosphate of DNA. Stacey et al. (1958) reported that the most reactive site in DNA is the phosphate group and contended that this was because it is the only readily accessible group by which the molecule can be attacked–all other reactive sites being hidden by alpha helical structures. They hypothesized that initial alkylation of DNA is of the phosphate group and that the triester so formed in turn alkylates a nitrogen atom in one or more of the moieties (in this case guanine and adenine). The quarternized purine then rapidly splits off the DNA, leaving a sugar phosphate residue. (The finding in this study that guanine and adenine are equally as effective as guanosine triphosphate and adenosine triphosphate would suggest that added guanine and adenine react with intracellar phosphates prior to their incorporation in damaged DNA.)

Further studies of the damage and repair processes, demonstrated that in exposed spores DNA, RNA and protein synthesis impairment could be overcome by guanine and adenine supplementation of the synthetic medium (see Fig. 4 and 5). Though ETO treatment definitely impaired RNA and protein synthesis, it is believed that such impairment was an indirect effect and that the main effect was most likely on DNA. Damaged DNA could bring about a disturbed process of transcription and translation, with the result that RNA and protein synthesis would be disturbed. Changes in purine and pyrimidine sequences could also cause abnormalities in RNA and protein synthesis. Additional evidence bearing on this point is needed to support a more definite conclusion.

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EFFECT OF MONOVALENT AND DIVALENT IONS UPON THE GERMINATION OF BACILLUS SPORES IN THE PRESENCE OF NISIN

SUMMARY—The effect of a monovalent ion like Na and divalent ions like Cu, Fe, Mn, Mg and Ca on the germination of spores of Bacillus stearothermophilus, B. cereus *and* B. megatherium *both in the presence and absence of nisin was investigated. In the absence of nisin, Cu, Fe and Mn ions were more inhibitory than Ca and Na ions for the germination of all three kinds of spores. At 1M, Mg increased germination of different spores 3–9%. In the presence of nisin, both Ca and Mg increased the germination of* B. stearothermophilus *by 10.8 and 14.2%, of* B. cereus *by 30.4 and 19.9%, and* B. megatherium *by 6.6 and 30.9%, respectively. The rest of the ions increased inhibition of germination by nisin. The increase in germination by Ca and Mg was correlated with the release of DPA and is discussed.*

INTRODUCTION

NISIN is known to inhibit the germination of all types of spores, but so far the mechanism of inhibition is unexplained. The general opinion is that the outgrowth of germinated spores is inhibited (Trammer, 1964). Trammer has also pointed out that nisin itself does not bind to the surface of the spores of *Bacillus stearothermophilus*. According to Hitchins et al. (1963) nisin prevents the rupture or lysis of spore coats but not the germination of the spores of *B. cereus* and *B. subtilis*. Recently many reports appeared dealing with the reversal of the effect of surface active antibiotics like nisin and vancomycin on vegetative cells by divalent ions like magnesium (Russell and Thomas, 1966; Gupta, 1969). The monovalent and divalent ions have also been shown to influence the germination of spores. According to Curran et al. (1943) spores contain more cations than the vegetative cells. Foerster and Foster (1966) reported increased germination of spores of the *Bacillus* species in the presence of ions. Hyatt and Levinson (1961) and Levinson and Sevag (1953) found manganese and a number of other anions to stimulate the germination of *B. megatherium* spores. Rode and Foster (1962) showed that in the absence of ions, spores of *B. cereus* and *B. megatherium* QMB 1551 showed decreased or poor germination. NH_4^+ has been shown to be essential for the germination of *B. subtilis* spores by Wolf and Mohmond (1957). Gibbs (1967) reported *Cl. bifermentans* required sodium for spore germination.

In the present report the effect of monovalent and divalent ions upon the germination of spores of some *Bacillus* species both in the presence and absence of nisin is reported.

EXPERIMENTAL

Organism

Bacillus stearothermophilus NCA 1518 was obtained from Alpin and Barrett Ltd., Yeovil, Sommerset, England. *B. cereus* and *B. megatherium* were laboratory strains. These cultures were maintained on nutrient agar at 4°C.

Nisin was obtained from Alpin & Barrett, Ltd., Yeovil, Sommerset, England.

Culture media

In addition to the usual nutrient broth/agar, Thorpe's (1960) medium was used for sporulation.

Preparation of spores

Spores were prepared according to the procedure of Thorpe (1960). After 8–10 days' incubation at 55°C in the case of *B. stearothermophilus* and 45°C in the case of *B. cereus* and *B. megatherium*, the spores were harvested. The stock suspension of spores was made in sterile water as described by Trammer (1964). Spore count was made according to Vinter (1957).

Effect of ions on germination of spores

Nutrient broth was tubed in 9 ml lots in 15 × 125 mm tubes and sterilized. To 9.0 ml sterile broth 1.0 ml of spore suspension was added and the number of spores and vegetative cells in 0.01 ml of the suspension determined by differential staining technique. Various concentrations of ions (e.g., Ca and Na as chlorides; Cu, Mn, Mg and Fe as sulphates) were added to the tubes containing 100 μ/ml nisin or no nisin. The tubes were incubated at 37°C in the case of *B. stearothermophilus* and at 28–30°C in the case of *B. cereus* and *B. megatherium* for 24 hr. Again the number of spores and vegetative cells in 0.01 ml of the suspension was determined by differential staining and the percentage of germination calculated.

Determination of DPA release

DPA release from spores treated with nisin or without nisin in the presence or absence of various ions was studied by the method of Janssen et al. (1958).

RESULTS & DISCUSSION

THE EFFECT of different ions at various concentrations on the germination of spores of *B. stearothermophilus, B. cereus* and *B. megatherium* is expressed in Table 1. It was found that the presence of Cu, Fe and Mn inhibited the germination of

Table 1—Effect of salts upon the germination of Bacillus spores

Salts	Percent germination at various concentrations (molar)							
	0.0	0.01	0.05	0.08	0.1	0.5	0.8	1.0
Bacillus stearothermophilus								
$CaCl_2$	61.0	52.0	50.5	45.6	40.6	31.8	20.2	18.5
$CuSO_4 \cdot 5H_2O$	61.0	6.4	5.1	2.0	3.2	1.08	1.07	1.05
$MgSO_4 \cdot 7H_2O$	61.0	63.1	69.8	68.7	69.4	70.1	69.0	69.9
$FeSO_4 \cdot 7H_2O$	61.0	3.23	4.2	7.5	7.2	7.6	7.7	7.5
$MnSO_4 \cdot H_2O$	61.0	4.35	3.0	9.5	15.4	22.0	21.9	14.0
NaCl	61.0	18.4	17.5	17.9	18.9	24.0	14.3	14.3
Bacillus cereus								
$CaCl_2$	52.0	52.0	55.0	31.0	40.0	39.0	39.0	39.5
$CuSO_4 \cdot 5H_2O$	52.0	15.3	20.4	16.7	21.2	21.3	20.7	21.6
$MgSO_4 \cdot 7H_2O$	52.0	37.3	51.1	52.9	55.0	55.4	56.1	55.3
$FeSO_4 \cdot 7H_2O$	52.0	19.4	20.5	29.4	34.1	29.7	26.5	27.0
$MnSO_4 \cdot H_2O$	52.0	13.6	14.1	17.0	17.2	17.04	16.5	16.9
NaCl	52.0	18.9	20.5	22.9	30.5	31.2	35.4	34.9
Bacillus megatherium								
$CaCl_2$	54.0	4.0	31.0	32.0	39.0	33.0	31.0	21.0
$CuSO_4 \cdot 5H_2O$	54.0	8.7	9.5	9.9	9.5	8.2	9.3	8.8
$MgSO_4 \cdot 7H_2O$	54.0	46.6	50.8	50.9	52.8	57.2	58.0	53.3
$FeSO_4 \cdot 7H_2O$	54.0	5.2	5.4	5.9	7.3	7.4	7.7	8.6
$MnSO_4 \cdot H_2O$	54.0	17.0	17.4	18.5	18.2	18.8	18.9	19.0
NaCl	54.0	15.0	18.1	20.2	21.3	19.5	19.9	20.0

Table 2–Effect of salts upon the germination of Bacillus spores in the presence of 100 μg/ml of nisin

Salts	Percent germination at various concentrations (molar)						
	0.0	0.01	0.05	0.08	0.1	0.8	1.0
Bacillus stearothermophilus							
$CaCl_2$	6.8	11.4	15.3	15.6	17.2	17.2	17.6
$CuSO_4 \cdot 5H_2O$	6.8	3.2	2.0	4.1	3.0	3.6	3.0
$MgSO_4 \cdot 7H_2O$	6.8	21.2	23.4	20.8	21.2	19.8	21.0
$FeSO_4 \cdot 7H_2O$	6.8	4.2	3.9	4.8	3.0	3.4	4.0
$MnSO_4 \cdot H_2O$	6.8	3.0	3.7	3.6	4.0	2.9	3.2
NaCl	6.8	3.9	3.8	4.0	3.7	3.0	4.2
Bacillus cereus							
$CaCl_2$	5.0	29.2	31.3	30.0	33.4	36.3	35.4
$CuSO_4 \cdot 5H_2O$	5.0	6.4	6.6	5.9	5.3	6.0	5.9
$MgSO_4 \cdot 7H_2O$	5.0	25.3	26.2	21.6	25.8	24.9	24.9
$FeSO_4 \cdot 7H_2O$	5.0	3.2	3.6	4.0	3.9	5.2	5.0
$MnSO_4 \cdot H_2O$	5.0	2.9	3.2	3.5	3.0	3.3	3.5
NaCl	5.0	4.0	3.8	3.6	3.7	3.8	3.6
Bacillus megatherium							
$CaCl_2$	5.7	15.1	11.2	14.3	12.6	10.8	12.3
$CuSO_4 \cdot 5H_2O$	5.7	4.0	4.1	3.9	3.7	4.8	3.9
$MgSO_4 \cdot 7H_2O$	5.7	31.1	32.3	32.0	36.0	29.0	36.6
$FeSO_4 \cdot 7H_2O$	5.7	3.3	3.9	5.2	4.9	4.8	5.0
NaCl	5.7	5.2	4.3	5.6	5.2	5.0	4.9

all three kinds of spores. Ca and Na were less inhibitory. There was a slow but definite increase in the germination of spores of *B. stearothermophilus* with an increase in Mg concentration. However, Mg decreased the germination of the spores of *B. cereus* and *B. megatherium* at 0.01–0.05% concentration above which germination increased.

Results summarized in Table 2 show that in the presence of 100 μ/ml nisin Ca and Mg ions increased spore germination. The increase by Ca ions observed in the case of *B. stearothermophilus, B. cereus* and *B. megatherium* was about 10.8, 30.4 and 6.5% respectively at 1M concentration and with respect to Mg ions the increase in germination of spores of *B. stearothermophilus, B. cereus* and *B. megatherium* was 14.2, 19.9 and 30.9% respectively at 1.0M concentration. The results indicate that Mg and Ca ions compete with nisin, thereby lessening the harmful effect of nisin on spore germination. In the presence of other ions like Cu, Fe, Mn and Na nisin was more inhibitory for the germination of spores.

If Mg and Ca help in the germination of spores in the presence of nisin then they must also help in the release of DPA. Results of studies on DPA release from spores treated with nisin in the presence or absence of Ca or Mg ions are summarized in Table 3. It can be seen that Mg or Ca helps in the release of DPA whereas in the presence of nisin only very little DPA is released. Therefore, it is concluded that the nisin inhibits the spore germination by preventing DPA release.

Increasing concentrations of Ca were observed to decrease germination of all three kinds of spores (Table 1). But when spores were treated with nisin, it helped to counteract the inhibition of spore germination by the antibiotic. Mg ions are a real stimulant (Table 1 and 2), in conformance with the findings of Foerster and Foster (1966). Russell and Thomas (1966) and Gupta (1969) have reported that the increase of germination of spores treated with antibiotic in the presence of ions may be due to competition between the two for the same site. The observed inhibition of spore germination by Mn ions is in contradiction with the earlier reports of Levinson and Sevag (1953). They have shown that Mn ions stimulate a number of spore enzymes. According to Rode and Foster (1966) the ionic conditions of bacterial spores affect their germinability. Ca, Sr and Ba are most effective in conditioning spores for germinability. Ions may possibly play a role by altering the spore coat proteins. NaCl was observed to be inhibitory for all three kinds of spore germination in the presence and absence of nisin (Table 1 and 2). However, Iandolo and Ordal (1964) reported that an increase in NaCl in the presence of CaDPA decreased the lag in the germination response and that complete germination occurred more rapidly as the concentration was increased. Such effect was also observed by Rode and Foster (1962).

Murty et al. (cf. Lee and Ordal, 1963) reported that various cations like Cu inhibited the germination of *B. cereus* spores. Lee and Ordal (1963) found that the germination of activated spores of *B. megatherium* was inhibited by copper, iron, silver or mercury salts. According to them the inhibition resulted in the disintegration of spore coats. However, cation salts of $CaCl_2$, $MnCl_2$, $NiSO_4$, $CoCl_2$ and $MgSO_4$ were not inhibitory for the germination. In this study besides Fe, Cu and Na, Mn was also inhibitory for spore germination.

Table 3–Release of DPA in the presence and absence of nisin and Ca and Mg ions

Organism	Nisin[a] (μ/ml)	Ions[a]	DPA released (μg)
B. stearothermophilus	100	0.5M Mg	72
	100	–	0.05
	–	–	53.0
B. cereus	100	0.05M Ca	22.5
	100	–	3.5
	–	–	43.5
B. megatherium	100	0.8M Mg	28.5
	100	–	3.5
	–	–	37.5

[a]Dash indicates the absence of antibiotic or ions.

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APPLIED SCIENCE and ENGINEERING

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SPIKED-PLATE FREEZE DRYING

SUMMARY—The acceleration of freeze-drying rates using spiked plates was observed experimentally and found to reduce overall drying times by as much as 24 hr. Experiments were conducted using different spike diameters, sample thickness, spike materials and heater plate temperatures. Weight loss and temperature profiles were obtained as a function of drying times. These experimental results were used to develop a simplified mathematical model for predicting drying rates which will be of use to the designer of spiked-plate freeze-drying systems. Results obtained using the mathematical model indicate spike diameter, spike spacing and plate temperature have significant effect on the drying rate, whereas sample thickness and spike material are not significant.

INTRODUCTION

CONDUCTION methods for supplying heat to the phase change front in freeze drying were proposed by Hay (1955) and later improved by Forrest (1962). These methods consist of using conduction to supply heat to the sublimating ice phase. A major problem associated with this technique for thick products was the continuously increasing dry layer which acted as a very good thermal insulator between the surface and the sublimating ice phase. This problem was overcome by Brynko and Smithies (1958). The process consisted of placing the beefsteaks between two spiked heater plates. The spikes thus transmit heat by conduction from the heater plate through the dried outer layer into the frozen core and thus increase the drying rate. Ballantyne et al. (1958) conducted acceptability studies of beef freeze dried using spiked plates and found them to be as acceptable to taste panels as meat conventionally freeze dried using radiant heating. Mink and Sachsel (1961) presented a mathematical model which predicts drying times of meat placed on spiked plates.

The spiked plate system presented by Brynko and Smithies (1958) exhibited handling problems when attempts were made to use the spiked plates in existing freeze-drying equipment. Therefore, the current investigation was undertaken to develop a feasible system which could be used with conventional freeze-drying equipment without major modification of the freeze dryer. Studies of the internal drying problem were made experimentally and a mathematical model was developed to evaluate the effect of the design parameters on drying rates.

EXPERIMENTAL

Spiked plate

The experiment conducted by Brynko and Smithies (1958) used a spiked plate system in which spikes were alternately attached to the top and bottom plate. Stripper plates were placed between the plates and meat to aid in the removal of the meat at the completion of freeze-drying. Electric resistance heaters were placed on the back of each plate and the pins were secured in place by a force fit in holes drilled in the plate. The entire spiked plate unit was cumbersome and difficult to use in existing radiantly heated freeze-drying units. As a result of the alternating top and bottom plate mounted spikes, the placement of the meat sample between the plates was difficult. The stripper plates acted as a resistance to thermal radiation which may be added to the meat sample from the heated plate.

An effort was made in the current investigation to eliminate some of the mechanical difficulties exhibited by Brynko and Smithies' design. The spiked plates for these experiments were constructed from two 1/8-in. thick aluminum plates with holes drilled in one plate for the spikes. The spikes were inserted and the plates were bolted together to secure the spikes in place. The spikes were constructed from different cold drawn materials in different diameters and lengths. One end of the spike was sharpened and the other enlarged to prevent the spike from falling out of the holes in the first plate. The plate was easily constructed in thin wafers and the problem of sample placement was eliminated. The stripper plates used by Brynko and Smithies were eliminated because it was decided that means for product removal could be provided externally to the freeze-drying equipment upon completion of the drying process. There appears to be little advantage gained from having two spiked plates when considering heat transfer to the product. When using spikes of high thermal conductivity (such as aluminum) the advantage gained from two plates in increased heat transfer appears to be small when considering the handling problems created with the second plate. Also, when only one plate is used the upper exposed surface of the product can gain radiant heat from the platen above each tray in the freeze dryer. When no stripper plate is used between the product and the spiked plate during the drying process, radiation from the plate will add heat to the product in addition to the heat added by conduction through the spikes.

The spiked plate was 10 in. × 10 in. for these experiments; however, much larger plates could be used. The spikes were constructed from

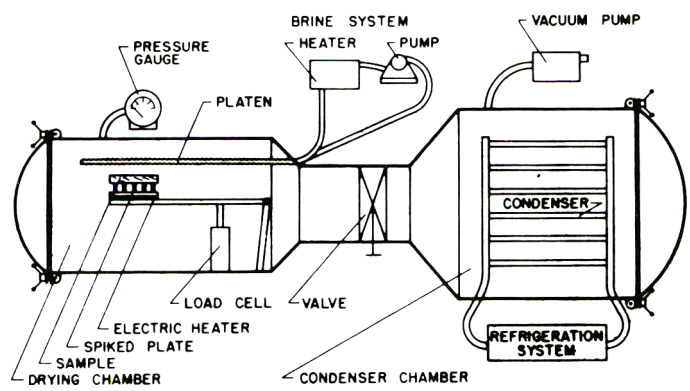

Fig. 1—Spiked-plate freeze-drying apparatus.

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three different diameter rods: 1/16, 3/32, and 1/8 in. and were 1-1/2–2 in. long. The 1/16 in. diam pins were made from stainless steel having a thermal conductivity of 9 BTU/hr-ft-°F. Three different plates were constructed to accommodate the three different spike diameters. The spike spacing was 1/2 in. for each of these plates.

The spiked plate was placed on a 1700 watt electrical heater which was then placed on a load cell just below the bottom heating platen in a freeze dryer as shown in Figure 1. The heater was controlled by a variable transformer. The electrical heater was used because it could be easily suspended from a load cell. Under normal production procedures the spiked plates would be placed directly on the heating platens in the freeze dryer.

Instrumentation

Temperature measurement. Temperatures were measured at locations within the meat, on the spikes, plate and platen using 36 guage copper-constantan thermocouples. The wire was supplied by Thermoelectric Corp.,Saddle Brook, N.J. on special order and was individually coated with teflon. The thermocouples were constructed by a resistance welding process. The thermocouples were placed on the spikes, plate and platen by using a fine-pointed punch to make a hole along the surface and placing the thermocouple junction in place, then the junction was peened into the surface of the metal. The emf produced by these thermocouples was recorded with a 24-point recording potentiometer. The thermocouples were calibrated using mercury in glass precision thermometers.

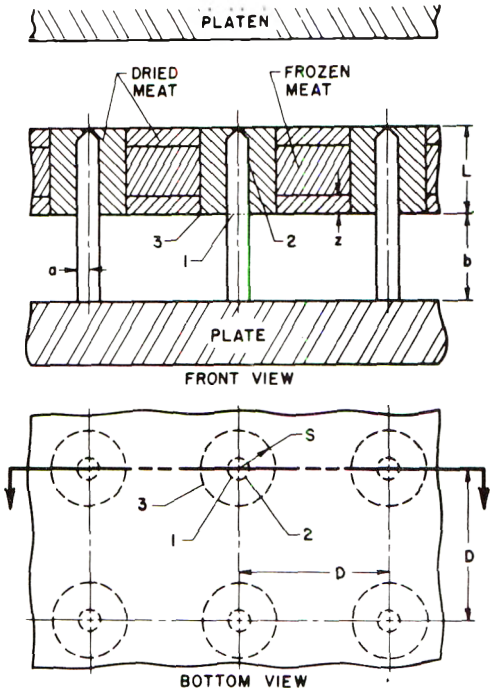

Fig. 2–Spiked-plate drying model.

Weight measurement. A force transducer supplied by Schaevitz Engineering, Pennsauken, N.J., type FTA-5-10 with a range of ± 10 lb was used on a cantilever pivot beam to increase the sensitivity of the weight change. The system is shown in Figure 1. The resolution of the load cell was less then 1g. The load cell was driven with a Schaevitz Engineering signal conditioner model SCM-025 having a ± 10 Vdc output. The signal conditioner output was measured with a digital voltmeter. Calibration was accomplished before each test run by placement of known weights on the load cell system. The linearity of the load cell was tested by Schaevitz Engineering and found to be less than 0.12% deviation over the full range of the impressed voltage. The overall weight loss of each sample was checked with a Mettler P1200N balance by weighing before and after drying.

Total pressure measurement. The total pressure of the drying chamber was controlled to 1 torr and this pressure was determined using a Wallace and Tiernan absolute pressure guage. Calibration of the gauge was conducted prior to each experimental run and immediately following.

Sample preparation

The beef used was obtained from a "top of the round" cut of U.S. Choice beef. The entire cut was frozen in a 0°F food freezer and samples were sawed out of the frozen meat using a Globe meat saw. The samples were cut such that the grain direction was parallel to the spikes when the sample was placed on the spiked plate. The samples were cut to approximately 3 × 4 in. and to the thickness required for each experiment. The edge effects were minimzed by properly sizing the sample such that the edge of the sample was just at the center of a row of pins. Very little over-hang of the sample was allowed behind the last row of spikes on each side. The sample was allowed to thaw on a stainless plate in a refrigerator at 40°F. This thawing procedure was necessary to allow insertion of thermocouples within the meat. For production purposes this thawing step may be eliminated. The meat was placed on the spikes by forcing the spikes down on the stainless plate allowing the spikes to come just through the top surface of the meat. Thermocouples were placed between the spikes on the top and bottom surfaces of the sample. Thermocouples were also placed between the spikes half way between the top and bottom surface of the sample. Three thermocouples were placed in this manner within the meat 2 in. from the side, thus placing the thermocouples approximately in the center of the sample. Following placement of the thermocouples the spiked plate holding the sample was placed in a food freezer at 0°F and allowed to refreeze for at least 6 hr prior to the experiments.

ANALYTICAL INVESTIGATION

A mathematical model is developed to predict drying time for spiked-plate freeze drying in which the product surface temperature and spike temperatures vary from the initial frozen product temperature to a temperature slightly less than the plate and platen temperature. The plate and platen temperature is constant for this analysis. The model is shown in Figure 2.

Heat added by the fin to the product through conduction is found by considering the heat per unit time crossing boundary 1,

$$\dot{q}_1 = k_p A_p \frac{(T_H - T_p)}{b} \qquad [1]$$

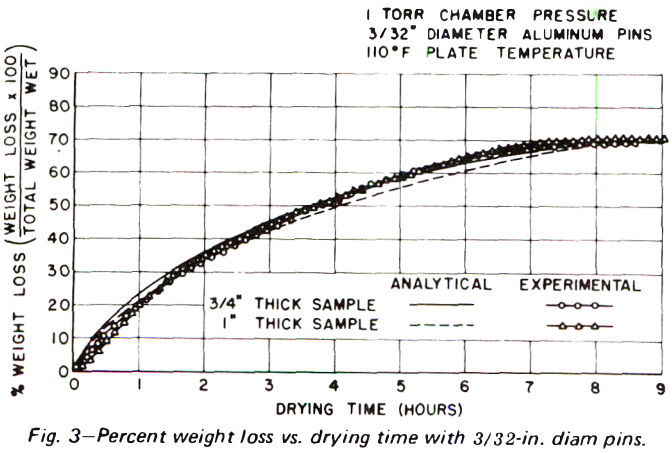

Fig. 3–Percent weight loss vs. drying time with 3/32-in. diam pins.

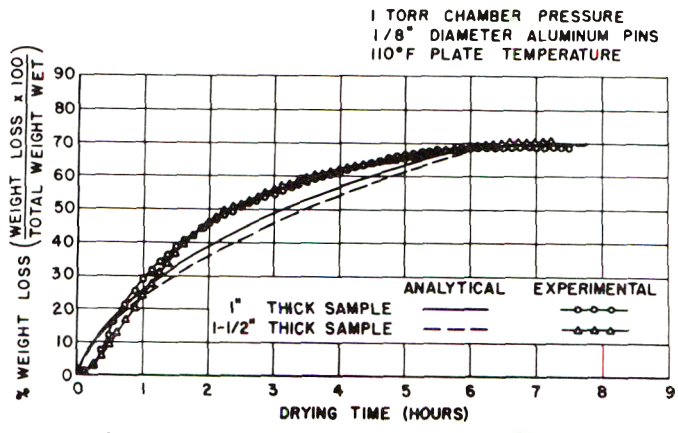

Fig. 4–Percent weight loss vs. drying time with 1/8-in. diam pins.

It is assumed that the pin is at a constant temperature T_p at any location within the meat. This assumption is reasonable as will be illustrated when the experimental data are presented in a later section. The heat leaving the pin into the sample at boundary 2 is

$$\dot{q}_2 = \frac{2\pi \cdot k_{mn} (T_p - T_f)L}{\ln(s/a)} \qquad [2]$$

assuming a radial heat flow around the pin into the dried portion of the beef. Thus at any instant of time these two heat flows should be equivalent. Equations [1] and [2] are solved for the pin temperature within the meat,

$$T_p = \frac{T_f + \gamma(\ln s/a)T_H}{1 + \gamma \ln s/a} \qquad [3]$$

where γ is a constant. The energy required to sublimate a differential element ds at the phase change front (position 3) per unit time is

$$\dot{q}_3 = 2\pi\, L\, s\, \rho\, \lambda\, H \frac{ds}{dt} \qquad [4]$$

Neglecting the energy needed to raise the temperature of the dried region, $\dot{q}_3$ will equal $\dot{q}_2$ and equations [2], [3] and [4] can be combined to yield

$$\frac{ds}{dt} = \frac{\gamma(T_H - T_f)}{B(s + \gamma s \ln s/a)} \qquad [5]$$

In equation [5] γ, B and a are constants which are independent of time and radial position. The temperature of the ice phase front T_f depends on the total and partial pressure of the water vapor within the drying chamber and is nearly constant if the chamber pressure remains constant throughout the process. Thus, in equation [5] only s and t are variables. Integrating equation [5] by parts,

$$t = \frac{B}{\gamma(T_H - T_f)} \left| \frac{(s^2 - a^2)(2 - \gamma)}{4} + \frac{\gamma s^2}{2} \ln s/a \right| \qquad [6]$$

Equation [6] gives the time required for the phase change front to reach a radial position s.

Heat is supplied by thermal radiation to the sample from the plate and platen. To account for this heat a mathematical analysis of slab freeze drying developed by Cho and Sunderland (1970) is used (see Appendix). The drying time required for the phase change position to reach a depth z within the slab due to thermal radiation from the plate and platen is given by

$$t = \frac{\lambda\, \rho\, H}{T_H - T_f} \left| \frac{z^2}{k_{mp}} + C_2 z + (C_3 - C_1 C_2) \ln\left(\frac{z + C_1}{C_1}\right) \right| \qquad [7]$$

With equations [6] and [7], all the heat inputs to the sample are considered. Most of the heat input is obtained from conduction through the pins; however, to obtain accurate weight loss data from the analysis, consideration must be given to the addition of thermal radiation from the plate and platen. Should length of drying be the only concern in the analysis, the use of equation [6] is all that is required. The controlling variable for total drying time is the radial drying depth s, and the slab drying found from equation [7] is not necessary. For example, the time required to dry a 1-in. thick slab is 30 hr with a 110°F plate temperature and the heat transfer from the pins will dry the same sample in about 6–8 hr, depending upon the pin diameter and spacing.

The method of calculating percent weight loss involves calculating the depth of penetration of the phase front from equation [6] and for the same time determining the depth of drying z obtained from equation [7]. From Figure 2 it may be observed that drying proceeds from the pins and from the surface between the pins. Thus, there is a continuous changing of the area of the frozen meat exposed to the pin and to the product surfaces. An account is made for this in the analysis by reducing the area of heat transfer from the surface of the sample to the frozen section in an amount equal to the radial area dried around the pin. The reduction of the slab heat transfer area was chosen because the pin drying is much faster

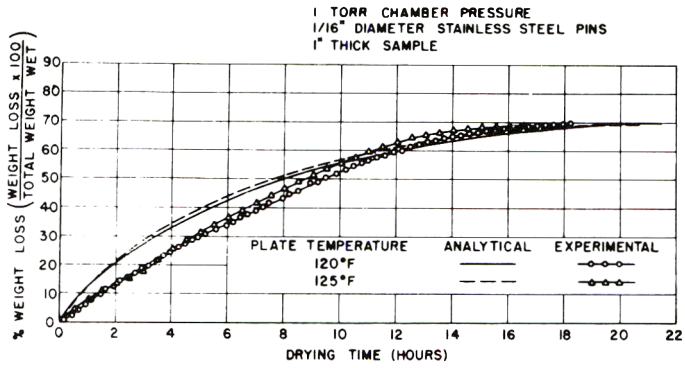

Fig. 5—Percent weight loss vs. drying time with 1/16-in. diam pins.

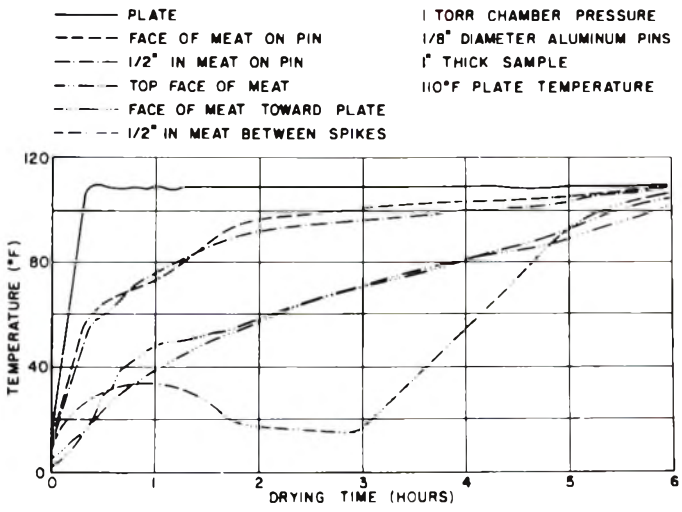

Fig. 6—Temperature throughout sample vs. drying time with 1/8-in. diam pins.

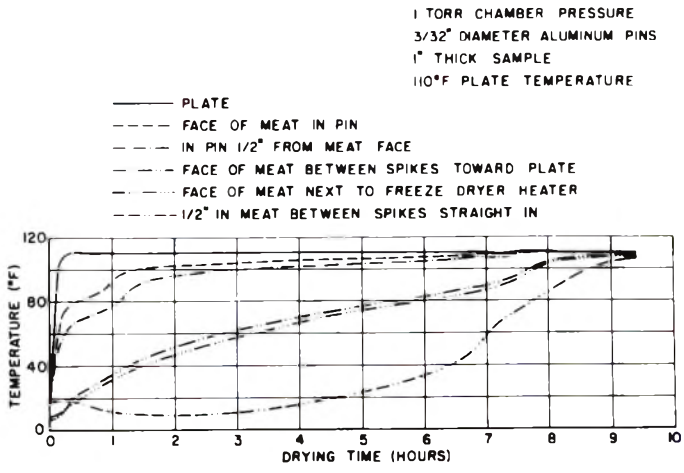

Fig. 7—Temperature throughout sample vs. drying time with 3/32-in. diam pins.

than the slab drying and this area is changing much faster than that of the pin. The phase change front receeds radially from the pin and simultaneously a phase change front receeds from the free surface. Using the volume created by the equivalent radial drying diameter and the meat thickness, and considering the radial and slab drying volumes, a simple calculation was used to determine weight loss from the following equation:

$$\% \text{ wt loss} = \frac{100\,\lambda(\text{vol dried by pin} + \text{vol dried by radiation})}{\pi(D^2 - a^2)L} \quad [8]$$

This equation gives the weight loss at a specific drying time at which the dried volumes of pin and slab were obtained. The volume dried by the pin was determined from equation [6] and the volume dried by radiation from plate and platen was found by using twice the value obtained from equation [7]. By calculating each volume from drying distance s and z at the same drying time t in equations [6] and [7] the weight loss was found at any time.

RESULTS & DISCUSSION

WEIGHT LOSS as a function of drying time is presented in Figures 3–5 for each set of test conditions. The drying chamber total pressure was maintained at 1 torr during each experiment.

The temperatures of the plate and platen are the same and are indicated on the figures. The spiked plates having the aluminum pins were operated at 110°F. These pins have a high thermal conductivity so that the pins in the meat reach a temperature of about 100°F very rapidly. Should a higher plate temperature have been used, scorching the dried beef around the pins may have resulted. The scorch temperature for beef may vary from sample to sample, but a surface temperature of 100°F will not result in damage to the beef. Brynko and Smithies (1958) used a plate temperature of 125°F and observed some damage due to scorching of the surface of the meat in contact with the pins. Plate and platen temperatures with the stainless pins were held at 120°F and 125°F. An inspection of Figure 5 reveals that a difference in plate and platen temperature of 5°F makes very little difference in drying rate using the stainless pins.

The results of the 3/32-in. diam pin using different sample thicknesses of 3/4 and 1 in. shown in Figure 4 indicate that the sample thickness has little effect on drying rate. The same result is indicated with the 1/8-in. diam pins using 1 and 1-1/2-in. thick samples.

The difference in terminal percentage weight loss ranges from 68–71% indicates the variation of the initial moisture and fat content of the samples.

The time at which no further drying takes place is difficult to determine as indicated by Figure 4. The data imply 6 hr as the drying time required; however, from 6–7 hr of drying, there is a change of 0.5% in weight loss indicating further drying of the sample. This same general result holds with the other methods of drying. Several tests were conducted where drying was terminated at 6 hr as in the case of the experiment indicated in Figure 5. The sample was dissected and inspected for sections of undried beef. In these tests, no wet sections were found by visual inspection. Therefore, the end point was chosen to be the time after which the percentage weight loss was less than 0.5%. For example, in Figure 5, the time required to dry the sample would be 6.5 hr.

Temperature distribution

The temperatures measured during the experiments are presented in Figures 6, 7 and 8. These temperatures are very useful in developing a model to describe the drying process. It may be observed from Figures 6 and 7 with aluminum pins that the temperature differences of the pins within the meat are very small. However, with the stainless pins shown in Figure 8, the temperature distribution in the pin is much larger. This should have an adverse effect on the results of the mathematical model due to the assumption of a uniform temperature of the pin in the meat.

A comparison of the drying times indicated in Figures 3 and 4 and the times at which the thermocouples in the center of the meat sample between the spikes shown in Figures 6 and 7 gives an indication of the end point. If the drying time at which the temperature starts to approach that of the plate and platen shown in Figure 6 is compared to the percent weight change data of Figure 4, a good correlation may be obtained for determining the end point of drying.

Comparison of analytical and experimental results

The calculations based on the models developed for radial and slab drying are compared with the experimental weight loss data using the following physical properties of the samples:

Properties			Reference
ρ	=	57.4 lbm/ft^3	Dickerson (1968)
k_{mp}	=	0.049 BTU/hr-ft-°R	Laboratory
k_{mn}	=	0.0193 BTU/hr-ft-°R	Laboratory
H	=	1488 BTU/lbm	Dyer et al. (1966)
λ	=	0.70	Laboratory
ϵ	=	0.74	McCulloch and Sunderland (1970a)
T_{sc}	=	100°F	Hatcher et al. (1971)
T_f	=	5.5°F	Hatcher et al. (1971)

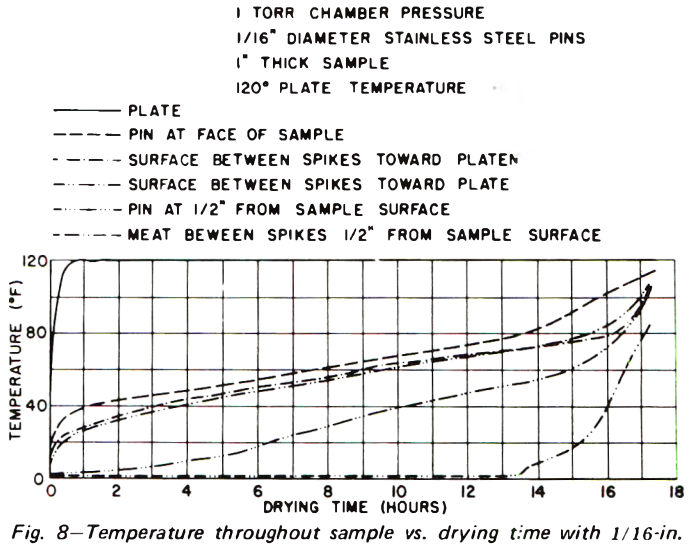

Fig. 8–Temperature throughout sample vs. drying time with 1/16-in. diam pins.

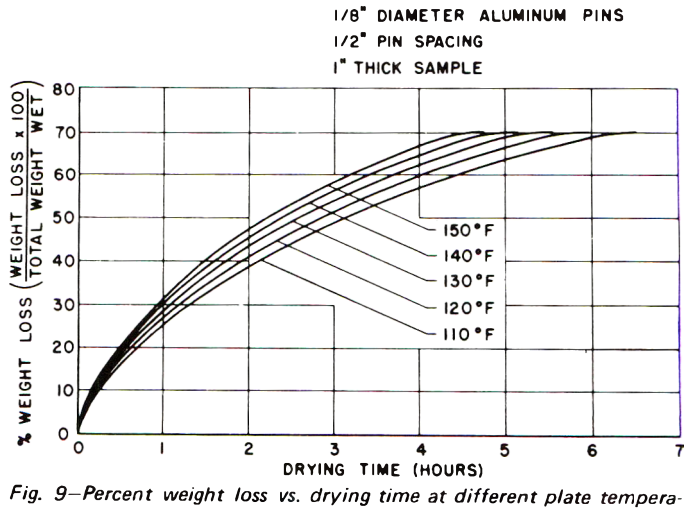

Fig. 9–Percent weight loss vs. drying time at different plate temperatures using mathematical model.

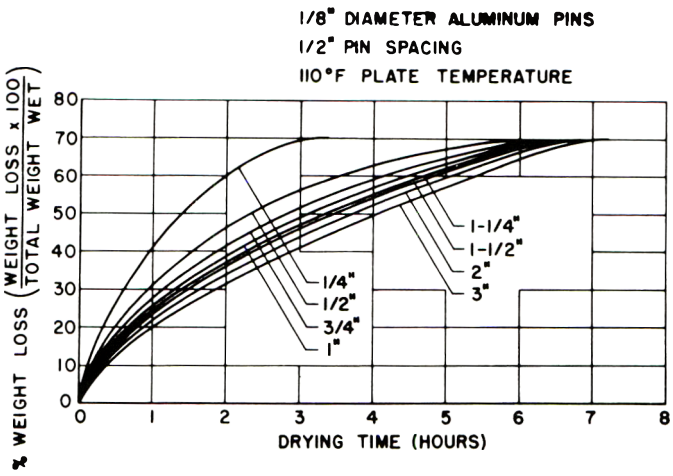

Fig. 10—Percent weight loss vs. drying time at different sample thickness using mathematical model.

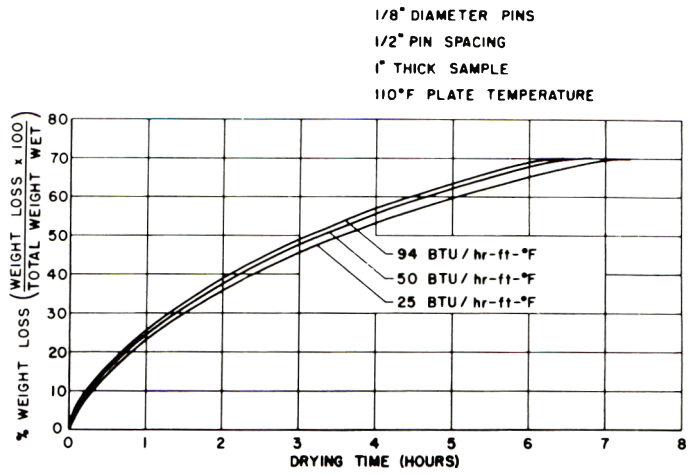

Fig. 11—Percent weight loss vs. drying time at different pin thermal conductivities using mathematical model.

The freeze-drying laboratory at North Carolina State Universtiy is presently engaged in research to determine the thermal conductivity of freeze-dried beef under conditions where the partial pressure of water vapor is equivalent to that existing during actual drying. The conductivity normal and parallel to grain direction is measured with a guarded hot plate. An experimental method is used to measure the porosity of freeze-dried beef. The data referenced as laboratory was obtained using this equipment.

The comparison of the analytical results to the experimental data is shown in Figures 3–5. The assumption of an isothermal pin within the beef though not valid for the stainless pin does not affect the results of the model as shown by the close agreement of the analytical and experimental results of Figure 5. The model appears adequate for design purposes within the range of pin conductivities of 9–94 BTU/hr-ft-°F. This range of conductivities covers most materials used for pin construction. Should a material having a conductivity of less than 9 BTU/hr-ft-°F be used there may be little advantage of the spiked plate method over other forms of heat addition such as radiant heating.

The choice of radial drying distance s for use in the model to predict the end point of drying is important. There appeared to be three choices: the diagonal center between opposing squares of spikes (the last point to dry in the cube of meat between the spikes); the radial distance formed by a circle equivalent in area to the area between four opposing spikes; or the radial distance equal to half the distance between the spikes. The last of these radii seemed to give the best agreement between calculated and experimental results.

Mink and Sachsel (1961) presented the following equation for predicting the total drying time:

$$t = \frac{3\sqrt{3MD^2L}}{8\pi k_f(T_H - T_f)a}$$

This equation predicts that the drying time is proportional to the square root of the sample thickness whereas our results indicate that the drying time is nearly independent of thickness for samples greater than 3/4-in. thick. This equation also uses the thermal conductivity of the frozen region. Our studies show that drying takes place around the spikes so that the thermal conductivity of the dried region seems to be more relevant. Although this equation adequately predicts the experimental results of Brynko and Smithies (1958), it gives errors as much as 60% for the data presented in the current investigation. Thus it does not apply to as many situations as the equations we have presented.

Extension of model to design considerations

The mathematical model is used to investigate the following design variables: plate temperature, product thickness, pin thermal conductivity, pin spacing and pin diameter. The properties of the sample are the same as those used in the previous section.

Plate temperature. The results of the model using different plate temperatures are shown in Figure 9. The 150°F plate temperature reduces the drying time to 4.7 hr from 6.4 hr for a 110°F plate temperature. Thus, the plate temperature has a large effect on drying time. However, the use of plate temperature above 110°F results in reduced product quality. Also, the product may be thermally damaged around the pins because of the high pin temperature with high plate temperatures. Thus, elevation of the plate temperature to achieve faster drying should be done only with consideration of resulting product quality.

Product thickness. The effect of sample thickness on drying rate becomes very small when thick samples are used as shown in Figure 10. For example, the 1-in. thick sample dries in 6.7 hr and the 3-in. sample dries in 7 hr. Thus, this design parameter has little effect on drying rates and is of small importance with thick products when spiked plate drying is being considered.

Pin thermal conductivity. The results of different pin thermal conductivities is illustrated in Figure 11. A change of pin conductivity from 50 to 94 BTU/hr-ft-°F affects the drying time very little; however, using pins having a thermal conductivity of 9 BTU/hr-ft-°F (data not shown on graph) increases the drying time by 50%. The similarity of the drying times for conductivities of 94 and 50 BTU/hr-ft-°F suggests that a special coating on the pins might not substantially increase drying times and would offer reductions of the sticking force of the product on the pins.

Pin spacing. The pin spacing has a drastic effect on drying rate as shown by Figure 12. With the 1/4-in. spacing, the distance between pins is only 1/8 in. with 1/8-in. diam pins. This configuration gives very fast drying; however, obvious problems result from this close spacing such as product acceptability and damage due to difficulties of removing the spikes after drying is complete. With a pin spacing of 1 in. the drying time is only slightly influenced by the spikes. After

considering the heat transfer and the sample product removal problems, the writers would recommend a pin spacing of about 1/2 in. for most applications.

Pin diameter. The effect of this design variable on drying rate is shown in Figure 13. A small change of pin diameter has a large effect on product drying time. Considering the 1/8-in. diam pins, a change of ± 1/32 in pin diam changes the drying time ± 20%. The selection of pin diameter may be influenced by the desired physical appearance of the finished product, resulting from the holes created by the pins.

In conclusion the pin diameter, spacing and plate temperature have a large effect on drying time and should be considered when designing spiked-plate freezer driers. The pin conductivity and product thickness are of little importance when considering drying times.

NOTATION

A_p cross sectional area of pin, ft^2
a radius of pin, ft
b distance of sample above plate, ft
B $\frac{\rho \lambda H}{k_{mn}}$, a constant, $(hr^\circ R)/ft^2$
C_1 constant defined by Eq. [19], ft
C_2 constant defined by Eq. [20], hr-ft^2-°R/BTU
C_3 constant defined by Eq. [21], hr-ft^3-°R/BTU
D distance between center of pins, ft
h heat transfer coefficient for radiation defined by Eq. [14], BTU/ft^2-hr-°R
H latent heat of sublimation of frozen beef juices, BTU/lb_m
h_o heat transfer coefficient at z = 0, BTU/ft^2-hr-°R
h_∞ heat transfer coefficient at z = ∞, BTU/ft^2-hr-°R
h_{sc} heat transfer coefficient at $z = z_{sc}$, BTU/ft^2-hr-°R
k_f thermal conductivity of frozen beef, BTU/ft-hr-°R
k_p thermal conductivity of pin, BTU/ft-hr-°R
k_{mp} thermal conductivity of dried beef parallel to grain direction, BTU/ft-hr-°R
k_{mn} thermal conductivity of dried beef normal to grain direction, BTU/ft-hr-°R
L length of pin within sample, ft
M energy of sublimation for one cubic foot of beef, BTU/ft^3
$\dot{q}$ heat transfer rate, BTU/hr the subscript on $\dot{q}$ denotes locations indicated in Figure 2.
s radial interface position, ft
t time, hr
T temperature in dried region, °R
T_f temperature of interface and frozen beef, °R
T_H plate and platen temperature, °R
T_p pin temperature, °R
T_s surface temperature of beef between pins, °R
T_{sc} scorch temperatures of beef surface, °R
x distance from exposed surface of sample, ft
z slab interface position, ft
z_{sc} drying depth when sample surface is at the scorch temperature, ft
α thermal diffusivity of dried region, ft^2/hr
β temperature gradient, °R/ft
ϵ total emittance
γ $\frac{k_p A_p}{2\pi k_{mn} Lb}$, dimensionless constant
λ porosity, ratio of pore volume to total volume, dimensionless
ρ density of ice at given pressure, lb_m/ft^3
σ Stefan-Boltzmann constant BTU/ft^2-hr-°R^4

APPENDIX

Cho and Sunderland (1970) have determined a simple and convenient approximate solution to the radiation boundary condition for slab freeze drying using thermal radiation from a heater at constant temperature. The following analysis applies this mathematical model to the slab drying of the product.

If the influence of convection of the vapor is neglected, the energy equation for the dried region is

$$\alpha \frac{\partial^2 T}{\partial x^2} = \frac{\partial T}{\partial t}, \quad t > 0, 0 < x < z \qquad [9]$$

The three boundary conditions are

$$T = T_f \text{ at } x = z, t > 0 \qquad [10]$$

$$k \frac{\partial T}{\partial x} = -\lambda \rho H \frac{dz}{dt} \text{ at } x = z, t > 0 \qquad [11]$$

and

$$-k \frac{\partial T}{\partial x} = \epsilon \sigma (T_H^4 + T_s^4) \text{ at } x = 0, t > 0 \qquad [12]$$

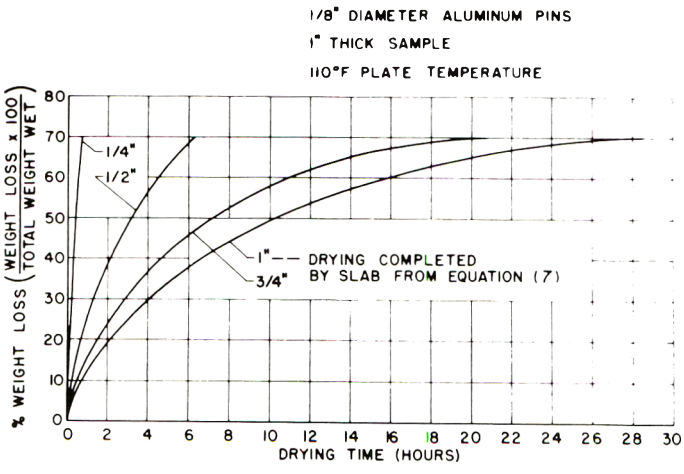

Fig. 12–Percent weight loss vs. drying time at different pin spacing using mathematical model.

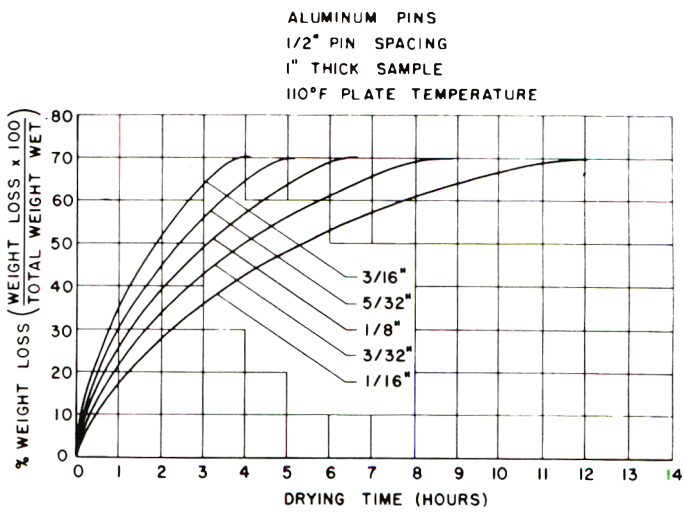

Fig. 13–Percent weight loss vs. drying time at different pin diameters using mathematical model.

Equation [12] is changed to

$$k_{mp} \frac{\partial T}{\partial x} = h(T_s - T_H) \quad [13]$$

where h is defined by

$$h = \epsilon \sigma (T_H^2 + T_s^2)(T_H + T_s) \quad [14]$$

Since T_s is a function of time, h is also a function of time. It has been shown in other work such as McCulloch and Sunderland (1970b) that the surface temperature is a function of depth of drying, thus a function of time indirectly through the dried thickness. Therefore, an assumption is made for h in terms of z, which is used to obtain a closed form solution for the drying position as a function of time. Thus, assume that

$$h = \frac{z + C_1}{C_2 z + C_3} \quad [15]$$

where C_1, C_2 and C_3 are constants obtained from the three conditions:

$$h = h_o = \epsilon \sigma (T_H^2 + T_f^2)(T_H + T_f) \text{ at } z = 0 \quad [16]$$

$$h = h_\infty = 4 \epsilon \sigma T_H^3 \text{ at } z = \infty \quad [17]$$

$$h = h_{sc} = \epsilon \sigma (T_H^2 + T_{sc}^2)(T_H + T_{sc}) \text{ at } z = z_{sc} \quad [18]$$

Solving equations [16–18],

$$C_1 = \frac{h_o}{h_\infty} \frac{h_{sc} - h_\infty}{h_o - h_{sc}} z_{sc} \quad [19]$$

$$C_2 = \frac{1}{h_\infty} \quad [20]$$

$$C_3 = \frac{z_{sc}}{h_\infty} \frac{h_{sc} - h_\infty}{h_o - h_{sc}} \quad [21]$$

Assuming a "quasi-steady" solution in which a linear temperature profile is assumed which satisfies equations [10] and [12],

$$T = T_f + \beta(X - z), \; 0 < x < z \quad [22]$$

Therefore from equation [13] we obtain

$$\beta = \frac{h(T_H - T_f)}{k_{mp} + hz} \quad [23]$$

From equation [11],

$$\frac{h(T_H - T_f)}{k_{mp} + hz} = \frac{\lambda \rho H}{k_{mp}} \frac{dz}{dt} \quad [24]$$

Integrating,

$$t = \frac{\lambda \rho H}{T_H - T_f} \int_0^z \left(\frac{z}{k_{mp}} + \frac{1}{h}\right) dz \quad [25]$$

Substituting equation [15] into [25] and performing the indicated integration, we obtain

$$t = \frac{\lambda \rho H}{T_H - T_f} \left[\frac{z^2}{k_{mp}} + C_2 z + (C_3 - C_1 C_2) \ln \left(\frac{z + C_1}{C_1}\right) \right] \quad [26]$$

The scorch depth (the depth of drying at which the surface reaches the scorch temperature) z_{sc} is obtained from equation [22] by observing that at x = 0, the following relation is obtained

$$T_x = T_f - \beta z \quad [27]$$

Then from equation [12] we obtain

$$T_s^4 + \frac{k_{mp}}{z \epsilon \sigma} T_s - \left(T_H + \frac{k_{mp}}{z \epsilon \sigma} T_f\right) = 0 \quad [28]$$

Thus, the drying depth z_{sc} at which the surface temperature reaches the scorch temperature T_{sc} is found from equation [28] to be

$$z_{sc} = \frac{k_{mp}}{\epsilon \sigma} \frac{T_{sc} - T_f}{T_H^4 - T_{sc}^4}$$

The approximate solution given in equation [26] was shown by Cho and Sunderland (1970) to yield errors less than 1% for predicting the interface position.

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VACUUM-PUFF FREEZE DRYING OF TROPICAL FRUIT JUICES

SUMMARY—A study was made to develop high quality juice powders from four tropical fruits. Guava and mango puree, passion fruit and pineapple juice were dehydrated in a vacuum-puff freeze-drying process. Sucrose in amount (25–40%) approximating nectar formulations was mixed with single-strength juice or puree samples, blended 2 min, and frozen. Freezing temperatures of various samples were measured in a constant temperature bath of −21.6° F. Samples initially frozen in blast freezers at −40° F or 0° F with 0.25–1.0 lb/ft² were vacuum-puffed and freeze dried on aluminum trays at 50μ Hg and 120–130° F shelf temperature for 6–9 hr to 1–2% moisture. Puffing was affected by (1) rapid freezing of the juice-sucrose mixture; (2) artificially entrapping some gas in the mixture before freezing; (3) changing the pressure or temperature in the chamber to effect gas expansion; and (4) increasing the mixture viscosity to limit bubble sizes. Results of drying over 160 different samples showed that: (1) added sucrose to give a 40 ± 5° Brix in the mixture resulted in a stable "puffed" structure; (2) several permissible calcium salts and silica at 0.1–0.5% (w/w) added to the mixture before drying, improve the free-flowing properties of the dried product. Process variables are discussed and typical sample compositions given.

INTRODUCTION

TROPICAL fruits are exotic in flavor, but their juices and purees are seldom available in dehydrated powder form. Pineapples grown in Hawaii, for example, are exported fresh or processed as canned fruit, canned juice or frozen juice concentrate.

Citrus juice concentrates were produced during World War II in powdered form in the U.S. by spray, drum, vacuum-puff, and freeze drying (USDA, 1962). From the late 1940's to the 1950's, vacuum drying, including vacuum freeze drying, received considerable attention and studies. Reviews and discussions of vacuum drying of various fruit and vegetable juices have been presented by Legault (1954), Talburt (1961), and Ponting et al. (1964). Notter et al. (1959) studied factors affecting behavior of fruit and vegetable juices during vacuum-puff drying, and found that fruit juices containing a high proportion of sucrose were easier to dry than those containing invert sugar.

Foam-mat drying and the Microflake process were new developments in juice dehydration in the 1960's. Hertzendorf and Moshy (1970) made a comprehensive and critical review of various techniques of drying food foams, concluding that limited commercialization of foam drying was mainly a cost-quality problem.

Berry and Veldhuis (1968) pointed out that considerable difficulty has been experienced in achieving desired storage stability with foam-mat dried orange powders. The addition of pineapple to orange was found to improve the storage stability at elevated temperatures.

The adaption of freeze drying to citrus or other fruit juices has been slow when compared with other freeze-dried food products. Burke and Decareau (1964) cited storage stability as the main problem. Berry and Froscher (1968) pointed out two other reasons: (1) difficulty in freeze drying solutions containing high proportions of sugars (low eutectics); and (2) the relatively low value of citrus solids when compared to other products.

Berry et al. (1967), and Berry and Froscher (1968) studied freeze drying of citrus juice by grinding various frozen sucrose solutions and grapefruit and orange juice of 10–60° Brix into two particle size ranges (ca. 20 and 60 mesh), and then freeze drying these frozen particles along with frozen slabs. It was observed that frozen juice particles of 60 mesh dried easier and more rapidly than the other two forms of samples and moisture removal was more nearly complete at lower °Brix. Also, no significant differences were found in storage stability of freeze-dried citrus juices regardless of type of atmosphere which the dried material first contacted or type of atmosphere in which the dried material was packaged. All freeze-dried samples developed flavor changes which were significantly different from the controls (stored at −21°C) in about 4 wk.

That the freezing point of a solution decreases progressively with higher soluble solids concentration is well recognized, which means that a fruit juice with added sugar will freeze at a temperature appreciably lower than that of an unsweetened juice. If kept above its freezing temperature, or loaded onto a warm platen, a juice-sugar mixture will puff easily under vacuum. A typical time-temperature curve was given by Strashun and Talburt (1954) for vacuum-puff drying a 60° Brix orange concentrate. A vacuum-puffed juice structure might have the disadvantages of having many large bubbles, a barrier to heat transfer and possibly losing some volatiles during puffing and drying.

Techniques for fruit juice dehydration mentioned above appear to have some problems and limitations, mainly in flavor and storage stability. Besides economics, a desirable dehydration technique for fruit juices should, therefore, result in flavor stability and free-flowing characteristics of the finished products. Sucrose and several permissible anti-caking agents could be useful to achieve these goals. In addition, if a vacuum technique is to be employed in puffing and drying, stability of the "puffed" structure during drying will also be important.

Combining vacuum puffing and freeze drying might conceivably produce a juice powder having some of the advantages of each process. A major consideration for vacuum-puff freeze drying is effecting puffing of a fruit juice under vacuum so that the bubbles are kept relatively small, and the entire puffed-juice structure frozen within a short period of time by evaporative freezing. To achieve this combination process and to assure storage stability and free-flowing properties, suitable amounts of additives can be incorporated into the product to be dried.

MATERIALS & METHODS

Sample preparation

Four tropical fruit juices and purees were used in this study: guava, mango, passion fruit and pineapple. Some of their characteristics, including the varieties used, are shown in Table 1. After being prepared by slicing, pulping and finishing, all samples were pasteurized at 190–200° F for 1 min, frozen in No. 2 (307 × 409) or No. 10 (603 × 700) cans at 0° F then thawed as needed.

Additives used included: sucrose (cane, granulated, commercial grade); corn syrup solids and malto-dextrin (80.3% and 77.5% solids respectively, 24–37% D.E., Hubinger Co., Koekuk, Iowa); tricalcium phosphate (reagent grade, Matheson, Coleman & Bell); calcium oxide (powder, analytical grade, Mallinckrodt); calcium silicate (purified, City Chem, N.Y.C.) and synthetic silica (Micron-sized, Grade 68, Syloid 224, W. R. Grace).

Frozen juice or puree samples were thawed and mixed with various amounts of sugar, calcium salts and silica in a blending jar. The amount of sugars added varied from 10–40% wet wt; the amount of calcium salts varied from 0–0.15% wet wt; and the amount of synthetic silica used was 0–0.5% wet wt. The mixtures were blended for 2 min in a Waring Blendor. The samples were transferred to aluminum trays with copper-constantan thermocouples

Table 1–Some characteristics of tropical fruit juices and purees

Fruit	Guava	Mango	Passion fruit	Pineapple
Generic Name	Psidium guajava L.	Mangifera indica L.	Passiflora edulis L.	Ananas cosmosus M.
Variety	Beaumont	Haden	Flavicarpa	Smooth Cayenne
Form	Puree	Puree	Juice	Juice
°Brix	6.5–9.0	14–17	15–18	11–14
pH	3.0–3.5	3.9–4.4	2.9–3.1	3.0–3.5
% Acid (as citric)	0.5–1.5	0.3–0.4	1.9–3.2	0.5–0.9

Table 2–Composition of some vacuum-puff freeze-dried tropical fruit juice samples

Fruit	Juice/sucrose wt ratio	Additive, percent by weight $Ca_3(PO_4)_2$	CaO	$CaSiO_3$	SiO_2
Mango	75/25	0.15	0.10	0.10	–
	66.5/33.5	0.15	0.10	–	0.50
Guava	60/40	0.05	0.05	0.05	–
	65/35	0.15	0.10	–	0.40
Passion fruit	60/40	0.15	0.10	0.05	–
	66.5/33.5	0.10	0.10	0.10	–
Pineapple	60/40	0.10	0.10	–	0.20
	66.5/33.5	0.10	0.10	0.10	–

planted at points varying from 1/8–1 in. from the bottom of the trays. Samples were frozen in a forced convection freezer at either −40°F or 0°F before being loaded into the freeze dryer.

Several typical formulations from more than 160 tested are shown in Table 2.

Freezing temperature determination

The freezing temperatures of various samples were measured in a heat-sink of constant temperature, low boiling bath. A large reservoir of Freon-12 (dichlorodifluoromethane, N.B.P. −21.6°F, E.I du Pont) in a wide-mouth Dewar flask (7 in. diam) provides rapid freezing of samples having a freezing point as low as 3°F, e.g. passion fruit-sucrose mixture with 56% total soluble solids (T.S.S.). 150g of sample at room temperature was placed in a 6 oz can (202 × 314) with a copper-constantan thermocouple at the geometric center of the liquid volume (ca. 2 in. diam × 3-1/4 in. high). The thermocouple was connected to an electronic, automatic cold junction compensating, 6-point Honeywell temperature recorder, calibrated at −21.6°F with Freon-12 and at 32°F with ice-water mixture. The can, covered with aluminum foil, was lowered into the Dewar flask until the top was about 1/2 in. above the Freon-12 level. Cooling and freezing continued until sample temperature reached −20°F.

Vacuum-puff freeze drying conditions

With the condenser of the freeze dryer operating at −60°F, sample trays were transferred from the freezer into the three-platen Model 15 RePP (a division of Virtis Co., Gardner, N.Y.) freeze dryer. The platens were precooled to 30–50°F. After loading, the chamber was evacuated immediately with a mechanical vacuum pump. Generally, it took 5 min to reach 3.5 mm Hg in the chamber, and another 5 min to 1.0 mm Hg or lower. The juice or puree samples puffed slowly and the structure gradually stabilized. Temperature of the sample rose 10–15°F, then dropped to below 0°F within 10 min. When the puffed foam and its temperature stabilized, radiant and conductive heat at a temperature of 120–130°F was supplied to the platen for drying. Chamber pressure was maintained at 25–50μ Hg during the drying cycle. drying cycle.

Sample temperatures, monitored throughout the drying cycle, became asymptotic at 100–110°F. Drying was continued for 2 hr after the sample temperature reached 100°F. Drying rates were determined gravimetrically by removing samples from the freeze dryer at various time intervals. Samples were easily scraped from the trays with a spoon and filled directly into glass jars at ambient temperature and humidity (70–80°F, 60–90% RH) without grinding or classifying.

RESULTS & DISCUSSION

THE FREEZING points of various juice mixtures were measured in a Freon-12 heat-sink through conductive heat transfer. The final freezing point of a sample is taken as the temperature at which solidification of the mixture is essentially complete, i.e. the intercept between the freezing (fusion) curve and cooling (post-freezing) curve of the frozen material. Below the final freezing point, the rate of temperature change is a function of the thermal diffusivity of the frozen mixture. Several typical freezing curves obtained with this technique are shown in Figure 1. These include solutions with T.S.S. of 45% guava-sucrose, 46.8% passion fruit-sucrose, 45% soluble coffee and 45% sucrose (w/w) for purposes of comparison. The entire cooling and freezing process requires about 25–60 min.

With Freon-12 boiling constantly at −21.6°F, the temperature gradient between the sample and the heat-sink is large and reproducible. Heat transfer is achieved by the turbulence of the boiling bath. These factors resulted in standardizing the rate of heat removal, and uniform freezing of the samples. Figure 2 shows the freezing temperatures of various fruit juice-sucrose mixtures as a function of their soluble solids concentrations.

Table 3 shows data of the freezing zone and the time of phase change of various juice mixtures, representing removal of heat of fusion in the sample. These data enabled interpretation of the state of the mixture when puffed under vacuum.

Figure 3 shows sample temperature profiles for the first 6 hr of drying for three samples: a passion fruit juice-sucrose mixture, a guava-sucrose mixture and a sucrose solution, all with 45–47% T.S.S. It can be seen that temperatures of the samples rose 10–15°F during loading and initial pump-down, but remained

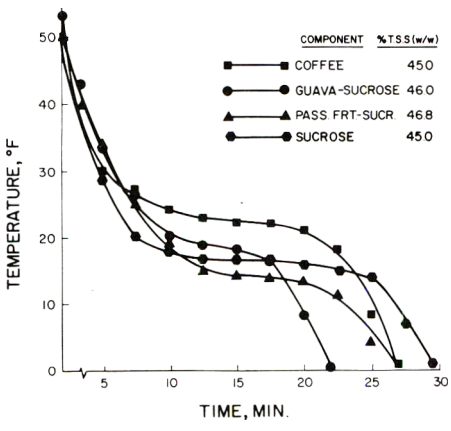

Fig. 1–Freezing curves of some tropical fruit juice-sucrose mixtures frozen in a Freon-12 bath at −21.6°F. (Sucrose and coffee solutions included for comparison.)

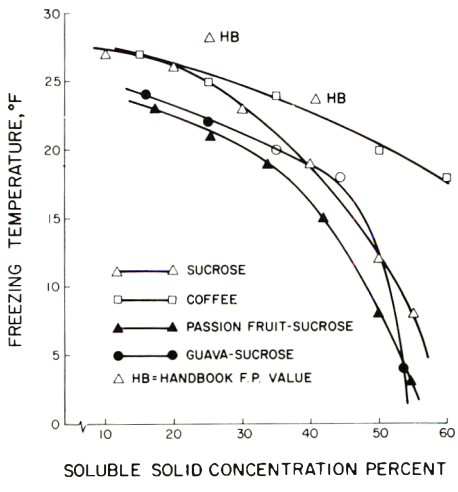

Fig. 2–Freezing temperatures of various tropical fruit juice-sucrose mixtures as a function of soluble solids concentration.

Table 3–Freezing zone and time of phase change of various fruit juice-sucrose mixtures measured in a constant temperature bath of –21.6° F

Base component, %	Mixture T.S.S., %	Freezing zone, °F	Time of phase change, min
Guava			
100	6.8	31–26	6.5
90	16.1	28–24	5.9
80	25.2	27–22	8.0
70	35.1	24–20	9.5
60	44.4	22–18	10.5
50	53.6	8–4	12.7
Mango			
100	16.9	27–25	6.8
85	29.4	24–21	8.6
75	37.7	20–18	9.8
65	46.0	19–16	10.0
55	54.3	8–5	16.6
Passion Fruit			
100	17.4	28–23	6.8
90	25.7	26–21	7.7
80	33.9	23–19	8.0
70	42.2	17–15	8.9
66.5	45.1	16–13	10.6
65	46.8	15–13	11.1
60	50.4	12–8	11.8
55	54.6	5–3	14.2
Pineapple			
100	12.5	27–24	14.5
85	25.6	26–23	16.2
75	36.9	23–20	17.0
65	43.1	19–16	18.9
55	51.9	15–12	23.9
Sucrose			
10	10.0	31–27	13.9
20	20.0	28–26	15.1
25.5	25.5	26–24	15.5
30	30.0	25–23	16.2
40	40.0	22–18	18.6
50	50.0	14–12	20.7

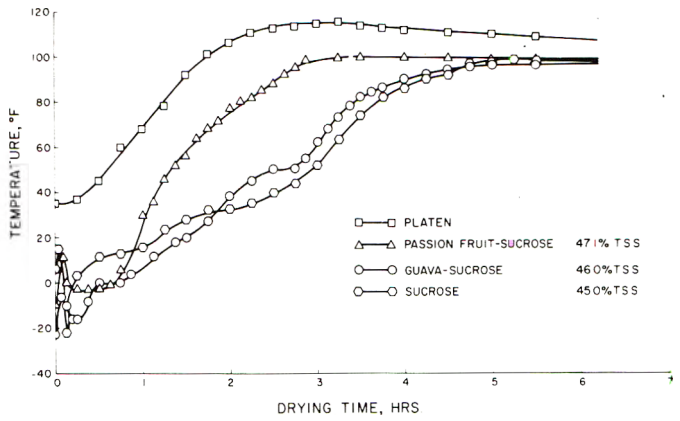

Fig. 3–Temperature profiles of two tropical fruit juices and one sucrose sample during vacuum-puff freeze-drying cycle; platen temperature 120–130° F and 50μ Hg.

below their freezing temperatures. Initial sublimation of water vapor cooled the sample to below 0°F, which was appreciably lower than the freezing temperature of the sample. When puffing stopped and the juice structure stabilized, heat was then supplied to the platens (120–130°F) for freeze drying. After the sample temperature has reached 100–110°F, drying was extended to allow a safety factor of a couple of hours in the absence of a better end-point indicator.

Limited degree of blending during preparation followed by rapid freezing of a juice-sucrose mixture to below its freezing point allows sufficient air to be entrapped in the mixture. When the sample was transferred from the freezer to the freeze dryer, part of the sample would tend to warm up due to the temperature gradient in the surroundings. Under rapid pump-down in the freeze dryer, the entrapped air expands and creates bubbles in the juice mixture, making the juice structure somewhat less rigid, more elastic and more likely to foam. Puffing for freeze drying, therefore, is probably influenced by a combination of factors: (1) rapid freezing of a low freezing mixture; (2) artificially entrapping some air (or gas) in the mixture before freezing; (3) changing the pressure or temperature in the surroundings of the frozen mixture to effect gas expansion in the mixture; and (4) making the mixture sufficiently viscous so that when slightly warmed up, the bubble sizes of the foam could be limited.

Heat transfer could be more efficient with smaller bubbles produced under the vacuum-puffing conditions described here than is the case of larger bubbles produced from the regular vacuum-puffing technique. Small bubbles formed at low temperature should result in a uniform composition, a uniform ice front and reasonably uniform conductive heat and mass transfer. Expanding from a small to a large bubble, the increase in surface area is $(\Delta r)^2$ and the increase in volume is $(\Delta r)^3$, where r is the radius of a bubble. Assuming an average of threefold increase in bubble size from that obtained in vacuum-puff freeze drying to that in vacuum-puff drying, the change in surface to volume ratio from the small bubble to the large bubble will be a decrease of from 3 to 1. Therefore, to achieve efficient heat transfer in drying, keeping the bubble size small seems to be an advantage in terms of having a large surface to volume ratio. In discussing the relationship between bubble size and drying time in the Microflake process, Hertzendorf and Moshy (1970) stated that "drying time was found to be proportional to the square root of the bubble size. Therefore, increasing the average bubble size from 25–100μ in diameter doubled the drying time required to attain 2% moisture. Foam stability is quite important, and even a slight collapse of the foam can increase drying time by 25–50%."

Figure 4 shows drying curves in residual moisture content vs. drying time of passion fruit juice-sucrose samples with 47.1° Brix at three different loading densities: 1.0, 0.50 and 0.25 lb/ft². It is apparent that lower loading densities resulted in higher drying rates. However, should the structure be allowed to collapse during drying (e.g. interrupted or insufficient vacuum), case hardening will occur and the material will be dried in a slab form, resembling somewhat ordinary freeze drying. Re-puffing will be quite limited in the absence of entrapped air, and drying is not likely to be complete as illustrated in Figure 4 with 0.25 lb/ft² of loading density of passion fruit-sucrose mixture. This points up one of the main problems for freeze drying of fruit juices, namely, a slab of frozen juice is difficult

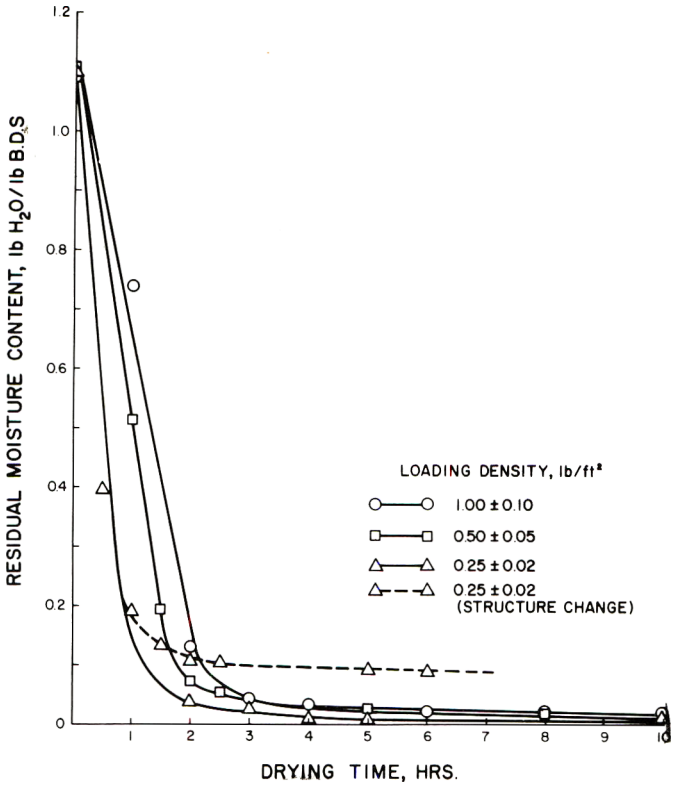

Fig. 4—Drying curves of passion fruit-sucrose samples with 47.1° Brix at three loading densities; dotted curve shows effect of foam collapse on a sample dried at loading density of 0.25 lb/ft².

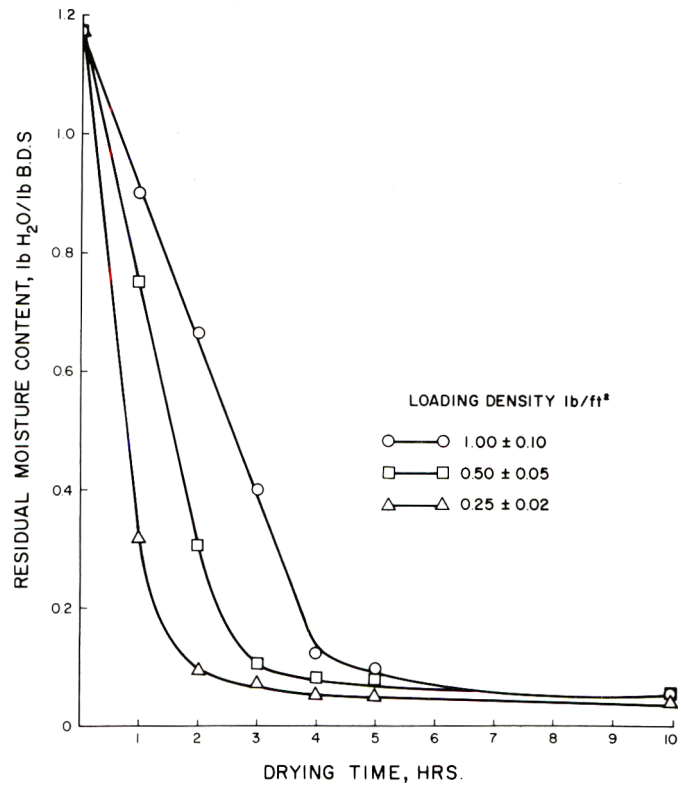

Fig. 5—Drying curves of guava-sucrose samples with 46.0° Brix at three loading densities.

to dry to completion. Figure 5 shows drying curves for guava-sucrose mixtures at 46° Brix. There is a constant rate period for both the passion fruit and the guava samples, and the length of the period is directly related to the loading density. From Figures 4 and 5 it can also be seen that a passion fruit-sucrose mixture dries faster than guava-sucrose mixture at the same loading density. The reason for this difference requires further study. Considering the efficient use of a freeze dryer and the possibility of incipient melting, a loading density of 0.50–1.0 lb/ft² would probably be considered optimal.

Rey and Bastien (1962) reported their study on freeze drying single-strength orange juice. Regarding the question of possible interstitial melting, they felt that "most of the irregular behavior of the juice is due to the fact that, during freezing, some of the interstitial fluids do not reach an adequate solidity. Their viscosity increases and they look like a solid, but as soon as the temperature increases, they begin to soften and give birth to a net of more or less viscous or sticky material which delays sublimation and often prevents an adequate desorption of water molecules. Actually in the course of drying, it is frequent to see an enormous bubble all over the tray." (Their orange juice sample was 10 mm thick frozen on metallic trays to −50°C before freeze drying.) These statements would imply that a juice sample might not always be completely solidified even at temperatures below its freezing point.

Earlier experimental runs led to the following observations: (1) guava puree, mango puree, passion fruit juice and pineapple juice did not by themselves vacuum-puff to give a satisfactory structure for drying. The bubbles were large, and the "puffed" structure was glassy and difficult to dry to completion; (2) with the same degree of blending during sample preparation, the puffing depended largely on the type and amount of soluble solids added; and (3) the expected hygroscopicity of the dried sample dictated that in-package desiccant be used, or anti-caking agents be added to the sample before or after drying.

Based on these observations, the considerations for a satisfactory dehydrated juice powder were: (1) to use it as a juice base with good color and flavor; and (2) to maintain its free-flowing characteristics. Sucrose and several permissible calcium salts and silica were found useful to achieve the desired properties. Some difficulty was experienced in dissolving corn-syrup solids and malto-dextrin when they were partial substitutes of sucrose in the sample. Also, the flavor of dehydrated products appeared to have changed somewhat as a result of adding the corn-syrup solids or malto-dextrin to the mixture before drying. Since they have been used successfully in other juice and beverage products, further studies of their use in tropical fruit juice dehydration would be worthwhile.

Sucrose appears to play several important roles in the juice mixture for vacuum-puff freeze drying. Besides helping to reduce the hygroscopicity of the juice powder, it helps keep the volatile components in the fruit juice and thus helps retain the flavor and aroma. In developing a grape juice powder, Eskew et al. (1954) found sucrose useful in preventing off-flavor development, and retaining the grape juice essence. Rey and Bastien (1962) postulated a mechanism for sugar as "an absorbant body which strongly binds the aromatic components to the dry material," and demonstrated in a series of their experiments in which acetone was added to an Earle-glucose solution for freeze drying. They found that the percentage of residual acetone was dependent only on the amount of glucose present in the solution.

The third role played by sucrose in this process is believed to be that of influencing the puffing and stabilizing the

"puffed" structure by minimizing surface sublimation of the frozen mixture when pressure in the chamber was reduced. This is because the presence of a relatively high concentration (25–40%) of dissolved sucrose in the juice increases the viscosity of the juice-sucrose mixture appreciably when cooled. From 77–32°F, for example, the viscosity of a 40% sucrose solution increases about threefold to 14.8 cp, and that of a 60% sucrose solution increases more than fivefold to 238 cp (Hodgman, 1954). Subsequent rapid freezing of the juice-sucrose mixture results in a fairly uniform mass. Limited initial surface sublimation would also help minimize chances for case hardening, thereby making drying more uniform and complete.

The criterion of adding permissible calcium salts and silica to the juice mixture was to use the minimal amount to achieve the free-flowing property of the dried product during storage. Results from this study showed that all four tropical fruit juices and purees can be made into a satisfactory dehydrated product by adding suitable amount of sucrose (25–40%), up to 0.15% tricalcium phosphate, 0.10% calcium oxide and 0.10% calcium silicate to the wet mixture.

The juice-sucrose powders were dried to 1–2% moisture (based on vacuum drying at 130°F for 24 hr), then filled and stored in glass jars at prevailing temperature and humidity, 70–80°F and 60–90% RH. The glass jars were capped manually; no in-package desiccant was added. Formulations shown in Table 2 represent typical vacuum-puff freeze-dried samples out of 57, all of which retained a free-flowing property after 10–16 months. All of these samples have maintained good color and solubility in cold water. A formal taste panel evaluation has not been carried out, but selective taste tests showed that some flavors are still excellent, some have changed slightly, probably due to oxidation in the glass container.

In terms of the amount of sucrose added to the products, the formulations used here are comparable to those of the tropical fruit nectar or nectar base. Single-strength juice, especially passion fruit, carries sufficient aroma and flavor, but each of these four juices and purees is generally consumed in sweetened form. The juice powders produced here have the following apparent bulk densities in gm/ml: guava, 0.38; mango, 0.39; passion fruit, 0.23; and pineapple, 0.24. They are probably considered low as compared with other similar products. No control of the bulk density was attempted in this study. However, for possible commercial adaptation, some degree of densification would seem necessary.

CONCLUSIONS

BASED on experimental results obtained from, and observations made on the vacuum-puff freeze-drying process, tentative conclusions can be made as follows: (1) Vacuum-puff freeze drying may be a useful process for producing quality dehydrated juice powder. Proper control of both the initial product temperature, and the degree of vacuum will be necessary to obtain adequate, but not over-puffing; and (2) Factors affecting this process are: the chemical and physical nature of the initial material; the amount of gas in, and the amount and type of sugars added to the material; the initial product temperature; the rate of change and degree of vacuum; and product loading density.

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EFFECT OF RADIO-FREQUENCY ENERGY AT 60 MHz ON FOOD ENZYME ACTIVITY

SUMMARY—The effect of radio-frequency (R-F) energy at 60 MHz on peroxidase, polyphenolase, pectinesterase, catalase and alpha-amylase activity was determined in water solutions of purified enzymes as well as in apple juice, milk and sweet potato extract. The apparatus used consisted of an R-F generator coupled to an evaporator and condenser. Temperature was kept constant at a desired level by evaporating water from the system. Results showed that while the enzymes were partially or totally inactivated by heat at 70°C, or 65°C (149°F) in the instance of milk, exposure to R-F energy at 20°C caused essentially no loss of enzyme activity. Within the limits of the experimental conditions, pH and enzyme concentration were not factors in relation to lack of effect of R-F energy on enzymes. Overall, the results indicate that per se, R-F energy at 60 MHz does not have any significant effect on the enzymes studied, aside from that caused by the heat effects of R-F energy.

INTRODUCTION

THE BASIC PRINCIPLES of microwaves, particularly in the R-F region, have been studied for a number of years. Goldblith (1967) extensively reviewed the subject. The electronics of R-F energy, as well as its application, were described in a volume edited by Okress (1968). The effects of R-F energy on biological systems have been presented in a monogram edited by Peyton (1961).

Izar and Moretti (1933) experimented with solutions of pepsin exposed to frequencies of 20, 37.5 and 75 mc/sec. No effect was found at 20 and 75 mc/sec; however, an increase in proteolytic activity was noted at 37.5 mc/sec. Copson (1954) reported complete inactivation of pectin-methyl-esterase in orange juice concentrate at a frequency of 2,450 mc/sec.

Bach et al. (1962) studied the effects of R-F energy on alpha-amylase in two groups of experiments. One was conducted by exposing enzyme samples to variable R-F frequency under constant-temperature conditions; the other, to a constant frequency while the temperature was varied. They found a partial inactivation of the enzyme at certain combinations of temperature and frequency. Cook and Smith (1964) exposed trypsin solutions to magnetic fields and found an increase in enzymatic activity. Edwards (1964) exposed flour to R-F energy at 2450 mc/sec and reported that 2-min treatments inactivated amylases and denatured the gluten. Bach (1965) suggested possible mechanisms by which biological systems may be influenced by R-F energy. Takashima (1966) studied the effects of R-F energy on alcohol dehydrogenase and DNA. He found no alteration in the activity of the enzyme or on the structure of DNA even by the prolonged irradiation at high field intensity.

Nyrop (1946), using a frequency of 20 mc/sec, killed 99.5% of *Bacillus coli* in liquid medium after 7 sec of exposure. He claimed 99.98% kill in 10 sec in an improved apparatus. Brown and Morrison (1954) found no significant lethal effects on bacteria in aqueous solutions except that of heat. Carroll and Lopez (1969) studied the effects of R-F energy at 60 mc/sec on microorganisms in several systems. They found no lethal effects of R-F energy per se and no synergistic effect of R-F energy and heat upon microorganisms.

Mellon et al. (1930) studied the effect of short electric waves on diphtheria toxin. They found a partial inactivation of the toxin in as short a time as 15 min. They concluded that the effect was due to R-F energy radiation, which excluded the possibility of inactivation due to heat. Nyrop (1946) also reported that foot-and-mouth disease virus was completely inactivated by R-F energy after a 10-sec treatment. The apparatus and the experimental design used were not described.

Bach et al. (1960) exposed human gamma-globulin to R-F energy in the range of 10–200 mc/sec. They observed a change in the paper electrophoretic patterns, and also in the antigenic reactivity. They concluded that R-F energy had produced an unfolding effect on the protein helix which resulted in loss of specificity.

As shown above, the literature is controversial on the subject of effect of R-F energy on biological systems. The main objective of this research work was to determine whether the activity of certain enzymes found in foods is affected by R-F energy per se, independent of temperature increases. The study was done in model enzyme systems and in foods. The enzymes under investigation were peroxidase, polyphenolase, pectinesterase, alpha-amylase and catalase. The foods used were milk, apple juice and sweet potatoes.

EXPERIMENTAL

Radio-frequency energy apparatus

The apparatus used for generating R-F energy at 60 MHz and for exposing enzymes to its effect was described in a previous paper by Carroll and Lopez (1969). Constant temperature was achieved by having the system under a selected vacuum, allowing small amounts of water to evaporate. The condensed vapor was collected and measured. Equivalent amounts of distilled water were added to the system to maintain a constant volume of approximately 4 liters. Electric heating elements were wrapped around the steel pipes. The amount of heat delivered was varied by a Powerstat variable transformer. The sample was heated by: a) applying R-F energy; b) using the heating elements or c) both means simultaneously. All surfaces in contact with the experimental samples were glass or stainless steel.

Preparation of samples

Salt-free, lyophilized peroxidase was purchased from Nutritional Biochemicals Corp., Cleveland, O. The following purified enzymes were purchased from Sigma Chemical Co., St. Louis, Mo.: Catalase stock C-100, 2× crystallized; alpha-amylase type 1-A, crystalline; polyphenolase, Grade III and pectinesterase, P-4750. The enzyme solutions were prepared by weighing specific amounts of the above-mentioned enzymes, adding distilled water and adjusting the pH to 7.0 with phosphate buffer. The pH of raw apple juice was modified by addition of sodium hydroxide solution and by adjusting with citric acid. The milk used was unpasteurized. A sweet potato extract was prepared by blending 200g of sweet potatoes with 400 ml water. This was diluted 4:100 for the sample solution. All of the purified enzyme solutions and food enzyme systems were prepared within 2 hr before testing and held refrigerated until used.

Exposure to R-F energy

Each sample was introduced into the apparatus as follows: First, the vacuum pump was turned on until a vacuum of approximately 27 in. was achieved in the system. The feed valve was opened and the liquid transferred by vacuum suction into the evaporation chamber. When 4 liters were introduced, the valve was closed and the circulating pump turned on for about 1 min to ensure further mixing and to eliminate empty spaces in the pipes.

The R-F generator was allowed to warm up for 5 min. At that time the R-F energy was applied and the current to electrodes adjusted to between 280 and 300 ma, D.C. After 10, 20, 30 and 40 min of circulation, the R-F energy was cut off, the circulating pump stopped and

the vacuum broken. Samples were collected and refrigerated. For the reason explained in the following paragraph, 10, 20, 30 and 40 min of circulation correspond respectively to 2.87, 5.75, 8.62 and 11.50 min of actual R-F exposure. Control samples were run for the same periods of time and under the same temperature and pressure, except without R-F energy. Temperatures used were 20°C (68°F) and 70°C (158°F). When the higher temperatures were used, the apparatus was brought up to the desired temperature by circulating an adequate amount of sample solution using the heating means described. After the temperatures were reached, this solution was discarded and a fresh solution was introduced. Thus, systems were not affected by temperature changes while the temperature was being raised.

The electrodes occupied only a portion of the apparatus. The sample was exposed to R-F energy only while it was flowing through the electrodes, and not while flowing throughout the total pipeline circuit. Circulation time refers to the total time that the sample was circulated in the apparatus. Time exposed to R-F was the time the sample was actually irradiated while circulating in the apparatus. Exposure time was calculated by estimating the percentage of time a sample of 4 liters was irradiated while circulating in the apparatus, as follows:
The flow rate through the electrode was:

1.15 liters/min

Then 4 liters were exposed:

$$\frac{1.15}{4} = 0.2875 \text{ or } 28.75\% \text{ of circulation time.}$$

Analytical procedures

A. Determination of peroxidase activity. The activity of peroxidase in all the systems was determined according to the method described by Luck (1963). The change in optical density (O.D.) was observed in a Bausch & Lomb Spectronic 20 colorimeter at intervals of 30 sec for 2 min. The activity was expressed as k''/ml.

B. Determination of polyphenolase activity. Polyphenolase activity was measured by the method of Ponting and Joslyn (1948), modified as follows. The sample was buffered and 0.5 M catechol was added. Instead of an Evelyn colorimeter, a Bausch & Lomb Spectronic 20 colorimeter was used. Observations were made at 420 mμ. O.D. readings were taken at 1-min intervals for 20 min. The activity was expressed as change in optical density per minute (O.D./min).

C. Determination of pectinesterase activity. Determination of pectinesterase was by the method of MacDonnell et al. (1945) modified as follows: 25 ml of sample was pipetted into a beaker equipped with a magnetic stirrer and pH electrodes. The pH was adjusted to 7.5 with 20% sodium hydroxide solution. NaCl was added to a 0.15 M concentration. After an addition of 0.5% N.F. pectin, the pH was adjusted with 0.1 sodium hydroxide. Optimum activity occurred during the first 5 min. Activity was expressed as pectinesterase units (P.E.-μ/ml × 10^{-4}).

D. Determination of catalase activity. It is customary to express catalase activity of foods as "Katalase Fahigkeit," which is the catalase activity (k) per gram of food (Kf = k/g). Catalase activity determination in apple juice was carried out according to Official Methods of

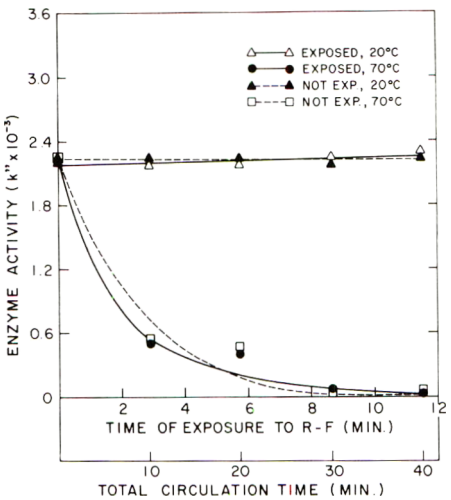

Fig. 1–Peroxidase activity (k'') in raw apple juice exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

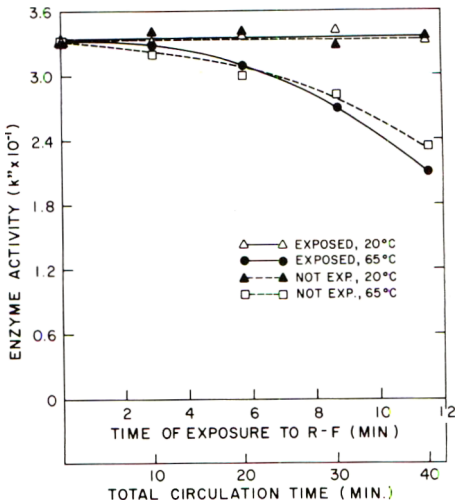

Fig. 2–Peroxidase activity (k'') in unpasteurized milk exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 65°C (149°F).

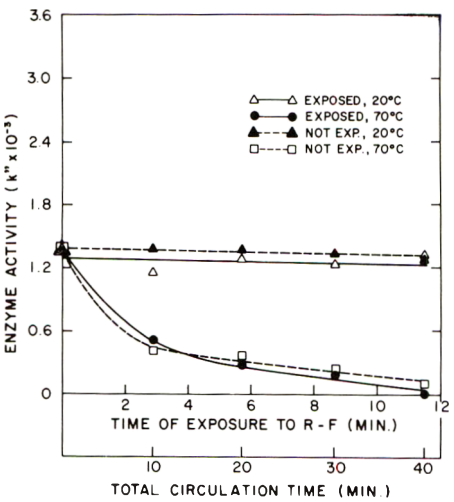

Fig. 3–Peroxidase activity (k'') in a buffered solution containing 0.1 g/ml purified peroxidase exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

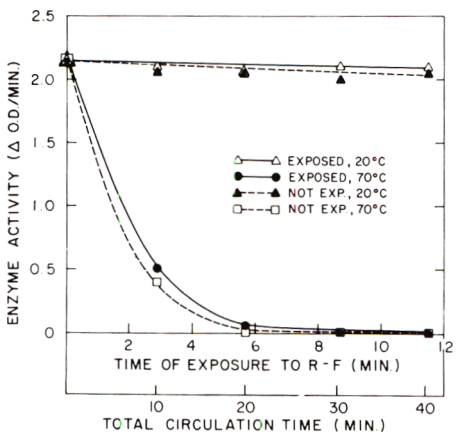

Fig. 4–Polyphenolase activity (Δ O.D./min) in raw apple juice exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

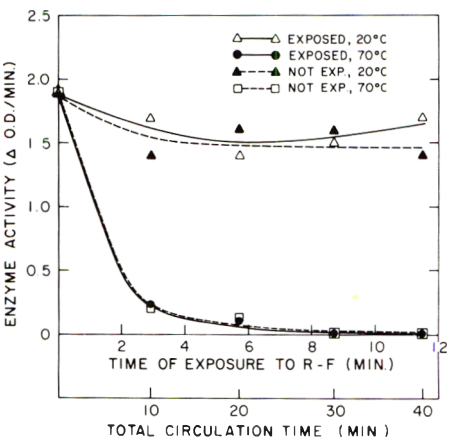

Fig. 5–Polyphenolase activity (Δ O.D./min) in purified enzyme solutions exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

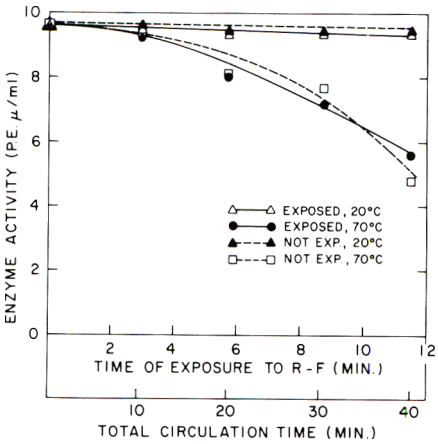

Fig. 6—Pectinesterase activity (P.E. μ/ml) in raw apple juice exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

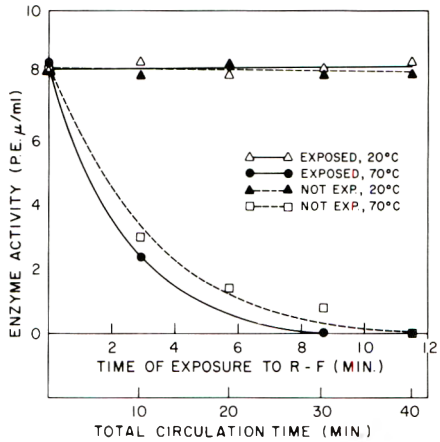

Fig. 7—Pectinesterase activity (P.E. μ/ml) in purified enzyme solutions exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

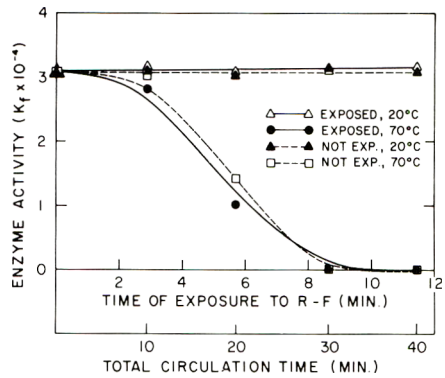

Fig. 8—Catalase activity (K_f) in raw apple juice exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

Analysis of the A.O.A.C. (1965). Catalase activity in purified enzyme solutions was determined spectrophotometrically according to the method described by Luck (1963), since it is more accurate for purified solutions.

E. Determination of alpha-amylase activity. The activity of alpha-amylase was determined by the method of Bernfeld (1955).

RESULTS & DISCUSSION

THE R-F ENERGY irradiation apparatus employed for this research afforded excellent controls for the temperature and current delivered to the electrodes. It also afforded an effective means for heating the samples. The temperature and pressure in the system, as well as the current delivered to the electrodes, were continuously observed and controlled. Thus, these factors were always known, and duplication of an experiment was possible.

Five enzymes were investigated in their purified form as well as in foods. With the exceptions of alpha-amylase and pectinesterase, there are no reports in the literature on the effects of R-F energy on these enzymes. Addition of a buffer to purified solutions was necessary because of the arcing effect that resulted in the capacitor when no ions were present. Peroxidase, polyphenolase, pectinesterase, catalase and alpha-amylase activity data are presented in Figures 1 through 11. None of the enzymes irradiated by R-F energy at 20°C (68°F) for different periods of time lost any significant activity. Duplicate control samples held at the same temperature but which were not irradiated with R-F energy had essentially the same enzyme activities as the irradiated samples. Similar experiments, but with a treatment temperature of 70°C (158°F) showed substantial loss of activity both in the irradiated and nonirradiated samples.

Thus, the results showed that the only samples which lost enzyme activity were those heated to a temperature sufficient to produce partial or total loss of activity of the enzymes. Enzymes irradiated with R-F energy but maintained at 20°C (68°F) did not significantly lose activity. R-F energy irradiation done at temperatures at which enzymes are heat stable did not have any significant inactivating effect which could be attributed to R-F energy per se.

A second phase of this research was conducted to determine whether enzyme

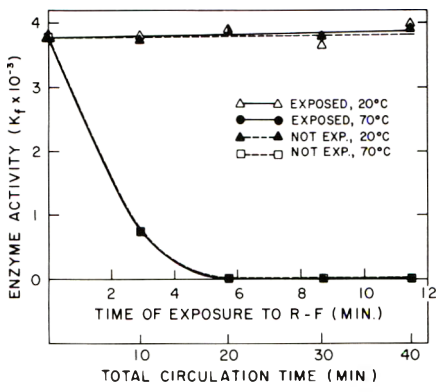

Fig. 9—Catalase activity (K_f) in buffered solutions of purified catalase exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

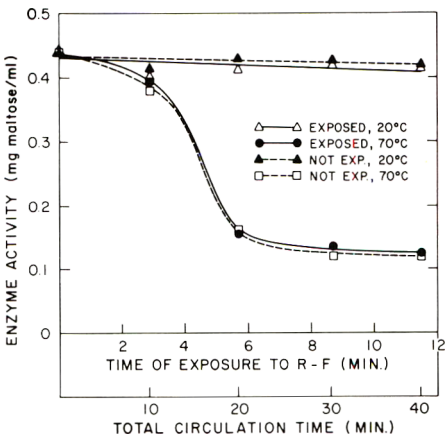

Fig. 10—Alpha-amylase activity (mg maltose/ml) in sweet potato juice exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

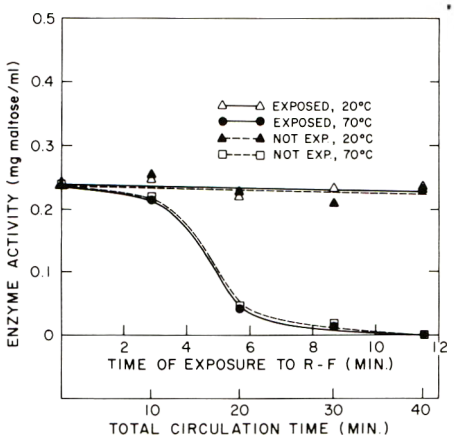

Fig. 11—Alpha-amylase activity (mg maltose/ml) in buffered solutions of purified alpha-amylase exposed and unexposed to R-F energy irradiation for various periods of time at 20°C (68°F) and 70°C (158°F).

concentration and pH value are critical factors in relation to R-F energy effects on enzymes. Purified peroxidase solutions were prepared at concentrations of 0.1, 0.01 and 0.001 μg/ml.

Essentially complete inactivation occurred at 70°C (158°F) with or without exposure to R-F energy, while at 20°C (68°F) there was no significant loss of activity under any conditions. Therefore, there was no reduction of enzyme activity at any of the enzyme concentrations studied that could be attributed to R-F energy per se.

Apple juice at pH 3.8, as well as the same juice with pH adjusted to 5.5 and 7.0, was subjected to R-F energy treatments. At each pH, the effects of R-F energy on the apple juice peroxidase, polyphenolase and pectinesterase were studied at 20°C (68°F) and 70°C (158°F). No effect was evident at any pH at 20°C (68°F), while at 70°C (158°F) there was a substantial inactivating effect on each enzyme. There was no reduction of enzyme activity at any of the pH values studied that could be attributed to R-F energy per se.

Overall, the results indicate that per se, R-F energy at 60 MHz does not have any significant effect on the enzymes studied, aside from that caused by the heat effects of R-F energy.

The effects of microwaves at other frequencies on enzyme activity should be investigated. Results obtained in the course of the work presented here indicate that in industrial applications the effects of R-F energy and microwaves on enzymes in food systems would only be those caused by elevation of the food temperature to a level such that enzyme activity is affected. The time-temperature effects on enzyme activity appear to be the same regardless of whether the heat originates from absorption of radio-frequency energy or is applied by other means.

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PREDICTION ON THE RELATIVE DIELECTRIC LOSS FACTOR IN AQUEOUS SOLUTIONS OF NONFAT DRIED MILK THROUGH CHEMICAL SIMULATION

SUMMARY—The relative dielectric loss factor in aqueous solutions of nonfat dried milk has been studied through chemical simulation of its major constituents. This study shows that milk dielectric loss cannot be predicted simply on the basis of its chemical composition. It does, however, provide a conceptual basis for milk modeling which leads to a more general understanding of food dielectric properties. The focal point in milk simulation was shown to be a complex relationship of solute-solute and solute-solvent interactions which reduce the dielectric loss factors to levels substantially lower than those predicted by considering only chemical composition. These include both binding and nonbinding interactions of the solutes. The binding effects are believed to result from the incomplete dissociation of milk salts in a saturated ionic environment and are reflected by a reduction of specific ion activities in solution. Nonbinding effects, on the other hand, are seen to result from the formation of hydrated ion complexes. These effects, in conjunction with chemical composition, are shown to completely determine the dielectric loss level in dried milk solutions.

INTRODUCTION

THE ABILITY to predict dielectric loss in foods on the basis of their chemical composition would offer an attractive means to estimate heating characteristics of foods for a wide variety of microwave processing applications. One means of evaluating this possibility is the chemical simulation of specific food systems based on their composition as determined by proximate analysis. A recent review of the literature shows very few studies have been made in the dielectric properties of foodstuffs, particularly at microwave frequencies allocated to commercial processing (i.e., 915 and 2450 MHz). One of the earliest dielectric measurements was by Dunlap and Makower (1945) to determine the moisture of carrots. Measurements of foodstuffs from 100–40 MHz were made by Ede and Haddow (1951). They reported heterogeneous heating effects which gave rise to "runaway heating" in microwave thawing of meats and fish. Similar measurements at 10–200 MHz were made for frozen and defrosted meats and fish by Bengtsson et al. (1963). Microwave measurements were made for foods of high water content by de Loor and Meijboom (1966), for potatoes and commercial fats and oils by Pace et al. (1968a; b) and for ground beef by Van Dyke, Wang and Goldblith (1969). During this period, related studies of chemical compounds had also been made by a number of investigators. Although these studies were not directly concerned with the dielectric properties of foods, these compounds are frequently encountered in food products. These include measurements of water by Collie et al. (1948); aqueous ionic solutions by Hasted et al. (1948); long-chain fatty acids by Buchanan (1954); and of amino acids, peptides and proteins by Takashima and Schwan (1965).

It is the intent of this paper to present results of the chemical simulation of a food product at a microwave frequency close to the frequency of major commercial interest in the United States. Milk was chosen for study since it can be chemically simulated in aqueous solutions by the use of chemical analogs. In addition, studies of this type are believed to be dielectrically applicable to a number of other foods which might be processed in batch or continuous-flow microwave heating applications. The simulation of the physical and chemical properties of milk and its constituents is based on data presented by Jenness and Patton (1959). The molecular basis of dielectric properties is reviewed by Von Hippel (1954). These are known to determine the energy coupling by a dielectric material in an electromagnetic field which, in turn, determines power absorption and temperature rise of the material and depth of penetration by the field. The relationships between dielectric properties and heating characteristics of foods are reviewed by Goldblith (1967). While results of this study are based solely on measurements at 3000 MHz and a temperature of 25°C, the effects of frequency and temperature variation are currently being investigated and will be reported in a subsequent paper.

EXPERIMENTAL

Milk analogs

Samples were prepared over a wide range of concentrations with maximum levels well above those normally found in reconstituted milk and diluted to lower levels with distilled water. The concentration range of the various components are shown in Table 1.

Nonfat dried milk

Nonfat dried milk was manufactured freshly by the low-temperature drying of condensed skim milk, sealed in amber glass bottles and stored at −20°C. Proximate analysis of the product showed total solids of 97.6% consisting of 0.8% milkfat, 8.1% ash content, 34.1% protein and 54.6% lactose.

Chemicals

Sodium caseinate was purchased from Matheson, Coleman and Bell of Cincinnatti, Ohio. D-(+)-lactose (monohydrate) was purchased from J. T. Baker, Phillipsburg, N.J. Inorganic chemicals of analytical reagent grade were purchased from various commercial houses.

Proximate analysis methods

Protein and ash content were determined in accordance with AOAC (1965) methods; total solids and milkfat by the method of Mojonnier and Troy (1925) and sodium by the method of Baird Associates (undated). Lactose was estimated by difference. Values were averaged from duplicate analyses.

Dielectric measurements

Standing wave measurements were made at a temperature of 25°C and a frequency of 3000 MHz. The method and the equipment used have been previously described by Pace et al. (1968a; b).

Table 1—Chemical compounds and concentrations used in dielectric measurements

Component	Chemical analog	Range of concentration studied (g/100 ml)			
Ash	Sodium chloride	0.5	1.0	1.5	2.0
Protein	Sodium caseinate	1.5	3.0	4.5	6.0
Carbohydrate	Lactose	2.5	5.0	7.5	10.0
Dried milk	–	4.5	9.0	13.5	18.0
Synthetic milk	Mixture of analogs	4.5	9.0	13.5	18.0

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Table 2–Chemical composition of milk salts analog

Compounds	Concentration (mg/100 ml)
Calcium phosphate	190
Calcium chloride	275
Sodium citrate	177
Potassium citrate	130
Magnesium chloride	32
Magnesium sulfate	12
Total	816

Data reduction

Dielectric properties were determined from standing wave measurements by means of a Fortran IV program for data reduction and was performed at the M.I.T. Computation Facility on an IBM 360 digital computer.

Milk salts analog

The distribution and concentration of ions found in normal milk salts were simulated by a mixture of the composition shown in Table 2. A stock solution of this mixture was prepared at a ten-fold concentration and adjusted to pH 2 for solubilization. Sodium chloride at 1.0% was also prepared at pH values to 12 to determine possible effects of pH on dielectric properties in the simulation studies of milk analogs.

Free cation measurements

Ion activities were measured by ion-selective electrodes for sodium, potassium and calcium manufactured by Corning Glass Works, Medfield, Mass. Potential measurements were made against a Corning silver/silver chloride reference electrode at controlled levels of pH and ionic strength. Measurements were calibrated by standard dilutions of chloride salts with ten-fold scale expansion on a PHM26C pH meter manufactured by the Radiometer Corp., Copenhagen, Denmark.

Total monovalent cation measurements

Total sodium and potassium levels were measured on a Model 143 Flame Photometer manufactured by the Instrumentation Lab., Lexington, Mass. with calibration by standard dilutions of chloride salts.

Total divalent cation measurements

Total calcium and magnesium levels were measured on a Model 303 Atomic Absorption Spectrophotometer manufactured by the Perkin-Elmer Corp., Norwalk, Conn. using calibration by standard dilutions of chloride salts.

Isoelectric precipitation

An aqueous solution of nonfat dried milk at pH 6.4 was reduced to pH 4.4 with concentrated hydrochloric acid. The resulting precipitate was removed in a Model BD-2 ultracentrifuge manufactured by the International Equipment Co., Needham, Mass. Centrifugation was at 100,000 × G for 2 hr.

RESULTS & DISCUSSION

VARIATION of the dielectric loss factor with respect to the concentration of the chloride salts of the major milk cations is shown in Figure 1. A linear relationship was obtained at low concentrations with increasingly nonlinear variation at higher concentrations. Dielectric loss factors were very nearly the same for all of the salts at concentrations of interest in milk simulation, i.e., up to 2% (w/v). Sodium chloride was then compared with the synthetic mixture of normal milk salts composition; results are shown in Figure 2. The dielectric loss behavior of sodium chloride was much the same as the synthetic mixture. A major disadvantage of the synthetic salts mixture was that its pH had to be reduced appreciably in order to dissolve all of the salts. This lower pH would alter substantially the properties of milk. However, loss measurements over a wide pH range with sodium chloride showed no variation of loss with pH. In view of these studies, sodium chloride was selected as an appropriate chemical analog for ash content in milk. Sodium caseinate and lactose were selected as the other analogs of milk on the basis that protein and carbohydrate content of milk are largely composed of these components. The sodium form of caseinates was chosen over the calcium form because of the generally lower binding activity of sodium as compared with calcium.

The relative dielectric loss factors of various materials measured at 3000 MHz and 25°C are graphically shown in Figure 3. In this figure the relative dielectric loss factors of aqueous solutions of sodium chloride, lactose, sodium caseinate and nonfat dried milk are presented individually. If one assumes that the dielectric loss factor of the aqueous milk preparation is composed of the sum of its component parts, consisting of sodium chloride, lactose and sodium caseinate, a predicted relative dielectric loss factor could be constructed and would be that shown as the dotted line in Figure 3. It can be seen that the actual loss factors are substantially lower than those predicted based on a linear additive relationship of the component parts.

To elucidate the reason for the discrepancy of the results shown in Figure 3, a series of studies was performed using a "synthetic" milk solution. This solution was prepared by combining the appropriate concentrations of sodium chloride, lactose and sodium caseinate. The relative dielectric loss factors at 3000 MHz and 25°C were again measured and are shown in Figure 4. The individual components' dielectric loss factors of the synthetic mixture are also shown in this figure. Here again, the assumption that one can predict the dielectric loss factor using the sum of the component dielectric loss factors was made and is shown as the dotted line in Figure 4. Once again it can be observed that the measured values are lower than those from prediction.

From the results shown in Figures 3 and 4, it was suspected that ionic binding effects may be responsible for the dis-

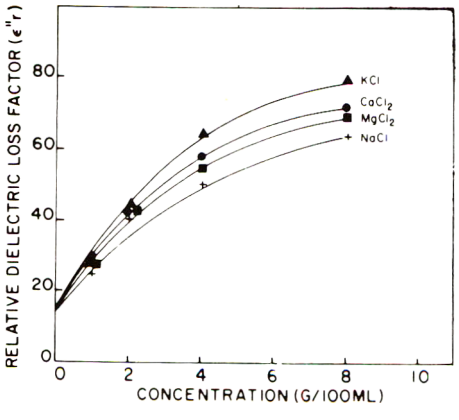

Fig. 1–Relative dielectric loss factor (E''_r) for chloride salts of major milk cations.

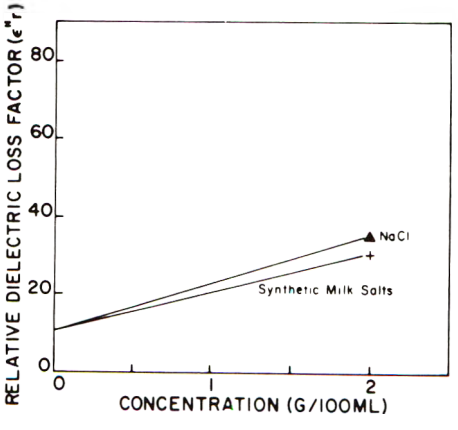

Fig. 2–Dielectric loss comparison of sodium chloride and a synthetic salt mixture.

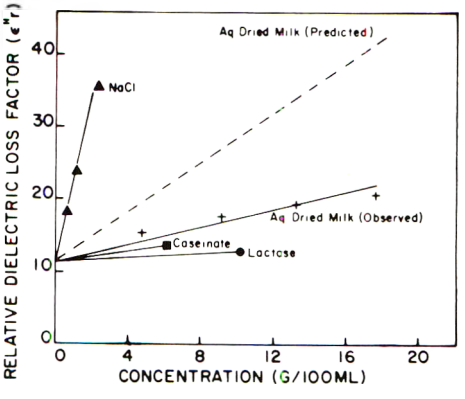

Fig. 3–Relative dielectric loss factor (E''_r) of aqueous dried milk and its chemical analogs.

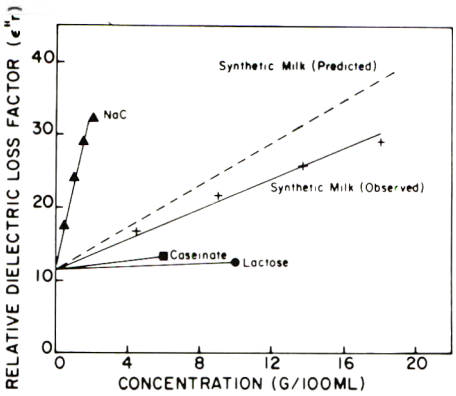

Fig. 4—Relative dielectric loss factor (E''_r) of synthetic milk and its chemical constituents.

crepancies in the prediction of dielectric loss factors. That is, if milk salts were not completely dissociated because of ionic saturation at the limit of solubility or if dissociated salts were bound by other milk constituents such as proteins, then the milk dielectric loss factor would be reduced to significantly lower levels than expected on the basis of chemical composition. A milk salts equilibrium between the colloidal and dissolved states had been demonstrated by the work of Verma and Sommer (1957) and others. In addition, the complexing of casein, calcium or magnesium, and phosphate particles to form milk micelles had also been shown by Noble and Waugh (1965) and Waugh and Noble (1965). Accordingly, specific monovalent and divalent cation binding levels were measured in dried milk solutions by comparison of total and free potassium and calcium levels.

In order to assess quantitatively the degree of solute binding in milk preparations, the following series of experiments was performed. An 18% (w/v) preparation of the dried milk preparation was analyzed for the respective levels of potassium and calcium which were either bound or free in solution. These two ions were selected as representative binding of monovalent and divalent cations which are present in milk. The total levels of the respective ions were determined through atomic absorption spectroscopy. The levels of free ions in solution were determined using ion selective electrodes. Two pH values of the milk sample were tested: pH = 6.4, normal pH of milk and pH = 4.4, the approximate isoelectric point of the caseins. The results of these studies are shown in Table 3. It can be seen that at normal pH of milk 6.4, 16% of the potassium and 71% of the calcium are in bound form. Interestingly, even at the isoelectric point (pH 4.4) the degrees of binding for these cations are very much similar to the previous findings. This would suggest an incomplete dissociating of the milk salts rather than direct binding onto milk proteins. Assuming the binding of other monovalent and divalent cations behaves quantitatively similar to potassium and calcium, it was estimated that approximately 33% of the total milk salts will be in the bound or undissociated form. This phenomenon on the undissociation of salts would therefore show a lower relative dielectric loss factor for an actual dried milk preparation as compared to a predicted value based on a simplified chemical simulation. However, the total level of undissociation of 33% cannot account for the entire discrepancy as shown in Figure 3. It was therefore reasonable to assume that the reduction of relative dielectric loss factor from the predicted value must be due to other factors in addition to association of the salts. To elucidate this unknown, the following experiments were performed, with accompanied results.

This series of experiments was performed in order to establish the level of salt binding in a synthetic milk preparation. The degree of dissociation in a synthetic milk preparation was analyzed because the dielectric loss factor prediction as shown in Figure 4 was in better agreement with measured values than that for solutions of nonfat dried milk. One could suspect that the level of salt dissociation in a synthetic system might be lower than that encountered with the dried milk preparation. The results of this study can be seen in Table 4.

The dissociation study of the synthetic milk was performed using an 18 wt % sample. This preparation is composed of 2% sodium chloride, 10% lactose and 6% sodium caseinate. It can be seen from the results in Table 4 that there was essentially no association of sodium in this synthetic milk preparation. One is therefore led to believe that the differences in the predicted to the measured relative dielectric loss factor for synthetic milk solutions are due to other factors than salt association.

A possible explanation in the reduction of the dielectric loss factors which were encountered in the nonfat and synthetic milk preparations is nonbinding solute interactions. In order to test whether a nonbinding effect which might reduce the relative dielectric loss factor is operative, a series of experiments was performed on the dielectric measurements of solute combinations. Although a large variety of combinations was examined, only a summary on the quantitative relationships of the solute combinations will be presented. These were: sodium chloride 2% + lactose 10%; sodium caseinate 6% + lactose 10%; and sodium chloride 2% + sodium caseinate 6%. These values represent the respective concentration relationships of the chemical analogs for a synthetic milk preparation.

The results on the relative dielectric loss factors at 3000 MHz and 25°C of the solute combinations are shown in Table 5. The loss factors are reported as a ratio of the predicted to the measured value. It can be seen that all of the solute combinations produced dielectric loss factors lower than what one would expect based on a weight fraction of the solute when measured individually. These findings along with the previous information in Table 4 all tend to support a reduction of dielectric loss factor due to possible solute-solvent and solute-solute interactions. This phenomenon has been described by Von Hippel (1965) in a discus-

Table 3—Association studies of salts of 18% (w/v) dried milk

	Concentration of cations					
	Potassium (K^+)			Calcium (Ca^{++})		
pH	Total (mg/100)	In solution (mg/100)	% Associated (%)	Total (mg/100)	In solution (mg/100)	% Associated (%)
6.4 (Normal)	280	234	16	123	36	71
4.4 (Isoelectric)	300	236	21	118	36	70

Table 4—Measurement of binding of solute in 18% synthetic milk preparation

			Sodium ion concentration		
Sodium chloride (%)	Lactose (%)	Sodium caseinate (%)	Total (mg/100 ml)	In solution (mg/100 ml)	Degree of association (%)
2	10	6	810	829	0

Table 5–Effect of solute interactions of synthetic milk preparation on the relative dielectric loss factors (3000 MHz, 25°C, pH = 6.1)

Solute mixture	Ratio of predicted to measured relative dielectric loss factor
2% Sodium chloride + 10% lactose	1.07
6% Sodium caseinate + 10% lactose	1.06
2% Sodium chloride + 6% sodium caseinate	1.07

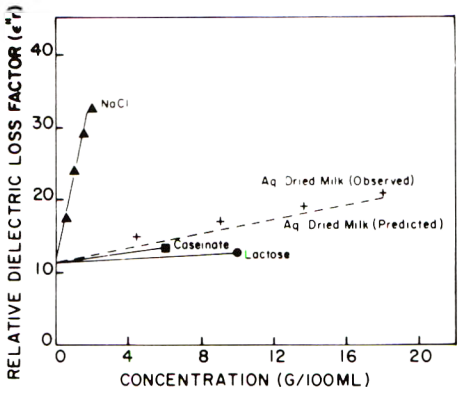

Fig. 5–Simulated relative dielectric loss factor (E''_r) for aqueous dried milk excluding binding and nonbinding effects.

sion of ionic conductance in aqueous solutions. In dilute solution, ions are well separated from each other with one or more layers of solvent molecules firmly bound to form a hydration sheath. As concentration is increased, the hydrated ions tend to aggregate into complexes whose size increases nonlinearly with concentration. This, in turn, would result in a nonlinear variation of the ionic component of dielectric loss with concentration. The dipole loss component is considered to be relatively unaffected by such complexes. The nonbinding effect may be visualized physically in terms of a drag force at the molecular level which varies with the cross-section of the hydration complexes. These complexes are then seen to be dragged through the solvent in the field direction of opposite polarity to that of the complex while the bulk of the solvent molecules orient themselves to the field by dipole rotation.

In view of the findings on the levels of solute association and solute-solvent interactions, it is now possible to formulate a model that quantitatively represents the dielectric behavior on the simulation of milk through its chemical analogs. In this model the components of milk which contribute toward the relative dielectric loss factors are the salts, protein (casein) and lactose. Those milk salts, which are partially associated, quantitatively represent a reduction of 33% of the relative dielectric loss factor as compared to its contribution when predicted from proximate analysis. The solutes which are in solution undergo further reduction in the dielectric measurement due to solute-solvent interactions. This reduction represents an additional 26% of the dielectric loss factor based on the proximate analysis of the milk sample. When both of these factors are considered, the behavior of the predicted relative dielectric loss factor with respect to concentration can now be adequately represented. The final correlations of the measured and predicted dielectric loss factors are now shown in Figure 5. It can be seen that an excellent agreement was obtained in predicting the dielectric properties of milk based on proper modifications of proximate analysis.

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INVESTIGATION INTO THE THEORIES OF DESORPTION ISOTHERMS FOR ROUGH RICE AND PEANUTS

SUMMARY—Several existing mathematical models are investigated with respect to their range of applicability to biological materials. It is found that a logarithmic plot of vapor pressure of water in the material vs. vapor pressure of pure water can best describe the sorption phenomenon. The general form of the equation is: $\ln P = f_1(W) \ln P_S + f_2(W)$. The functions $f_1(W)$ and $f_2(W)$ may differ significantly from material to material. The method of determining these functional relationships is described in detail for rough rice and peanuts. The comparison of experimental and computed values for peanuts shows that this method can predict the required sorption isotherm data within 2% deviation from the experimental values.

INTRODUCTION

MOISTURE sorption isotherms of heterogeneous biological materials represent the integrated hygroscopic properties of numerous constituents which vary from sample to sample for a given product. Intensive study is revealing ever increasing interrelationships of these isotherms to chemical, physical, thermodynamic and stability characteristics of dehydrated, dried, frozen and stored food products. Several phenomenon such as the effect of ambient relative humidity on products at constant temperature, effect of temperature changes on products at constant moisture content, microbial activities in storage of crops and extent of drying under constant ambient conditions are directly determined from the study of moisture sorption isotherms. The sorption isotherms may be classified in two categories: adsorption and desorption. This paper will be concerned only with moisture desorption isotherms of two food crops: rough rice and peanuts.

Since the publication of Henderson's (1952) semi-empirical isotherm equation, several investigators have tried to present more precise mathematical relationships to describe the equilibrium moisture content data. However, each of these relationships has been considered inadequate by subsequent investigators. This necessitated a thorough investigation into the existing models of desorption isotherms utilizing the data available in the literature.

EXPERIMENTAL

Characteristics of desorption isotherms

The desorption isotherms of biological materials are generally type II (Fig. 1) or sigmoid shape, depicting one inflection point, a linear portion and a reflection point. Some isotherms are of type III (Fig. 2) that behave parabolically or exponentially. Rough rice belongs to type II and peanuts fall in the type III class.

Our aim in this paper will be to test each model of desorption isotherms to determine if it will fit available data on the two crops. No attempt will be made to ascertain the validity of underlying theory of development of the models whether derived from empirical or thermodynamic considerations or based on capillary, kinetic or potential theories. We shall not restrict the scope of this investigation to models with a certain number of parameters.

Each of these models must be able to predict the shift in the curve due to temperature variation and compute either equilibrium vapor pressure ratio, ϕ, or equilibrium weight fraction water, W, in grains. Preferably it will be simple to use and offer no difficulty in evaluation of unknown parameters. The resulting deviations between the observed vs. calculated values will be small and consistent throughout the range of vapor pressure ratio from 5–95%.

We hypothesize that finite values of W exist in the vicinity of end points of the EWF curve and that in the limit W can be regarded to approach zero and infinity at zero and 100% relative humidity values,

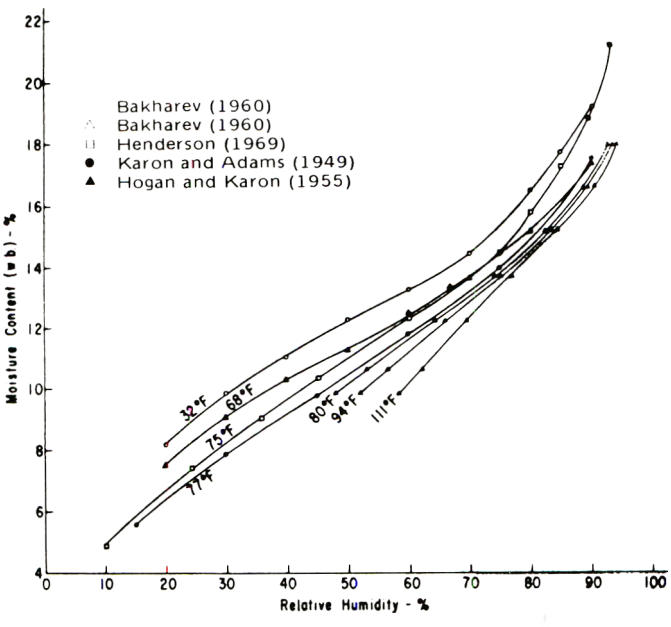

Fig. 1—Desorption isotherms for swollen rough rice by various workers.

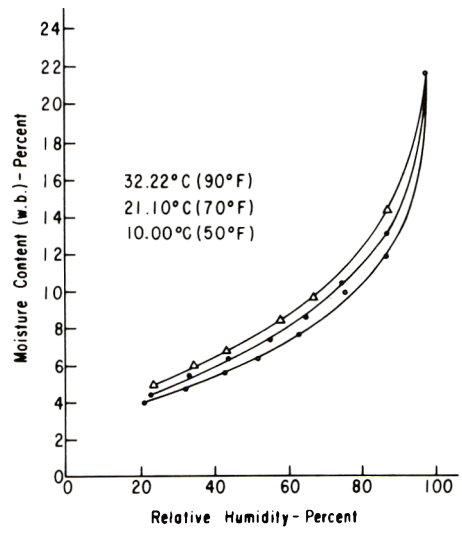

Fig. 2—Desorption isotherm of swollen peanut pods. (Data from Beasley, 1962.)

respectively. It is assumed that presently no known reliable data are available in the range 0–5% and 95–100% ϕ to disprove the hypothesis. Any data on desorption isotherms taken when the material is undergoing structural or chemical changes will be excluded from this investigation. The term temperature implies environment or material temperature.

Investigation

As we trace the trend in development of the isotherm equations, we find that Langmuir (1918) gave an equation to describe the monomolecular adsorption from the assumptions that adsorption is confined to definite points of attraction on the adsorbing surface and that adsorption is limited to a single molecular layer. By balancing the rate of adsorption on the surface against the evaporation he obtained an equation of the form:

$$V/V_m = bP/(1 + bP) \qquad [1]$$

This equation gives an isotherm that is hyperbolic in form (type I shape) rising rapidly at low pressures but becoming parallel to the pressure axis as bP becomes large compared with unity. The curve exhibits a saturation effect at high pressures, the adsorbed volume not increasing with a further increase of pressure. In the case of biological materials, its range of applicability is limited to vapor pressure ratios not greater than 0.20.

Later Brunauer et al. (1938) (BET) studied a wide variety of isotherm shapes of different materials and classified biological materials into the sigmoid or type II shape. They described the inflection point as the one where adsorption ceases to be confined to a monomolecular layer with adsorption beginning in the second layer long before the first layer is filled. This means that above the inflection point adsorption takes place concurrently in the first and second layers until the first layer is complete. By applying Langmuir's assumptions to the second and higher layers, they derived the following isotherm equation for multimolecular adsorption on a free surface:

$$V = \frac{C V_m P}{(P_o - P)[1 + (C - 1) P/P_o]} \qquad [2a]$$

Letting P/P_o be analogous to ϕ and V analogous to W it can be written in the form:

$$\frac{\phi}{W^s(1-\phi)} = \frac{1}{CW_m} + \frac{(C-1)}{CW_m}\phi \qquad [2b]$$

In effect the BET model assumes that a plot of $\phi/[W^s(1 - \phi)]$ against ϕ will be a straight line with a slope of $(C - 1)/CW_m$ and an intercept of $1/CW_m$. For most biological materials these lines are curved beyond vapor pressure ratios of 0.30.

Herkins and Jura (1944) proposed a simpler form of equation for the sorption isotherms, the validity of which is restricted to regions in which the adsorbed molecules form a condensed film. Assuming the process similar to that of condensation of vapor on a liquid surface he proposed the relationship,

$$\log (P/P_s) = B - (A/V^2) \qquad [3]$$

which will be linear if $\log (P/P_s)$ is plotted against $(1/V^2)$ giving an intercept B and slope –A. The Herkins and Jura model does yield a sigmoid shape for biological materials but differs from experimental values as much as 3–4% throughout the range of vapor pressure ratios. It does not describe the temperature dependence of the equilibrium moisture curves also.

Subsequently, Smith (1947) studied the behavior of the sorption of water vapor on high polymers and suggested that a model of the form,

$$W^s = W_b - W' \ln (1 - \phi) \qquad [4]$$

will adequately describe the sorption data with a linear relationship between W^s and $\ln(1 - \phi)$, W′ being the slope and W_b the intercept. Smith's model fits the data on biological materials beyond vapor pressure ratios of 0.20 with an error of 2–3%.

Each of the above models has had some degree of success in representing experimental data. However, none of these has been able to give consistently accurate results throughout the range of vapor pressure ratios at all temperatures.

A breakthrough was introduced by Henderson (1952) who gave a remarkably useful model relating temperature, relative humidity and weight fraction of adsorbate (water) in biological materials,

$$(1 - \phi) = \text{Exp}\,[-K'(W^d)^N] \qquad [5a]$$

It can be transformed in the linear form as,

$$\log [-\ln(1 - \phi)] = N \log W^d + \log KT \qquad [5b]$$

Where K′ is replaced by KT to account for the temperature variation. A log-log plot of W^d or W^s and $-\ln(1 - \phi)$ however, retains a slightly sigmoid shape which accounts for some error in reproducing experimental data. Also, it does not adequately describe the temperature dependence, since K and N have different values at different temperatures. It will be noted that due to the nonlinearity of the plot, two points cannot be used for constructing the full isotherm since the shape of the isotherm will depend upon the location where these two points were selected.

Becker and Sallans (1956) suggested a method, by graphically combining the BET, linear and Smith equations for obtaining the complete desorption isotherms. This method has some merit but it requires six constants to be evaluated separately (two each for the concave, linear and convex portions of the curve) at each temperature.

Haynes (1961) reported a remarkably simple yet accurate empirical equation to describe the sorption isotherm for biological materials at any temperature:

$$\ln(P) = C + C_1 \ln P_s + C_2 W^d + C_3 W^{d^2} + C_4 \ln P_s W^d \qquad [6]$$

He fitted the test data to this general equation by means of the modified Doolittle system to procure regression equations for each of the seven seed species. Correlation coefficients for six of these equations were in the range of 0.98621–0.99658, indicating that test data conformed to the derived equations. This model, however, required that five constants be determined for each material. Note that Haynes (1961) did not measure EWF beyond 80% to 85% relative humidity in his experiments.

Day and Nelson (1965) expressed the constants in Henderson's equation as functions of temperature which are valid for vapor pressure ratios up to 0.70. A separate equation for defining the maximum grain moisture was formulated by plotting W vs T for ϕ levels of 30%, 50% and 70% and extrapolating the slopes of these lines for ϕ = 100%.

The equations are of the form:

$$W_{sat} = P_1 (T)^{P_2} \qquad [7]$$

and

$$(1 - \phi) = \text{Exp}\,(-P_1 T^{P_2} (W)^{P_3} T^{P_4}) \qquad [8a]$$

Chung and Pfost (1967) presented an isotherm equation from the considerations of free energy changes during the sorption process as,

$$\ln(P/P_s) = -(A/RT)\,\text{Exp}(-BW) \qquad [9]$$

Equation 9 reveals that a semilog plot of $\ln(-\ln\phi)$ with W will be a straight line with a slope –B and an intercept of ln(A/RT). This equation gives a good correlation with existing data for values of ϕ between 30% and 70%.

Strohman (1965) and Strohman and Yoerger (1967) obtained an equation from Othmer's plot of the equilibrium weight fraction of water (EWF) data for corn. He described the slopes and intercepts of straight lines as some functions of moisture content,

$$\ln P = f_1(W) \ln P_s + f_2(W) \qquad [10a]$$

After authentic arguments he proposed an equation relating equilibrium and saturation vapor pressures of water in corn, to a characteristic vapor pressure, P_o, and another function of EWF,

$$\ln(P/P_s) = -\ln(P_s/P_o) f_3(W) \qquad [10b]$$

He used an additional equation to fit the Rodriguez-Arias data on corn by using a function originally proposed by Gallaher (1951) for the slope as,

$$f_1(W) = 1 + a\,\text{Exp}(bW) \qquad [10c]$$

and a function for the intercept as,

$$f_2(W) = c\,\text{Exp}(dW) \qquad [10d]$$

The additional equation was,

$$\ln(P/P_s) = a\,\text{Exp}(bW) \ln P_s + c\,\text{Exp}(dW) \qquad [10e]$$

Equation 10a is similar in form to Haynes equation, and equation 10b to Chung and Pfost's equation. Equation 10e did not fit experimental data outside the range of 10–85% relative humidity.

Several other workers have shown the linearity of Othmer plots for corn (Strohman, 1965; Rodriquez-Arias and Hall, 1963; Haynes, 1961)

blue lupine, crimson clover, rescue, sorghum (Haynes, 1961), wheat (Gallaher, 1961; Haynes, 1961), dehydrated eggs (Makower, 1945), rough rice (Agrawal, 1967; Hogan and Karon, 1955) and peanuts (Beasley, 1962). It should be possible to obtain a generalized solution of equation 10a for any material after considering the limitations on the form of the functions $f_1(W)$ and $f_2(W)$ and evaluating constants from experimental data.

Concurrent to this work Young and Nelson (1967) gave a mathematical basis for explaining the hysteresis phenomenon during the adsorption and desorption processes. They derived the equation by combining the BET and Smith equations and thus it includes the advantages of both. A serious limitation in addition to having temperature-dependent constants is that the equation is complex enough to prohibit its use in closed form mathematical solutions of problems.

Ngoddy et al. (1968) presented a moisture adsorption model based on BET and capillary condensation theories and claimed that the characteristic isotherms can be constructed through the variation of a single parameter. This analysis indicates that the isotherm shape is a function of pore structure of the product and is thus independent of temperature. The isotherm equation appears to be no less complicated than Young and Nelson's equations.

Chen (1969) presented an isotherm equation relating the drying parameters implicitly,

$$K - \ln\phi = m\,\mathrm{Exp}(-nW) \qquad [11a]$$

which can also be written as,

$$\phi = A\,\mathrm{Exp}[-m\,\mathrm{Exp}(-nW)] \qquad [11b]$$

where K, m and n are found to be temperature dependent parameters and A is close to unity. Equation 11a or 11b has the required properties of type II isotherms at the end points including a reflexion point. However, none of these equations permits the evaluation of the shift in the curve due to temperature variation. Later Chen (1970) expressed the parameters m and n as functions of temperature similar to equation 8a and showed that the root mean square error computed from equation 11c was smaller than the error given by equation 8b for the same data.

$$\ln\phi = -P_1 T^{P_2}\,\mathrm{Exp}(-P_3 T^{P_4} W) \qquad [11c]$$

Evidently equations 11a, 11b and 11c are improvements over the Henderson and Day-Henderson models but they add to the existing complexity in computation and do not give thermodynamic temperature dependence. Note the similarity between equation 9 and 11c. It appears that the Rodriquez-Arias data on corn are better represented by equation 10e than equation 11c.

Materials and methods of analysis

Extensive data in the literature are available on several materials of common interest. Rough rice and peanuts are selected because each of these fall in two separate classes of isotherms: namely, rough rice in type II (Fig. 1) and peanuts in type III (Fig. 2). We wish to investigate several theories outlined in the previous section on desorption equilibrium isotherms using the available data.

Day-Henderson model. The most widely used model is the Henderson model which after expressing the constants K′, and N as some functions of temperature can be written as,

$$\log[-\ln(1-\phi)] = \log P_1 + P_2 \log T + P_3 T^{P_4} \log W \qquad [8b]$$

A plot of equation 5b on logarithmic paper does not yield straight lines and K′ and N vary with temperature. It was found that the correlation coefficients using W^s and normal temperature are higher than those using W^d and absolute temperature.

The data for rough rice were arranged to represent the full curve from 10–95% relative humidity and the linear portions of the curves between 30–75% relative humidity. The values of the coefficients K′ and N were obtained from intercepts and slopes of each of these curves which are plotted in Figures 3 and 4 as functions of temperature. The best values of the constants P_1, P_2, P_3 and P_4 were found by a least square regression analysis in each case. It is noted from Figures 3 and 4 that the regression correlation coefficient is higher for the linear portion of the curve than for the full curve. The percent deviations in computing the weight fraction of water for swollen grain lie in the range of 4% for the linear portion and 6% for the full curve.

The data for peanut pods, hulls and kernels were analyzed in the full range of relative humidities. From this analysis it was found that the weight fractions W^s of the hull and kernel when added together gave more precise EWF for the whole pod than did the values computed

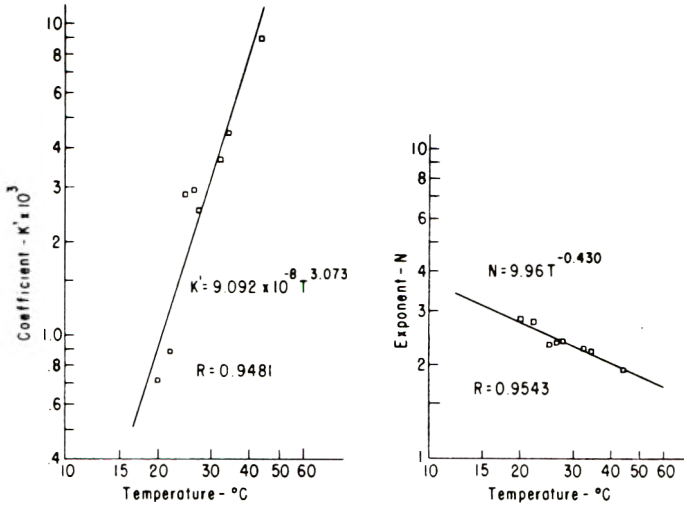

Fig. 3—Dependence of exponent-N and coefficient-K′ on temperature in Henderson's equation for 10–95% relative humidity. Weight fraction moisture of swollen rough rice was used for correlation.

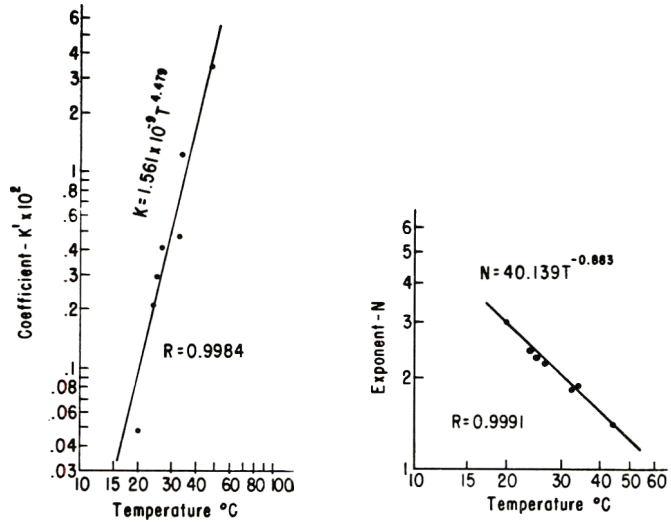

Fig. 4—Coefficient-K′ and exponent-N as functions of temperature in the range of relative humidities between 30–75%. Weight fraction moisture of swollen rough rice was used for correlation.

from the data on the pod alone.

Though the EWF for the whole pod lies between the values for the hull and kernel at all temperatures, K′ did not turn out to be a regular function of temperature which accounted for additional errors in EWF's. The values of constants P_1, P_2, P_3 and P_4 are reported in Figure 5 with deviations up to 4.5% as compared to experimental values.

Modified Chung and Pfost model. During the analyses of data it was noticed that if slopes and intercepts in equation 10a are expressed arbitrarily as $f_1(W) = a\,\mathrm{Exp}(bW)$ and $f_2(W) = c\,\mathrm{Exp}(dW)$ then the contribution of $f_2(W)$ is approximately equal to the average of ln(Ps) for the peanut kernel. This transforms equation 10a into the form,

$$\ln(P/Ps) = a\,\mathrm{Exp}(bW)\ln(Ps) \qquad [12]$$

Equation 12 is similar in form to equation 9 with $-(A/RT)$ analogous to $a[\ln(Ps)]$. The constants a and b were evaluated by minimizing the sum, S, in equation 13,

$$S = \sum_{i=1}^{n} [Pi/Psi - \mathrm{Exp}(a\,\ln Psi\,\mathrm{Exp}(bWi)]^2 \qquad [13]$$

The final forms of the equations are:

$$\ln(P/Ps) = 1.9682\,e^{-0.4063W}\ln(Ps) \qquad [12b]$$

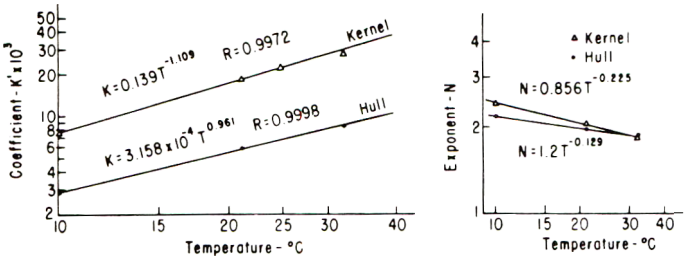

Fig. 5–Coefficient-K' and exponent-N as functions of temperatures for the kernel and hull of peanut. The pod EWF is found by adding the weight fraction EWF of each of the hulls and kernels. Weight fraction ratio of kernels and hull is 0.748 and 0.252 respectively (Karon and Hillery, 1949).

Table 1–Computed values of $f_1(W)$ and $f_2(W)$ from Othmer's plot for peanut pod[a]

W Wet basis %	$f_1(W)$ Slope (b)	$f_1(W)$-1	$f_2(W)$	Intercept (a)	$f_1(W) + f_2(W)$
6	1.2105	0.2105	−0.0984	0.9063	1.1121
8	1.1334	0.1334	−0.0127	0.9874	1.1207
10	1.0918	0.0918	0.0288	1.0293	1.1206
12	1.0699	0.0699	0.0584	1.0607	1.1283
14	1.0510	0.0510	0.0708	1.0733	1.1218
16	1.0378	0.0378	0.0851	1.0887	1.1229

[a]Beasley, 1962.

for peanut kernels and

$$\ln(P/Ps) = 2.3978\, e^{-0.232W} \ln(Ps) \qquad [12c]$$

for rough rice.

The computed value of relative humidities had 3.5% and 5% deviations from experimental values for peanut kernels and rough rice respectively. Equation 9 did not adequately satisfy the data for rough rice and peanuts. The parameters A and B were functions of temperature and the equation was valid only for the linear portion of the isotherms.

Modified Strohman model. Although Strohman (1965) lists several functions for $f_3(W)$ in equation 10b that might fit the isotherm data, he did not use any of them. By solving equation 10a for $f_1(W)$ and $f_2(W)$ and considering the limit as $W \to \infty$ and $W \to 0$, ie., at two extremes of the EWF curve, we find that,

$$\lim_{W \to \infty} f_1(W) = \lim_{P \to Ps} \frac{\ln(P_2) - \ln(P_1)}{\ln(Ps_2) - \ln(Ps_1)} \to 1 \qquad [14a]$$

and

$$\lim_{W \to \infty} f_2(W) = \lim_{P \to Ps} (\ln P) - \lim_{f_1(W) \to 1} (f_1(W) \ln Ps) \to 0 \qquad [14b]$$

When $W \to 0$ both P and Ps are finite hence $f_1(W)$ and $f_2(W)$ will also be finite. The task lies in determining these functions to satisfy the limiting conditions. These functions may vary from material to material, desired degree of accuracy, availability of data reduction facilities, and the humidity range of available data.

The values of $f_1(W)$ and $f_2(W)$ were evaluated from the Othmer plots for peanut pods. Table 1 clearly indicates that the summation $f_1(W) + f_2(W)$ is approximately a constant (K = 1.12). This means that when $W \to \infty$, $f_2(W) = K - 1$. This satisfies equations 14a and 14b keeping in mind that W in practice could never be infinite, hence $f_1(W)$ and $f_2(W)$ can not be one and zero, respectively. After plotting $f_1(W)$ against W it was found that a function of the form,

$$f_1(W) = 1 + aW^{-b} \qquad [14c]$$

very nearly satisfied the tabulated values and the limiting conditions. This finding permitted us to write equation 10a in the general form as,

$$\ln(P) = (1 + aW^{-b}) \ln(Ps) + K - (1 + aW^{-b}) \qquad [14d]$$

or

$$\ln(P/Ps) = aW^{-b} (\ln(Ps) - 1) + (K - 1) \qquad [14e]$$

The constants a, b and K were evaluated using an existing program which minimized the sum of the squares of the deviations between the observed vs. calculated values of ln(P/Ps) for the peanut pod data. The final equation is,

$$\phi = \mathrm{Exp}\,[4.215\, W^{-1.672} (\ln(Ps) - 1.0) + 0.119] \qquad [14f]$$

It may be noticed that the chosen function and equation 14f fits the data (Beasley, 1962), Table 2, remarkably well with deviations of less than 1%. Note that equation 14f can be solved directly for W.

Attempts were made to investigate the validity of models proposed by Young and Nelson (1967) and Ngoddy et. al. (1968) using data on rough rice. The required constants could not be easily evaluated without the expense of considerable computing time. Additional investigation may be necessary to arrive at the systematic procedure for evaluating the constants.

ϕ-W-T diagrams. To illustrate the interactions between temperature, relative humidity and moisture content, the data on rough rice and peanuts have been plotted in Figures 6 and 7. We shall refer to these figures as ϕ-W-T diagrams which will be useful in studying the relationships between relative humidity vs. moisture content at constant temperature and temperature vs. moisture content at a constant relative humidity. It may be noted from the ϕ-W-T diagram for rough rice (Fig. 6) that the moisture content decreases with an increase in temperature. Also the constant relative humidity lines on the ϕ-W-T surface are straight and nearly parallel to each other.

The ϕ-W-T diagram for peanuts shows three surfaces. One each for the hull, pod and kernel. It may be noted that at lower temperatures changes in moisture content are more erratic than at higher temperatures, yet the lines of constant relative humidity for each hull, pod and kernel are parallel and straight. Perhaps the effectiveness of this diagram lies in selecting an empirical relationship with coefficients that will best

Table 2 –Comparison of calculated and experimental values of relative vapor pressures against weight fraction water in swollen peanut pods at test temperatures

W Wet Basis %	32.22°C ϕ Cal. %	ϕ Exp.[a] %	Error %	21.1°C ϕ Cal. %	ϕ Exp.[a] %	Error %	10°C ϕ Cal. %	ϕ Exp.[a] %	Error %
6	48.30	48.00	0.62	42.10	41.80	0.71	36.20	36.00	0.55
8	66.80	66.00	1.20	61.30	60.00	2.12	55.09	55.00	0.16
10	78.33	78.00	0.42	74.10	73.00	1.48	69.40	68.80	0.86
12	86.38	86.70	0.37	82.70	82.60	0.12	78.90	78.80	0.12
14	93.23	92.00	1.31	88.76	89.00	0.27	85.59	85.80	0.24
16	95.51	95.20	0.32	93.10	93.00	0.11	90.40	90.40	0.00

[a]ϕExp. interpolated from Beasley, 1962; P_S at 32.21°C = 0.0487939 Kgf/cm²; P_S at 21.1°C = 0.025365 Kgf/cm²; P_S at 10°C = 0.0124361 Kgf/cm².

fit the data. We postulated that a polynomial of the following form would be adequate for mathematically describing the data on peanut hulls,

$$W^S = C_1\phi + C_2\phi^2 + C_3T + C_4\phi T \qquad [15]$$

where,

W_s = Weight fraction water of swollen hull, %
ϕ = Relative humidity, %
T = Test temperature, °C

A modified Doolittle technique was used to evaluate the constants C_1 through C_4 in equation 15. It was found that this polynomial fits the data with a regression correlation coefficient of 0.989 and standard deviation of 0.606. The final constants are,

$C_1 = 3112.1 \times 10^{-4}$ $\quad C_2 = -9.6 \times 10^{-4}$
$C_3 = -23.3 \times 10^{-4}$ $\quad C_4 = -13.1 \times 10^{-4}$

Equation 15 is similar to Haynes' model but it is more direct and can be used for closed form solutions.

A close analysis of Figures 6 and 7 reveals that the sorption isotherms can also be represented in the following general form:

$$W = g_1(\phi) + g_2(\phi)T \qquad [16]$$

Looking back in the literature we found that Henderson (1952) has plotted the moisture content data of shelled corn against temperature from 10–90% relative humidity. These form straight lines with nearly constant slopes. No attempt so far has been made to determine the functions $g_1(\phi)$ and $g_2(\phi)$ for any crop. It is possible that this ϕ-W-T model may overcome some of the shortcomings associated with existing ones.

DISCUSSION

SEVERAL difficulties were encountered in using the available data. Earlier workers (Coleman and Fellows, 1925) considered that the effect of temperature on EWF was negligible. Some of the data reported at 25°C (Breese, 1955; Coleman and Fellows, 1925; Haynes, 1961; Henderson, 1969; Karon and Adams, 1949) have invariably been taken at laboratory ambient conditions where the temperature does not always remain 25°C. Any change in temperature at the time of measurement directly affects the relative humidity but not the moisture content since the process of desorption from the kernel is slow. In effect, one is studying a very slow changing parameter (W) with the very fast changing parameters (ϕ and T). The errors in the measurement of ϕ-W-T largely depend upon the method used. Lal and Agrawal (1968) have summarized various methods for moisture measurement that have been internationally accepted as standards for different crops.

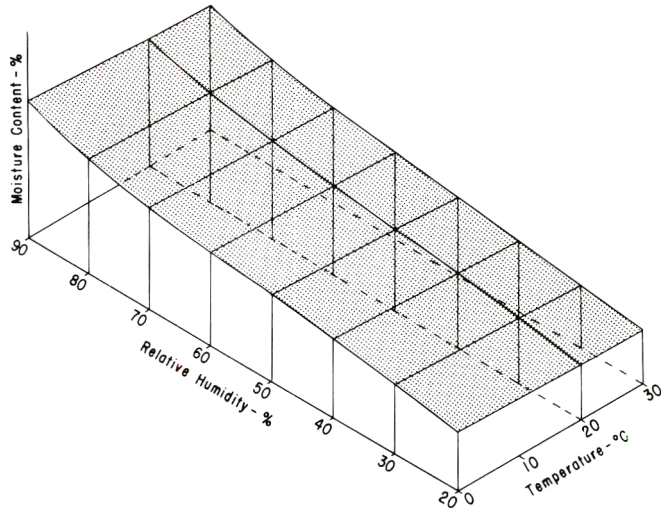

Fig. 6—Relative humidity, equilibrium weight fraction and temperature (ϕ-W-T) diagram for rough rice. (Data from Bakharev, 1960.)

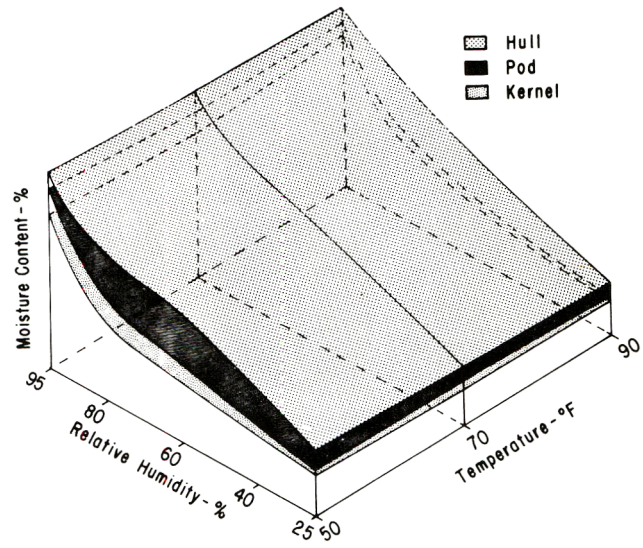

Fig. 7—Relative humidity, equilibrium weight fraction and temperature (ϕ-W-T) diagram for peanut hulls, kernels and pods. (Data from Beasley, 1962; Beasley and Dickens, 1963.)

Table 3—Range of applicability of various desorption isotherm models and their approximate grouping according to state of water present

Model	Range of applicability, ϕ% Good	Poor	Type of bond	Specifications
Langmuir (1918)	0–15	0–20	Polar[a] – I	Dry, hard, shrunken
Brunauer (1938)	0–20	0–30		microbicidal, rancid odor and flavor.
Herkins and Jura (1944)	25–55	20–90	Nonpolar – II	Optimum, dry surface,
Henderson (1969)	25–75	10–95		microbiostatic, normal
Day and Nelson (1965)	10–70	5–90		odor and flavor.
Chung and Pfost (1967)	30–70	25–80		
Smith (1947)	–	>20	Nonbonded – III	Moist, soft, swollen,
Becker and Sallans (1956)	–	5–95	(Also covers II and I)	microbial growth,
Haynes (1961)	–	5–95		off odor and flavor.
Polynomials	5–95	5–95		
Strohman (1965)	10–90	5–95		

[a]Rockland, 1969.

The ASAE yearbook (1966) even gives correlations between a few of these methods. Evidently, all the moisture methods cannot be used satisfactorily for every crop.

Most of these methods assume that the samples in the oven are dried to zero percent moisture content. This cannot be true, particularly in whole grain where bound water cannot be evaporated without effecting chemical changes in the structure of the seed. However, none of these moisture measuring methods are designed to effect chemical changes in the seed. Likewise, the relative humidity in the ovens, even when operated at 130°C, does not become zero, hence the moisture content cannot be zero.

The current science of moisture measurement is more accurate than the science of vapor pressure measurement and the major errors associated with desorption equilibrium data can be attributed to the relative humidity values. Therefore, the selection of a vapor pressure measurement method is extremely important in collecting data on moisture desorption isotherms. Lal and Agrawal (1968) surveyed a wide range of vapor pressure measuring devices that have been used in the past by several workers. It appears that the most satisfactory results were obtained by using the Dubrovin Manometer and Isotenscope method. Haynes (1961) in fact, outlines difficulties associated with some of these devices and shows the resulting isotherms that criss-cross each other. This difficulty, however, was overcome by the Isotenscope method. The ranges of relative humidity from 0–5% and 95–100% are not amenable to common measuring devices. The static method using the saturated salt solution has been the most unsatisfactory way of collecting data on moisture sorption isotherms.

Most of the desorption isotherm models considered in this study have had some success in representing experimental results, but none of these accurately predicts the effects of all the physical variable. It is hoped that Othmer's development can be used successfully for biological materials after selecting the proper functions of EWF in equation 10a from experimental data.

CONCLUSIONS

SEVERAL models proposed for evaluating the moisture isotherms of food products have been investigated using the available data on rough rice and peanuts. An approximate range of applicability of each of these models is tabulated in Table 3. The models are grouped so that they fall in a specific state of water bond and represent the physical and chemical conditions of food crops. It is concluded that most of the food crops will fall within these limits. At least two approaches (equations 10a and 15) of empirically defining the moisture desorption isotherms appear encouraging. Both of them have a very high degree of success. The third approach (equation 16) needs to be investigated further. Most of the published data on both products have been used in this analysis. The evaluated constants for rough rice, peanut pods, kernels and hulls will be very useful in future work.

NOMENCLATURE

EWF = Equilibrium weight fraction of water in grain. (Same as equilibrium moisture content.)

P = Characteristic vapor pressure may be P_w of water in seeds, Kgf/cm².

P_o = Reference vapor pressure, may be P_s or P_{ws}, Kgf/cm².

P_s = Vapor pressure of pure water at saturation, Kgf/cm².

P_w = Partial pressure of water vapor in the air, Kgf/cm².

P_{ws} = Vapor pressure of water in the saturated moist air, Kgf/cm².

$P/P_s = \phi$ = Vapor pressure ratio or relative humidity, decimals.

R = Gas constant, MKgf/Kg°K also correlation coefficient.

T = Temperature °C or °K.

V = Volume of adsorbate.

W = Average weight fraction of water in seeds, percent.

W_m = Constant in BET Equation defined as average weight fraction water required to form a unimolecular layer.

Superscripts s and d refer to swollen grain and dry grains. Subscripts o and s refer to initial and saturation conditions.

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EFFECT OF DIETARY FATS ON THE FATTY ACID CONTENTS OF CHICKEN ADIPOSE TISSUE

SUMMARY—150 broiler-type chicks were reared from hatching to 4 wk of age on a low-fat ration before being fed diets containing 10% of either corn oil, lard, beef tallow or hydrogenated coconut oil. Analyses of treatment effects were made at 2 wk intervals until the chicks were 10 wk old. The fatty acid content of extracted total lipids were characteristic of the dietary fats and dietary fatty acid patterns were incorporated into the adipose tissue within 2 wk after the experimental diets were fed. The total lipids when separated into solid fats and liquid oils at room temperature also reflected the fatty acid pattern of the experimental diets. Neutral triglycerides from the adipose tissue contained less linoleic acid and more palmitic and oleic acids than the total lipid fraction. High amounts of palmitic and oleic acids were observed in all of the fractions analyzed. No significant differences were found in total cooking loss, drip loss, taste preference, or TBA values of skin or adipose tissue among the 10 wk old chickens fed different experimental diets.

INTRODUCTION

THE COMPOSITION of the depot fat in fowl can be changed by ingesting large amounts of dietary fat (Cruickshank, 1934). Feigenbaum and Fisher (1959) concluded that dietary linoleic acid appears to exert the greatest influence on body and egg fat in hens. The changes brought about by dietary oils were qualitatively, but not quantitatively, related to their fatty acid composition. A number of other investigators [Machlin and Gordon (1961); Marion and Woodroof (1963); Mickelberry et al. (1966); Sell et al. (1968); and Schuler and Essary (1971)] have studied the effect of dietary proteins and fats on the fat composition of different organs of chicken or hens. Adipose tissue was analyzed along with muscle, liver and skin tissues in these studies. Sell and Hodgson (1962) proposed the possibility of direct deposition of unaltered dietary fatty acids as depot fat in chicken. More recently, Yoshida and Morimoto (1970) changed young chicks from lipogenic diets to lipolytic diets and vice versa, and concluded that changes in whole carcass fat level were rapid and reversible.

Carcass composition variation resulting from feeding of dietary fats has been shown to influence poultry flavor (Holdas and May, 1966; Carlson et al., 1957; Miller and Robisch, 1969; Miller et al., 1969), stability of carcass fat to oxidation (Marion and Woodroof, 1966; Marion et al., 1967; Schuler and Essary, 1971), tenderness (Schuler and Essary, 1971) and overall storage quality (Darrow and Essary, 1955).

This study was designed to study the turnover rate of dietary fat into depot fat in chicken adipose tissue together with detailed characterization of the lipid of the adipose tissue to gain insight in the mechanism of the metabolism of dietary fat in broiler chicks. Cooking loss, TBA values (2-thiobarbituric acid) and taste preference studies of frozen chickens from each dietary treatment were conducted.

EXPERIMENTAL

MALE BROILER chicks (150 White Rock Cross) were reared to 4 wk of age in electrically heated batteries. A starter ration (Table 1) low in fat but containing 1.5% safflower oil to meet essential fatty acid requirements was fed for the first 4 wk. At 28 days of age, 120 chicks nearest to the average weight were randomly assigned to treatments and fed 5 experimental diets (Table 1) in replicate for 6 wk.

After 2, 4 and 6 wk on the experimental diets, 4 chicks from each pen were sacrificed, processed, immediately frozen and stored at −20°C. All carcasses were thawed immediately before analysis and the abdominal adipose tissue was removed from the carcasses. The adipose tissue of 8 chicks from each treatment (4 per replicate) at each age were pooled as one sample and dried to a constant weight under vacuum. Total lipids were extracted from 20g samples using the Soxhlet method described by Borgman (1964). The feeds and the supplemented fats were extracted by the same method except the sample sizes were 40g and 5g respectively.

The extracted lipids were methylated by the methanol-HCl method of Stoffel et al. (1959). The fatty acid methyl esters were then fractionated on a Microtek MT-220 dual column gas chromatograph. Stainless steel columns (72 × 1/8 in.) packed with 15% Butanediol succinate polyester and 3% OV-101 on 100/120 mesh Chromosorb G, AW/DMCS, were used at an isothermal oven temperature of 195°C. The samples were introduced in 0.5–1.0 μl carbon disulfide. The fatty acids were identified by comparing the retention values with the authentic fatty acid standards (Hormel Institute, Austin, Minn.). Percentages of individual fatty acid methyl esters were calculated from the peak area computed by a CRS digital-type integrator (Infotronics Corp., Houston, Tex.) and checked by planimetric readings.

Part of the lipid samples were fractionated on preparative thin-layer plates of silica gel GF-254 to separate neutral triglycerides from other lipids. The loaded plates were developed in hexane:ethyl ether:acetic acid (90:10:2, v/v) for 3 hr. The fractions were detected with iodine vapor and recovered from the plates by methanol extraction on Soxhlet apparatus for 12 hr.

The total lipids of the adipose tissue were separated into solid fat and liquid oil fractions at room temperature by a modification of the method of Nagai and Nishikawa (1969). 40g of adipose tissue was cut into small pieces and refluxed with 150 ml deionized distilled water. After filtering, the aqueous layer was removed and the lipid fraction was allowed to stand at room temperature for 24 hr. Petroleum ether was used to facilitate all transfers quantitatively, and was removed by passing dry nitrogen through the sample at 37°C. At the end of the 24 hr period, the solid fats were separated from the liquid oils and the two were separated.

Whole 10-wk old broilers (6 wk on the experimental diets) which had been frozen for 4 months at −20°C were thawed at room temperature for 12 hr. The carcasses were halved, rinsed, drained and blotted dry. The left half of each carcass was wrapped in a heavy duty aluminum foil package and baked cavity side down at 178°C to an internal breast temperature of 85°C. Immediately after cooking, the package was tilted approximately 30° and the bottom perforated to allow the juices and condensate (drippings) to drain off (Moran and Orr, 1969). Cooking losses were expressed as the total percent weight loss and percent drip loss from the uncooked weight of the broiler half. Taste preference panels consisting of 10 members were conducted on breast muscle samples from each treatment using a 9-point hedonic scale from nine (like extremely) to one (dislike extremely). Skin and adipose tissues from the uncooked right broiler half were analyzed for TBA values following the method of Tarladgis et al. (1960). In several cases where 10g of adipose tissue was not available, 5g samples were used and the TBA value adjusted accordingly. Duplicate halves of broilers on each dietary treatment were evaluated for cooking losses, taste preferences and tissue TBA values. These results were analyzed statistically by analysis of variance and the significance of means tested by the least significant difference (LSD) method following Ostle (1963).

RESULTS

THE FATTY ACID contents of the total lipids extracted from the experimental rations are shown in Table 2. Note that the low fat control diet has a fatty acid pattern proximate to that of the corn oil supplemented diet. The weight gains of all groups were similar. The digestibility of the five feeds were all good.

Table 1–Composition of starter, basal and experimental rations

Ingredient	% content (g/100g feed) Starter ration	Basal ration	Fat supplemented
Corn starch	28.85	37.35	11.55
Dextrose	20.00	20.00	20.00
Soybean meal[a]	44.00	31.00	31.00
Phosphorus	2.75	2.25	2.25
Safflower oil	1.50	1.50	1.50
Salt	0.25	0.25	0.25
Trace minerals[b]	0.15	0.15	0.15
DL-Methionine	0.20	0.20	0.20
Vitamin premix[c]	0.30	0.30	0.30
Solka-floc[d]	2.00	2.00	22.25
Casein	–	5.00	5.00
Fat[e]	–	–	10.00

[a]Curaphos brand.
[b]Mineral mixture contains: Fe, 2%; Zn, 6.5%; Cu, 0.45%; Co, 0.05%; Mo, 0.01%; Mn, 5.03%; I, 0.44%; Ca, 14.56%.
[c]Vitamin premix provides per kg of feed: Vitamin A, 3,000 USP units; vitamin D_3, 750 IC units; vitamin E, 1 IU; vitamin B_{12}, 4 mg; riboflavin, 2.5 mg; niacin, 10 mg; d-pantothenic acid, 4 mg; choline chloride, 250 mg; menadione sodium bisulfied complex, 1 mg; folic acid, 0.1 mg; ethoxyquin, 25 mg.
[d]Non-nutritive cellulose.
[e]Fats used were either corn oil, lard, beef tallow or hydrogenated coconut oil.

Table 2–Fatty acid patterns of the total lipids obtained from the experimental rations

Fatty Acids[b]	Rations[a] LF	C	B	L	H
Saturated fatty acids					
8:0	–	–	–	–	7.11
10:0	–	–	–	0.03	6.18
12:0	0.32	–	0.08	0.03	39.68
14:0	0.10	0.01	3.33	0.83	16.75
16:0	19.49	14.14	32.18	27.94	10.33
18:0	0.98	2.58	14.75	12.48	7.42
Total	20.89	16.73	50.34	41.31	87.47
Unsaturated fatty acids					
14:1	–	–	1.14	–	–
16:1	1.47	0.79	2.92	1.92	0.47
16:2	–	–	0.64	–	–
18:1	56.67	33.91	39.83	46.78	8.86
18:2	20.97	48.33	5.13	9.99	3.20
18:3	–	0.24	–	–	–
Total	79.11	83.27	49.66	58.69	12.53

[a]Diet designations: LF = low fat basal diet; c = corn oil-supplemented diet; B = beef tallow-supplemented diet; L = lard-supplemented diet; H = hydrogenated coconut oil-supplemented diet.
[b]Number of carbons:number of double bonds.

From Table 3, it can be seen that the fatty acid contents of the total lipids from adipose tissue of chicken fed different dietary fats are quite different. However, the characteristics of each dietary fat were incorporated into the adipose tissue in less than 2 wk after the chickens were fed the experimental rations. The fatty acid contents and patterns for chickens of different ages receiving the same diet were very close to each other. The variations of individual fatty acids in the adipose tissue of the 6-, 8- and 10-wk old birds were within biological variation and experimental error. In chickens fed corn oil or hydrogenated coconut oil, a high content of linoleic acid or lauric acid was incorporated into the adipose tissues, respectively. High amounts of palmitic and oleic acids were observed in all adipose tissue. The combined percentage of palmitic and oleic acids in the adipose tissue was always higher than that in the feeds.

The fatty acid patterns of the neutral triglycerides separated from each total lipid extract were similar to that of total lipids in the corresponding adipose tissues (Table 4). Two points of interest were noticed. First, a higher percentage of saturated fatty acids was observed in the neutral triglycerides than in the total lipids; and second, a decrease in linoleic acid content was observed in all triglyceride samples in comparison with corresponding total lipid samples. The decrease

Table 3–Fatty acid patterns of total lipids from adipose tissue of chicken fed different dietary fats

	LF-2[a]	LF-4	LF-6	C-2	C-4	C-6	B-2	B-4	B-6	L-2	L-4	L-6	H-2	H-4	H-6
Saturated fatty acids															
8:0	–	–	–	–	–	–	–	–	–	–	–	–	0.06	0.06	0.10
10:0	–	–	–	–	–	–	–	–	–	–	–	–	0.77	1.36	1.40
12:0	–	–	–	–	–	–	0.02	0.02	0.04	–	–	–	19.24	18.09	22.71
14:0	0.16	0.29	0.31	0.52	0.10	0.23	1.89	2.16	2.15	0.51	0.75	0.24	10.10	15.22	15.43
16:0	35.01	36.76	34.03	23.10	19.87	21.43	37.91	28.50	27.84	31.02	30.05	30.25	26.94	25.20	23.41
18:0	8.20	7.84	2.69	4.31	4.08	5.83	7.78	9.14	9.50	5.78	5.42	6.28	5.69	8.52	6.38
Total	43.37	44.89	37.03	27.93	24.05	27.49	47.60	39.82	39.53	37.31	36.22	36.77	62.80	68.45	69.43
Unsaturated fatty acids															
14:1	–	–	0.11	–	–	–	0.18	0.28	0.38	–	–	–	0.33	0.37	0.45
16:1	5.18	5.37	7.84	0.74	0.25	2.54	2.33	2.78	2.94	2.45	2.41	2.09	2.86	2.00	2.93
16:2	–	–	–	–	–	–	–	0.10	0.30	–	–	–	–	–	–
18:1	47.81	44.40	50.77	31.59	31.67	29.15	46.12	53.17	52.82	51.05	52.23	54.28	27.79	23.40	23.12
18:2	3.64	5.34	4.25	39.74	44.03	40.82	3.77	3.85	4.03	9.19	9.14	6.86	6.22	5.78	4.07
Total	56.63	55.11	62.97	72.07	75.95	75.21	52.40	60.18	60.47	62.69	63.78	63.23	37.20	31.55	30.57

[a]For designation of diets, see Table 2. The number following the diet indicates the number of weeks on the experimental diets, i.e., LF-2 = adipose tissue from chickens fed low-fat diet for 2 wk.

Table 4—Fatty acid patterns of neutral triglycerides from adipose tissue of chicken fed different diets

	LF-2[a]	LF-4	LF-6	C-2	C-4	C-6	B-2	B-4	B-6	L-2	L-4	L-6	H-2	H-4	H-6
Saturated fatty acids															
8:0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10:0	–	–	–	–	–	–	–	–	–	–	–	–	0.82	2.21	2.51
12:0	–	–	1.91	–	–	–	–	–	–	–	–	–	28.82	35.67	35.82
14:0	8.24	1.35	2.50	0.39	0.11	1.27	5.87	2.77	2.90	0.35	1.05	0.72	11.02	18.19	18.36
16:0	41.54	40.04	36.00	35.54	41.34	37.65	38.66	36.12	32.47	40.74	35.52	33.10	28.24	25.64	24.51
18:0	6.08	7.01	2.44	5.49	4.49	6.33	6.25	10.64	10.39	3.70	4.68	6.24	5.02	4.14	2.96
Total	55.86	48.40	42.85	41.42	45.94	45.25	50.78	49.53	45.76	44.79	41.25	40.06	73.90	85.85	84.16
Unsaturated fatty acids															
14:1	–	–	–	–	–	–	–	–	–	–	–	–	0.35	0.09	0.25
16:1	6.29	9.84	8.21	0.95	1.11	1.81	1.49	2.21	3.33	1.09	2.55	2.64	2.01	0.56	1.15
16:2	–	–	–	–	–	–	–	–	0.17	–	–	–	–	–	–
18:1	36.43	39.06	48.18	37.71	37.21	36.65	47.24	48.02	49.30	51.67	51.10	51.90	21.11	13.20	14.22
18:2	1.42	2.70	0.76	19.92	15.74	16.29	0.49	0.24	1.44	2.45	5.10	5.40	2.63	0.30	0.22
Total	44.14	51.60	57.15	58.58	54.06	54.75	49.22	50.47	54.24	55.21	58.75	59.94	26.10	14.15	15.84

[a]For designation of diets, see Table 2.

of linoleic acid was generally coupled with an increase in palmitic acid content. In chickens receiving a hydrogenated coconut oil-supplemented diet, the neutral triglycerides showed a large increase in lauric and myristic acids and a decrease in oleic and linoleic acids in comparison with the total lipids. In chickens fed a corn oil supplemented-diet, an increase in oleic acid was also observed in the same comparison.

From a practical viewpoint, the amount of solid fat relative to liquid oil in the chicken adipose tissues is important. The percentage of solid fats and liquid oils are influenced by dietary fat sources (Table 5). The fatty acid patterns of the solid fat and liquid oil fractions from adipose tissue of 10-wk old chicks are presented in Table 6. Although the oils always contain more unsaturated fatty acids than their solid fats counterpart, it is interesting to observe that there are over 50% saturated fatty acids in one oil sample and over 50% unsaturated fatty acids in two fat samples. The fatty acid content of the oils and fats seem to be the same but the quantities of fatty acids involved differ substantially. The differences are mainly in the palmitic and oleic acid contents except in the case of the corn oil and hydrogenated coconut oil samples in which linoleic and lauric acids also differ in the oil and fat fractions.

No significant differences ($P \leqslant 0.05$) were found for cooking losses, taste preference or TBA values among broilers from the experimental diets. Mean values for these analyses are given in Table 7 with the minimum values (LSD) required for significance at the 5% level of probability. Large values for within treatment variations (from analysis of variance) were likely the result of analyzing only two broilers from each dietary treatment. Larger sampling would be required to determine if the variation remains the same. Panel ratings showed a higher preference for the low-fat control broilers in comparison to broilers of the other dietary groups. Cooking losses and TBA values did not appear to be related to preference ratings. This may be due to the use of broiler muscle tissue (which is relatively low in fat content) for panel analysis.

DISCUSSION

RESULTS of this study suggest that most of the dietary fats were deposited in the adipose tissue with minor changes in their fatty acid contents. When the fatty acid contents of the dietary fats are suitable for direct deposition, the chicken seems to do so to conserve energy expenditures as in the case of corn oil-, lard-, and beef tallow-fed chickens. The direct deposition theory was mentioned by Sell et al. (1968) in their studies of hens. In the case of the hydrogenated coconut oil-supplemented ration, direct deposition of this oil onto the depot fat seems to be limited and some fatty acid transformations might have occurred. Feigenbaum and Fisher (1959) noticed that coconut oil produced a profound change in body fat but no change of consequences in the egg fat. Mickelberry et al. (1966) concluded that coconut oil influenced the adipose lipid more than liver fat. However, if one compares the fatty acid contents of the adipose tissue of chicks fed a hydrogenated coconut oil-supplemented diet to that of the feed, there are more changes quantitatively than the adipose tissue from chicks fed corn oil-, lard- or beef tallow-supplemented diets.

The overall changes of the fatty acid contents in all adipose tissues favor the accumulation of palmitic and oleic acids. High contents of palmitic and oleic acids in all adipose tissues analyzed were noticed by Miller and Robisch (1969) as well. The reasons for this have not been elucidated. Bottino et al. (1965) used isotope tracers and concluded that the increase of linoleic acid in body tissue

Table 5—Percentage of solid fats and liquid oils in total lipids

Samples[a]	% fats (w/w)	% oils (w/w)
LF-2	40.80	59.20
LF-4	29.58	70.42
LF-6	23.37	76.63
Average	31.25	68.75
C-2	9.40	90.60
C-4	12.33	87.67
C-6	11.74	88.26
Average	11.16	88.84
B-2	39.32	60.68
B-4	45.61	54.39
B-6	37.31	62.69
Average	40.75	59.25
L-2	35.61	64.39
L-4	41.83	58.17
L-6	22.60	77.40
Average	33.35	66.65
H-2	69.80	30.20
H-4	89.17	10.83
H-6	88.42	11.58
Average	82.46	17.54

[a]For diet designation, see Table 2.

was at the expense of fatty acids of endogenous origin. In low-fat controls, no linoleic acid was found in depot fats by Machlin and Gordon (1961).

Separation of total lipids into neutral triglycerides and phospholipids have been studied by Katz et al. (1966) and by Marion et al. (1967). The fatty acid contents from muscle, skin, and depot fat were the same. When compared with phospholipids, the neutral triglycerides contained more palmitic, oleic and linoleic acid but less longer-chain fatty acids. In this study, less linoleic acid was found in neutral lipids than total lipids, indicating a higher content of linoleic acid in phospholipids. The fact that the unsaturation of fatty acid in adipose tissue was influenced by the amount of unsaturated fatty acids in the diet, especially with 18-carbon fatty acids, was stressed by Schuler and Essary (1971). The physical state of the depot fats does not seem to depend on either the individual fatty acid or the total amount of saturated or unsaturated fatty acids.

Two observations from the taste preferences and cooking losses of broilers from the experimental diets (Table 7) should be noted. Although the TBA values of skin and adipose tissue were higher for the low-fat treatment, the taste panel showed a higher preference for the breast meat from this treatment. Rancid odors were not detected on cooking or noted by panelists. It has been stated that at a TBA value of one, off-odors become detectable in meat and meat products (Watts, 1962). Secondly, if the percent drip loss is ranked from least to highest, the following order is obtained: H-6 < B-6 < L-6 < LF-6 < C-6. The same order is obtained if the percentage of liquid oil in the adipose tissue is ranked from least amount to highest amount (Table 5), suggesting a relationship between the solids-to-liquid ratio of tissue lipid and the amount of drip occurring during cooking.

Table 6–Fatty acid patterns of solid fats and liquid oils

	LF-6[a]		C-6		B-6		L-6		H-6	
	fat	oil	fat	oil	fat	oil	fat	oil	fat	oil
Saturated fatty acids										
8:0	–	–	–	–	–	–	–	–	–	0.04
10:0	–	–	–	–	–	–	–	–	0.84	1.18
12:0	–	–	0.03	–	0.07	–	–	–	38.93	28.34
14:0	0.02	0.63	0.55	–	1.87	0.64	2.62	1.90	14.73	11.88
16:0	59.20	32.32	51.85	19.10	30.02	29.06	31.28	26.95	23.89	18.69
18:0	4.38	3.54	3.77	2.22	16.85	4.97	14.80	8.43	4.08	4.43
Total	63.60	36.49	56.20	21.32	48.81	34.67	48.70	37.28	82.47	64.56
Unsaturated fatty acids										
14:1	–	0.03	–	–	–	0.02	0.09	0.22	0.08	0.88
16:1	2.60	6.15	0.27	1.37	2.82	2.46	3.02	3.31	1.05	3.38
16:2	–	–	–	–	–	–	–	0.03	–	–
18:1	33.80	54.49	33.51	33.10	44.74	57.69	47.33	56.32	16.18	26.56
18:2	–	2.84	10.02	44.21	3.63	5.16	0.86	2.84	0.22	4.62
Total	36.40	63.51	43.80	78.68	51.19	65.33	51.30	62.72	17.53	35.44

[a]For diet designation, see Table 2.

Table 7–Cooking losses, preference ratings, and skin and adipose TBA values of chicken fed different dietary fats[a]

Evaluation	LF-6	C-6	B-6	L-6	H-6	(LSD[d])
Total cooking loss, %	31.88	33.50	34.12	33.94	33.88	(3.90)
Cooking loss as drip, %	8.55	9.46	7.16	7.46	5.93	(3.95)
Preference ratings[b]	7.2	5.8	6.7	6.6	6.4	(1.90)
TBA values[c]						
Skin	7.68	6.57	4.94	5.14	3.68	(4.44)
Adipose	5.54	4.70	3.58	4.74	4.68	(4.29)

[a]For diet designations, see Table 2.
[b]Panel scores range from 1 = dislike extremely to 9 = like extremely.
[c]mg malonaldehyde per 1000g tissue.
[d]Least significant difference (LSD) among treatment means at $P \leq 0.05$.

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FOOD PROTEIN FROM MEAT AND BONE MEAL

SUMMARY—Studies were undertaken to develop a nutritious and aesthetically attractive protein concentrate from commercial meat and bone meal. Flotation in carbon tetrachloride was used to separate meat residues from the bulk of the bone. The meat residues had moderate nutritional merit in rat feeding trials. Further improvement was achieved by size separation or by base extraction. The finer fraction from size separation had a more favorable amino acid balance and gave better growth rates and protein efficiency ratios (PER) than did the unfractionated material. Growth rates increased markedly as the amount in the diet was increased to give 15 and 20% protein. It had more than an additive effect on growth and PER as a supplement to wheat flour or whole corn. Extraction with base improved amino acid balance, growth response and flavor. Addition of 9% of the base-extracted material to rice or biscuits had little effect on flavor; a gray-red color remained prominant. Overall yields of the base-extracted material starting with meat and bone meal were about 34%. The protein content of the product was about 88% on a dry weight basis. Its PER when 2% methionine was added was 75% of that of casein (without added methionine) in a rat feeding experiment. The cost of production is estimated at about $0.13/lb when allowance is made for methionine addition and for sale of residues from the production process as an animal feed supplement.

INTRODUCTION

THE RENDERING industry salvages various by-products from meat processing as a source of fat. These include viscera, fat trimmings, bone scraps and fallen and unsound animals from the livestock dressing process and shop-fat, bone and grease from stores and restaurants. After the fat has been removed by a combination of cooking and applied pressure, the remaining residue is ground and sold to the animal feed industry as a source of protein and minerals under the name "meat and bone meal." This constitutes a satisfactory utilization of the material but considering world protein shortages it was decided to determine if a good quality protein concentrate could be recovered from the meal for use in human food. Total annual production in the United States is in excess of two million tons. The composition of meat and bone meal and other animal by-product meals has been reported recently by Doty (1969). The amino acid composition of meat and bone meal indicates that approximately half the protein is derived from collagenous material. The viscera proteins present are of high nutritive quality as shown by Olson (1970). If the muscle and viscera protein could be separated from the collagenous proteins in the meal, the protein concentrate should approach meat in nutritional quality. Additional requirements are removal of the bulk of the bone, removal of hair, improvement in taste and improvement of physical properties. Fractions removed in recovering a protein concentrate for human use will still have value as animal feed.

MATERIALS & METHODS

MEAT AND BONE meal used in this study was obtained from commercial lots prepared by the Van Hoven Co., St. Paul, Minn. The proximate analysis of two lots is shown in Table 1. The bulk of the work reported here was done with Lot B.

A first step in preparing the meat and bone meal for fractionation was in all cases extraction of residual fat. In the preparation of smaller lots of material, this was done by overnight extraction in a Soxhlet apparatus with ethyl alcohol. In the preparation of lots of several pounds size, extraction was carried out on a batch basis using two extractions with 1.5 vol of 60–90°C petroleum ether and four extractions with ethyl alcohol.

Fractionation

Three fractionation techniques proved of value. One was flotation in carbon tetrachloride as developed by Criswell, et al. (1964). This involved adding the fat-free meat and bone meal to CCl_4 and collecting separately the materials that float and the materials that sink to the bottom. The float fraction (MBF) was rich in protein and served as the starting point for all further work. To make certain that all CCl_4 was removed before use in feeding studies, the MBF material was placed in a vacuum oven overnight at 40°C.

One technique of subfractionating the MBF material was by size fractionation with standard screens and a RoTap apparatus. The second technique of subfractionation was by extraction with 0.1N NaOH. This was done by adding 10 ml of 0.1N base for each gram of MBF, stirring, allowing to stand 0.5 hr, centrifuging and washing the precipitate twice with 10 ml portions of water, followed by a 10 ml wash which was neutralized with HCl before centrifuging. The precipitate was dried in an oven at 50°C, ground in a meat grinder and then with a mortar and pestle. In preparing the large lots of material used for feeding studies, handling problems were underestimated and material remained in basic conditions for as long as 7 hr. Washing was also less thorough than in the standard preparations.

Amino acid analyses

To prepare samples for amino acid analysis, 100 mg of material was refluxed for 18 hr in 100 ml of 6N HCl with carbon dioxide bubbling through the solution to prevent any contact with oxygen. The volume was reduced nearly to dryness several times on a rotary evaporator, with addition of water after each evaporation to aid in removing residual HCl. The final solution was made to volume and aliquots taken for Kjeldahl nitrogen determination and determination of amino acids. The amino acid

Table 1—Composition of two lots of meat and bone meal

Constituent	Lot A %	Lot B %
Moisture	5.7	2.6
Fat	12.0	10.1
Ash	29.2	27.8
Protein	54.0	59.5

Table 2—Flotation of two lots of meat and bone meal

	Percentage on moisture and fat-free basis			
	Lot A		Lot B	
	Float fraction	Sink fraction	Float fraction	Sink fraction
Percentage of original mix	57	43	60.0	40.0
Ash content	7.7	61.2	6.5	67.8
Protein content	86.4	32.0	90.4	31.6
Percentage of ash of original mix	14.3	85.7	12.5	87.5
Percentage of protein of original mix	78	22	81	19

Table 3—Amino acid analyses of MBF and of fractions obtained from it by screening

	Grams of amino acid per 16 grams nitrogen			
	MBF	Fractions by screening Lot B		
	Lot B	< 24 > 60-mesh	< 60 > 115-mesh	< 115-mesh
Lysine	6.3	4.0	3.7	5.5
Histidine	2.2	1.3	1.1	1.9
Ammonia	1.2			
Arginine	6.6	6.6	6.7	6.1
Aspartic acid	7.3	6.1	7.2	7.7
Threonine	3.3	2.7	3.2	3.5
Serine	3.7	3.4	3.8	3.5
Glutamic acid	12.6	11.3	12.3	12.6
Proline[a]	7.0	7.8	7.8	6.6
Glycine	12.0	12.9	12.7	10.1
Alanine	7.0	7.0	7.1	6.6
Half-cystine	0.9	0.8	0.8	0.9
Valine	4.3	4.0	4.2	4.6
Methionine	1.5	1.3	1.4	1.6
Isoleucine	2.9	2.8	2.9	3.3
Leucine	6.3	5.4	6.1	6.6
Tyrosine	2.5	2.5	2.5	2.6
Phenylalanine	3.4	2.9	3.1	3.5
Tryptophan	0.5	0.4	0.5	0.8
Total	91.5	83.2	87.1	88.0

[a]Includes hydroxyproline

Table 4—Fractionation of MBF material by particle size

Fraction (mesh size)	Percentage of original MBF
> 24	5
< 24 > 60	22
< 60 > 115	25
< 115	48

Table 5—Effect of base extraction on amino acid analysis of MBF

	Grams per 16 grams N		
	MBF Lot B	Base extract	Residue
Lysine	6.3	5.3	5.5
Histidine	2.2	1.5	2.0
Ammonia	1.2	–	2.5
Arginine	6.6	8.8	5.8
Aspartic acid	7.3	5.9	7.4
Threonine	3.3	2.1	3.4
Serine	3.7	3.0	4.2
Glutamic acid	12.6	11.1	12.4
Proline[a]	7.0	9.1	3.7
Glycine	12.0	16.2	9.7
Alanine	7.0	7.0	6.5
Half-cystine	0.9	0	1.3
Valine	4.3	2.7	5.0
Methionine	1.5	1.0	1.6
Isoleucine	2.9	1.8	3.3
Leucine	6.3	3.8	7.0
Tyrosine	2.5	1.7	3.5
Phenylalanine	3.4	2.0	3.6
Tryptophan	0.5	0.1	0.9
Total	91.5	83.1	89.3

[a]Includes hydroxyproline

analyses were conducted on an amino acid analyzer by Dr. Irvin Liener of the Dept. of Biochemistry, University of Minnesota.

Tryptophan analyses were made using 21.4N H_2SO_4 and p-dimethylaminobenzaldehyde to effect color development (Spies and Chambers, 1949). An 18-hr period was allowed for development. $NaNO_3$ solution was added to complete color development. Corrections were made for color developed by H_2SO_4 with the protein in the absence of p-dimethylaminobenzaldehyde.

Feeding trials

Feeding trials were conducted using weanling male rats of the Sprague Dawley strain. Animals were individually housed and fed; water was available ad lib. Feed wastage was minimized by use of a floating disk with six half-inch holes to cover the feed in the feed cups. Daily weighings were made of feed consumption; weekly or twice weekly weighings were made of the rats. Eight rats were used in each dietary group.

Diets were mixed to contain the desired protein level, 8% fat (cottonseed oil), 5% minerals ("Jones and Foster Salt Mixture" from Nutritional Biochemicals Corp., Cleveland, Ohio), 1% fiber ("Alpha Cel") and vitamins (2% of "Vitamin Diet Fortification Mixture" from Nutritional Biochemicals). The fat, fiber and ash content of the protein source was taken into account in determining the amount of cottonseed oil, cellulose, or minerals to be added. Corn starch was used as a variable in bringing the diets up to weight. DL-methionine was added to all diets except the casein control in an amount equivalent to 2% of the protein. The objective of keeping fat, fiber and mineral content constant had to be slightly compromised in diets that used wheat and corn as protein sources because the low protein content of these materials reduced flexibility. The wheat flour used was one manufactured for general home use by Old Fashioned Millers Inc., St. Paul, Minn. It was sold under the brand name, "Big Shot." The corn was obtained from a local farmer and ground in the laboratory.

In calculating protein consumption and protein efficiency ratios, values of nitrogen content obtained analytically on the mixed feed were used as a basis for calculation. Nominal values of protein content were high in several instances because of greater than expected moisture gain. A factor of 6.15 was used in converting nitrogen to protein contents except for wheat and corn, where a factor of 5.70 was used.

RESULTS

Fractionation

CCl_4 flotation. Results of flotation of samples of two lots of meat and bone meal are given in Table 2. In each case, the material used had been subjected to drying and fat extraction. It is seen that about 59% of the weight floated and that the float fraction contained about 80% of the protein and 13% of the ash of the original meat and bone meal.

Screening. The meat and bone meal had been subjected to hammer-milling to effect size reduction by the manufacturer as part of his normal processing operations. It was noted microscopically that the finest particles of the MBF fraction had a different appearance than the coarser materials in that they were much more irregular in outline and had less sheen. It was therefore hypothesized that they might differ qualitatively. To test this hypothesis, a sample was graded for size and key fractions subjected to amino acid analysis. Values obtained are shown in Table 3, together with values obtained on the starting MBF material. The fractions were obtained from Lot B. The yield of the several fractions is shown in Table 4. In a larger scale preparation of fractions from the same starting material, 52% of the total was recovered in the < 115 mesh fraction. Inspection of the amino acid analysis data in Table 3 shows that the amino acid balance in the finest fraction is considerably more favorable than in the two coarser fractions. This is as would be expected if the coarser fractions contained more connective tissue material. The finest fraction had appreciably higher levels of tryptophan, lysine and histidine and moderately higher levels of phenylalanine, leucine, isoleucine, valine, methionine and threonine. Levels of proline and glycine are lower in the finer fraction than in the others.

Analytical data on the size fractions are consistent among fractions but inconsistent in some respects with analyses on the starting materials. The worst incon-

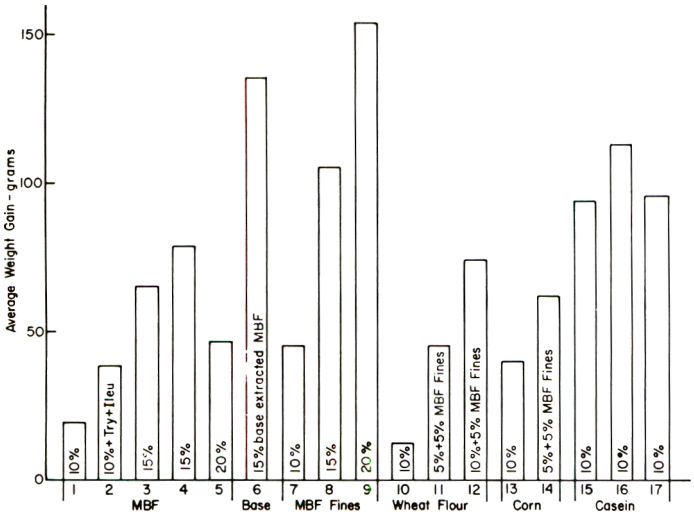

Fig. 1–Effects of dietary protein variations on growth rate of rats. All diets except casein diets had added methionine. Percentage figures refer to percentage of protein in the diet from the indicated source.

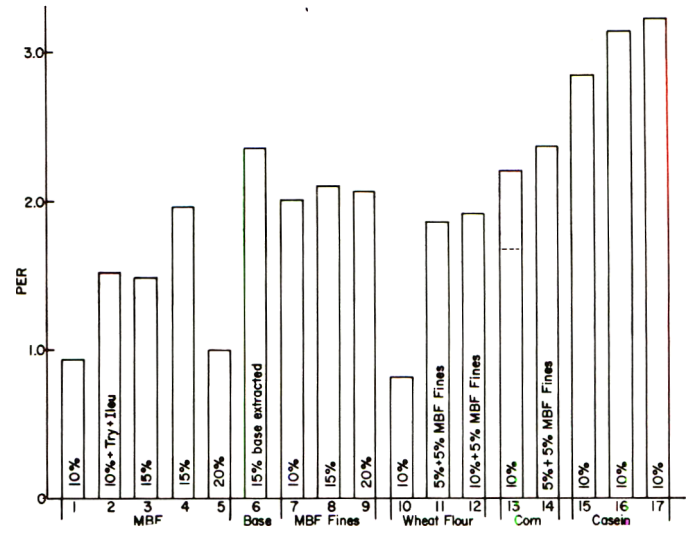

Fig. 2–Effects of dietary protein variations on protein efficiency ratio (PER) in feeding experiments with rats. All diets except casein diets had added methionine. The dotted line in Bar 13 retains two rats that were otherwise rejected as atypical because of a low feed consumption and lack of weight gain.

sistencies are in lysine and histidine, and it is suspected that the values for these two amino acids are in error in the analysis of Lot B of MBF. There are minor inconsistencies in the analyses on glutamic acid.

Base extraction. Extraction of MBF with base solution was initially undertaken as a means of removing undesirable flavors and is discussed in more detail in a later section. It was found in feeding trials that the base-extracted material gave an unexpectedly good growth response. Analysis of the residue and of the extract showed that the base extraction had indeed removed material of low nutritional quality, with consequent enhancement of the quality of the residue. Results of amino acid analyses are given in Table 5, together with a repetition of analysis on the starting MBF, for ease of comparison.

For the most part, changes in composition as a result of base extraction are internally consistent. Exceptions are lysine, histidine and glutamic acid. It is believed these lack consistency because of incorrectly high values for the starting MBF material. It is thought that, in actuality, the extraction caused little change in lysine and a slight increase in histidine. The most dramatic changes have been marked decreases in proline (+ hydroxyproline) and glycine concentrations and a marked increase in tryptophan concentration. Other changes are decreases in arginine and alanine, and increases in threonine, serine, half-cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. The fraction removed has a collagen-like amino acid composition.

Yields of residue after base-extraction have varied from 65–85% in 14 measurements. Preparation of two large lots (500g) gave yields of 65 and 68%. These lots were exposed to basic conditions for 6 hr, so yields may be low for that reason. Several small-scale preparations (2g quantities) have given recoveries of 73, 68, 70 and 70%. The higher yields were obtained using procedures involving less thorough washing. The effect of time of exposure to basic conditions on amount of material extracted with base was explored using the "fines" fraction. Yields were corrected for the part of the total loss that was due to changes in moisture content. Results are given in Table 6. They give little evidence that losses are increased by moderately prolonged exposure to basic conditions.

An experiment was conducted to determine the amount of material that could be extracted from MBF that had been carried through the entire base extraction and washing procedure. The material used was from one of the larger scale preparations and had involved a 32% loss of material in the initial base extractions. Carrying out the base extraction procedure a second time resulted in an additional 7% loss.

Analysis of two lots of base-extracted material have shown 81 and 81% protein as equilibrated with the room atmosphere and 86 and 90% protein on a dry weight basis. The ash content was 12.0 and 11.8% on a dry weight basis.

Hair removal. To produce an aesthetically acceptable product, hair must be removed. Removal of longer hairs is readily accomplished by screening or combing techniques but removal of short hairs is a more difficult problem. Removal by density differences was investigated and found unsuitable. A broad spectrum of densities were found to be present. Electrostatic removal showed some promise. The most successful technique was sequential screening. A fine fraction (< 120 mesh) could be recovered with very few hairs if only moderate vibration on a screen was used. The bulk of the hairs from the coarser fractions could be removed by causing the fraction to roll gently across a screen coarse enough that the rounded particles would readily drop through. Hair passes through much less readily. Cleanup can be accomplished by vigorous shaking of these coarser fractions on screens that they will not normally pass. The hair fragments upend and pass through lengthwise. In trials of such sequential screening, 91% of the MBF material was recovered in fractions containing 4.8% of the hairs on a number basis. The fraction of hairs retained on a weight basis was much less since the longer hairs were completely removed.

Table 6–Extraction of MBF materials with 0.1N NaOH

Exposure time min	Percent solubilized MBF– < 115-mesh
10	22
20	21
30	21
60	21

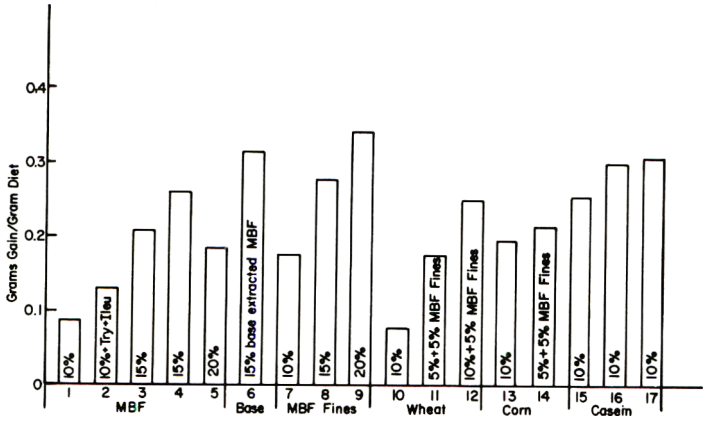

Fig. 3—Effects of dietary protein variations on the amount of weight gain per gram of diet in rat feeding studies. All diets except casein had added methionine.

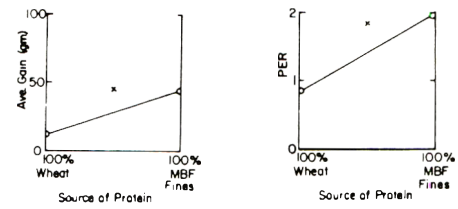

Fig. 4—Effects of substituting MBF fines for part of wheat protein on growth and protein efficiency ratio. Performance of 50—50 mixture indicated by X.

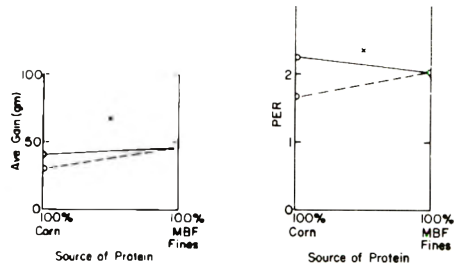

Fig. 5—Effects of substituting MBF fines for part of corn protein on growth and protein efficiency ratio. Performance of 50—50 mixture indicated by X. The dotted lines retain two animals that were otherwise discarded as atypical.

Physical properties

Solubility. Since one is dealing with a heterogeneous mixture of materials, solubility measurements have a different meaning than do solubility measurements on a pure compound. In many cases they measure the fraction of the whole that is of a particular class of structures rather than measuring solubility of the whole per unit weight of solvent.

Solubilities in water were determined as a function of temperature using 100 mg aliquots of MBF material and 50 ml of water. Solubility increased as a straight line function from 21% at 25° to 27% at 100°C. Solubility as a function of pH showed a minimum at pH 4 (10%) with increase to 19% at pH 2 and to 32% at pH 13. Solution occurred only very slowly and incompletely in 18 hr in 1N NaOH at 40°C. Solubility in 6N guanidine hydrochloride was less than 10% even after 3 days at room temperature.

Taste. MBF had a taste like that of overripe meat and would surely be judged objectionable by many people. Attempts to improve flavor included coating with gums, fats and zein, enzyme treatment, and extraction with acid, base and organic solvents. Extraction with base was by far the most effective treatment. Extraction with 0.1N HCl gave considerable improvement and even extraction with water was of some benefit. Base of 0.1N concentration was more effective than 0.05N base but more concentrated solutions, or longer periods of exposure than 10 min, or repeated extractions with base, or extraction with base and then with acid did not give further improvement. Almost no residual taste could be detected after extraction with base in coarsely ground material. Fine grinding uncovered some taste, which might be described as a chicken soup flavor.

An additional component of "taste" is mouth feel. Because it is insoluble and relatively nonhydroscopic, MBF has a degree of sandy feel. This is partially removed by fine grinding. It is unaffected by base extraction. The use of additives to remove the sandy feel in the mouth was investigated. Coating with guar gum or gum karaya was partially successful as was addition of disodium hydrogen phosphate. Coating with casein, soy protein, soy flour, soluble starch or a hydrogenated fat were not beneficial.

Nutritional value

Results of the three feeding experiments conducted are shown graphically in Figures 1, 2 and 3. These summary graphs include groups from all three feeding trials and it is not necessarily true that results from one feeding experiment to another are strictly comparable, but the replication was at least fairly good in instances for which data are available. Bars 15, 16, and 17 in the figures represent replication of the 10% casein diet; Bars 3 and 4 represent replication of the 15% MBF diet.

Animals receiving 10% protein from MBF made poor gains (Fig. 1), showed poor protein efficiency ratios (PER) (Fig. 2) and poor feed efficiency (Fig. 3). All were improved by increasing the protein content of the diets to 15%, but a further increase to 20% gave poorer performance than was attained at 15%. A level of 15% protein in the diet may represent the limit to which quantity of protein can substitute for protein quality with this material. An attempt to improve quality by adding tryptophan and isoleucine met with limited success. It is to be noted that methionine was added to all diets except the casein control diets.

When the fines fraction (< 115-mesh) of MBF was used as the protein source, better growth, PER values and feed efficiency were attained at all concentrations used than when the original MBF fraction was used. The PER value was raised to about 2.0. The growth rate was markedly greater when the amount of protein from the fines fraction was increased from 10 to 15 to 20% of the diet. 15% MBF fines protein gave a growth response similar to 10% casein protein. No differences in PER values were seen as protein level of the diet was increased using the fines fraction.

Base-extracted MBF material was used in only one diet and that at the 15% level. It gave the second highest rate of gain seen among the diets. Only MBF fines at the 20% level gave a greater rate of gain. The protein efficiency ratio was 2.35. It was exceeded only by casein.

In each case, the higher levels of gain were associated with high levels of feed intake.

The experiments with wheat flour and ground corn were undertaken to find if MBF fines have an additive effect on response when they are used together. It

is seen that both in the case of corn and wheat, use of 5% cereal protein and 5% of MBF fines protein has given a better response than when 10% of cereal protein was used alone. The relationships can be seen graphically in Figures 4 and 5. Wheat flour alone gave very poor rates of gain and poor protein efficiency ratios. Substituting MBF fines for half the wheat flour protein gave marked improvement in both criteria of performance. Performance was as good as with the same total amount of protein in the form of MBF fines. When 5% MBF fines were added to a wheat flour diet instead of being substituted for part of its protein, a further increase in growth rate was obtained. The PER value was increased only insignificantly. (See Fig. 1 and 2.)

Ground whole corn has given as good performance as MBF fines at the same protein level. The substitution of MBF fines for half the protein of ground corn has significantly increased the growth rate and given a PER value insignificantly better than ground corn.

Microbiology

It was expected that the combination of solvent extraction to remove fat and extraction with 0.1N base would kill any microorganisms present. This proved not to be the case. A relatively large fraction of both vegetative forms and spores survived. Alcohol extraction was the most effective treatment.

Food formulations

MBF and base-extracted MBF were tried in two food formulations. Rice and "Bisquick" (a commercial biscuit mix) were chosen as examples of a boiled and a baked food product of relatively bland taste and smooth texture in which any undesirable contributions of meat and bone fractions would be apparent. In all trials, meat and bone products were added at the 9% level by weight.

Additions to rice before cooking caused remarkably little change in taste. The base-extracted material changed the taste and aroma almost not at all. The material which had not been base-extracted contributed a slightly meaty taste. MBF fractions contributed a light brown color. A slight sandy feel was evident. The use of base-extracted MBF coated with guar gum eliminated the sandy feel and gave a product distinguishable from rice alone only in color.

Addition of the meat and bone fractions to Bisquick and then baking gave a clear-cut result. Use of MBF material that had not been base-extracted yielded a product of quite unacceptable taste. Use of base-extracted MBF had only a very slight effect on taste. The biscuits were bland; the only discernible effect on taste was masking of one of the favorable flavors appearing in biscuits to which additions had not been made. The flavor component so affected might be described as a buttered taste. Sandiness was not discernible. The base-extracted MBF fraction contributed a gray color.

DISCUSSION

ALTHOUGH it has some obvious limitations, the product obtained by flotation to remove the bone, screening to remove the hair, and base extraction to remove disagreeable tastes does show definite promise as a protein supplement for human use. It is of high protein content, nearly tasteless when used in cooked foods, is of fair nutritional quality, and supplements cereal proteins in more than an additive manner. The basis for the statement on successful supplementation of cereal proteins rests on work actually done with the fines fraction of MBF, but the base-extracted material gave an even better growth response in work done on it and appears to have a slightly better amino acid pattern than the fines fraction. Probably the greatest limitation of any MBF fraction in use in human foods will prove to be its color. Its color is sufficiently intense that it is likely to change the appearance of all foods except those that are initially highly colored in themselves.

The objective of the present study has been to determine whether a meritorious product for human use can be salvaged from the residues from the standard rendering process. Alternate approaches to salvaging protein from slaughtering and meat trimming wastes might avoid exposure to the heat of the usual rendering operation with its deleterious effects on physical properties. With some investment of labor, the more desirable and acceptable wastes for processing as a source of protein for human consumption could be kept separate from the less desirable ones.

Fractionation

The separation of bone and meat materials to give a fraction of high protein and low ash content (MBF) by flotation in CCl_4 worked very well indeed. Separation was accomplished in a few minutes even without application of centrifugal force. Hair removal by sequential screening seems reasonably efficient and should be accomplished even more expeditiously using commercial equipment.

Subfractionation of the MBF material to improve protein quality has been successfully achieved by two techniques. Fractionation by size achieved a remarkable degree of improvement in quality and would be worth further pursuit if it were not deemed essential that base extraction be conducted to remove unpleasant flavors. The base extraction in itself achieves an equally great or possibly greater improvement in protein quality and gave a better yield. It is, of course, true that further work might improve the yield of good quality material by size fractionation techniques. The yield of material of good protein quality taking the particular split that we explored was about 50% of the MBF fraction. The yield obtained by base extraction was about 70% of the MBF fraction.

Physical properties

The physical characteristics of MBF and its subfractions are those of a protein that has undergone denaturation and probably some cross-linking. It is largely insoluble in even such effective protein dispersants as guanidine hydrochloride or strong base. There is, at the same time, a fraction that amounts to from 20–30% of the MBF that is fairly readily soluble in acid or base or even in water. This fraction, as determined from analysis of the material extracted by base, is similar to collagen in amino acid distribution.

The most undesirable facet of the low solubility is the sandy texture that may be contributed to food formulation by an insoluble, nonhygroscopic ingredient. This property is, however, present in only moderate degree in finely ground material and is efficiently masked by 10% guar gum. Perhaps the physical property which will prove most limiting in the long run is the fairly intense grayish-brown color.

The taste of the MBF material without further treatment is considered probably too flavorsome for most palates. Even this strong flavor does not, however, come through strongly in some cooked foods. It does show up very adversely in others. It was only noticeable in cooked rice but was highly objectionable in biscuits made from Bisquick and MBF. Extraction of MBF with acid or even with water lowered the flavor intensity in some degree. Extraction with base proved even more successful and lowered the intensity to about that of a chicken soup. When base-extracted material was used in either rice or biscuits, the flavor of these foods was scarcely modified at all. Repeated extraction with base or extraction with base and then with acid did not further reduce the flavor in any impressive degree.

Nutritional value

In summarizing the nutritional value of the MBF material itself, one must conclude that it has less value as a protein source than does casein. This is true whether one looks at growth rates or at PER values. Increasing the percentage of MBF in the diet is only moderately helpful and at the 20% protein level appears to be deleterious. The protein quality of the MBF fraction appears to lie between that of the whole corn and

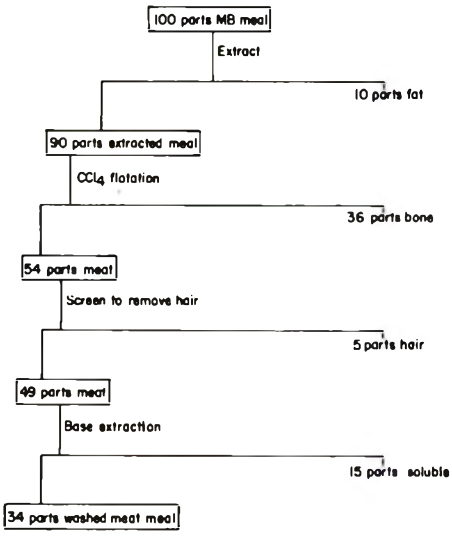

Fig. 6—Material balance in process for production of base-extracted MBF.

wheat flour materials we examined. A considerable improvement is achieved by using only the fines fraction from MBF. The improvement is evident both in growth response and PER values. The improvement would be forecast by inspection of the amino acid analysis itself. One can still not conclude that the fines fraction is equivalent to casein in protein nutritional value, but it is of respectable value and improved growth responses were obtained as the amount in the diet increased, at least up to a 20% protein level. Substituting it in wheat flour or ground corn diets for an equivalent amount of protein resulted in greater than additive effects. Adding it to wheat flour to enrich the total protein content of the flour resulted in an even greater effect on growth rate. Experience is less extensive with the base-extracted material but it did excellently in the single group in which it was used. The PER value was 78% of that of casein. The good response seen in the experiments in which it was added to the diet is reinforced by its amino acid analysis. Its amino acid pattern is even more favorable than that of the MBF fines fraction and the extract had the pattern of a very poor quality protein.

In judging the usefulness of a protein concentrate for human nutrition, the PER values give an indication of the efficiency with which a unit weight of the protein will be utilized. If, however, the protein is not so badly unbalanced that further enrichment of the diet with the protein source will result in a deleterious response, then the protein efficiency ratio is principally a factor that should be applied to the price of the protein in comparing it with other patterns. A protein of lower protein efficiency ratio must be used in larger quantity to achieve the same nutritional effect. The markedly greater growth rates seen as the MBF fines were added in increasing concentration gave promise that either the fines fraction or the base-washed material could be used in larger amounts to offset some deficiency in quality. The success of the MBF fines fraction in supplementing wheat flour and corn indicates usefulness in supplementing cereals generally. This is an area of general need. In comparing the amino acid analysis of the MBF fines fraction and the wheat and corn used in the diets, it seems likely that the supplementary effects came principally from the lysine content of the MBF fines fraction.

It is to be noted that in all cases the rate of gain and total food intake are well correlated. The animals that gained more did so because they ate more food. This could be a palatability factor, but it is a common observation that animals respond to feed quality by eating more of a high quality feed and less of a poor (Albanese, 1959).

Process considerations

The process steps and expected yields for the production of MBF base-extracted material are represented in Figure 6. Range-finding economic estimates show the cost of producing the base-extracted material to be about 18¢/lb, starting with meat and bone meal costing 4.5¢/lb. If allowance is made for resale of the by-products at 3.5¢/lb, the cost of producing the base-extracted protein concentrate is reduced to about 11.5¢/lb. Addition of methionine would increase the cost to about 13¢/lb and addition of guar gum, to 15¢/lb.

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USE OF GAS-LIQUID CHROMATOGRAPHY FOR QUALITY EVALUATION OF DRY EGG PRODUCTS

SUMMARY—Egg white solids and dried whole eggs were stored at 5, 24 and 43°C and analyzed and evaluated at regular intervals. Changes in aroma and flavor of dry egg products with storage at different temperatures have been recognized for many years. These flavor changes, indicative of egg product quality, could be followed with GLC headspace techniques and correlated with baking performance of the ingredient. Total peak area increased with time and temperature of storage while baking performance decreased. Changes in carbonyl composition as well as free fatty acid composition were also investigated and found to be indicative of baking performance.

INTRODUCTION

MAJOR ATTENTION of research in the field of dry egg products appears to have been directed towards improvement of storage life as indicated in a review by Lightbody and Fevold (1948). A comprehensive review of the literature and discussion of egg dehydration was presented by Bergquist (1964).

Dried egg products are used in many areas of food processing. The effect of moisture level, composition of the headspace gas and storage temperature on the shelf life of the products have been reported. Banwart and Ayres (1956) found that it is possible to store dried albumen containing 1.5, 3 or 6% moisture at 60 or 70°C for 90 days without significant change of functional properties, including angel food cake-baking properties. Carlin and Ayres (1951) held egg white solids at 22°C for 16 wk without detrimental effect on angel food cake-baking properties; at 40°C cake volume decreased 5% after only 2 wk. Baldwin et al. (1967) reported no loss of functional properties of spray-dried egg whites during 60 days at 54°C. Hawthorne (1943) used flavor changes to express product deterioration. The flavor score of egg products decreased by 36–75% from the initial score when stored at 98°F. Increased moisture levels at temperatures between 55–100°F resulted in an increased rate of deterioration as reported by White and Thistle (1943). Low moisture levels have been found to increase the shelf life of the product (Pearce et al., 1946).

Rolfes et al. (1955) reported that freeze drying had no detrimental effect on functional properties of egg white solids, but that spray drying increased beating times and slightly decreased cake volume. Brown and Zabik (1967) found that exposure time of egg albumen to preheating, spray drying, or high temperature storage brought about the following significant changes: decrease in surface tension, increase in specific gravity of both egg albumen foam and angel food cake batter, decrease in foam stability and decrease in volume of angel food cakes.

Kline et al. (1951a, b) identified a glucose-lipid interaction as the principal cause of off-flavor development of whole egg powders and reported in a later publication (1953) that acidification of whole egg powder retards glucose-induced phospholipid and protein deterioration.

Bennion et al. (1942) attempted to correlate results of a beating test and the baking quality of whole egg solids. Thistle et al. (1943) reported a fair correlation of the beating test with other quality tests such as fluorescence, solubility and pH. In each case, however, a baking test was required to establish the baking quality of the egg products with certainty.

Headspace vapor analysis of foods and food ingredients would eliminate the need for time consuming chemical, physical and rheological measurements. The possibility of using this technique for the quality evaluation of dry egg products was explored as well as changes in carbonyl composition and fatty acid composition as possible quality indicators of baking performance.

EXPERIMENTAL

STORAGE STUDIES were conducted using commercially available pasteurized dried whole egg solids and egg white solids. The whole egg solids were received in a cardboard container and transferred to screw-top glass jars, 5 lb per jar. The egg white solids were received in small cardboard containers, 3 lb per container and stored in these containers. Samples of these dry egg products were stored at 5, 24 and 43°C for 4 months. At regular intervals, moisture and pH of 3% suspensions were determined of samples stored at different temperatures.

Determination of total carbonyl compounds

Carbonyl compounds were extracted with chloroform and determined by the method of Lappin and Clark (1951). The optical density of the hydrazones was measured at 480 mμ using a Bausch and Lomb Model 20 spectrophotometer. A standard curve was prepared using butanal as the standard and results were expressed as ppm relative to the color of that standard.

Headspace volatiles

2 ml of water, 1.2g of sodium sulfate and 0.5g of the egg white solids were placed into 5 ml serum vials closed with rubber septa. These vials were heated for 15 min in a 60°C water bath and a 1 ml vapor sample was injected into the gas chromatograph.

This procedure was simplified for whole egg solids. 10g of whole egg solids were placed into 125 ml Erlenmeyer flasks which were stoppered and stored at 5, 24 and 43°C. At regular intervals, 2 ml vapor samples were taken from these flasks and injected into the gas chromatograph. A Hewlett-Packard Model 5750 gas chromatograph equipped with an 8-ft long, 1/8 in. OD column was used. The liquid phase was Carbowax 20 M (weight % 20) and the solid support was Chromosorb P (AW-HMDS) with a mesh size of 60/80. The carrier gas was nitrogen with a flow rate of 15 ml per minute. Column temperature was 98°C isothermal, injection port 195°C and the detector 245°C. The total area under the peaks was calculated by multiplying the height of each peak by its width at one-half height and adding the individual peak areas.

Fatty acid composition

10g of the dry egg products (whole egg solids and egg white solids) were extracted for 30 min with 100 ml of a mixture of 60:40 petroleum ether-diethyl ether. The ether extract, along with 100 ml of 1% conc sulfuric acid in methanol, was placed in a 300 ml round-bottom flask and refluxed for 1-1/2 hr. After cooling, the condenser was rinsed with 50 ml of distilled water. The total mixture was saturated with sodium chloride and transferred to a separatory funnel to collect the ether layer containing the methylesters. That was followed by three additional extractions with 5 ml of petroleum ether. All ether extracts were combined and evaporated to 10 ml final volume. Sodium sulfate was added to absorb any residual moisture.

Resolution and identification was accomplished using a Hewlett-Packard Model 5750 gas chromatograph equipped with a dual column hydrogen flame ionization detector. The column was 8 ft long, 1/8 in. OD packed with 60/80 mesh Chromosorb G AW-DMCS and coated with 7.5% HI-EPP 1 BP (diethylene glycol succinate). Injection port temperature was 195°C, column temperature 175° isothermal and the flame detector 265°C. The total peak area was calculated by multiplying the height of each peak by its width at one-half height and adding the individual peak areas.

Identification of the individual fatty acids was made by measuring relative retention times of pure commercial fatty acids separated under the same gas chromatographic conditions.

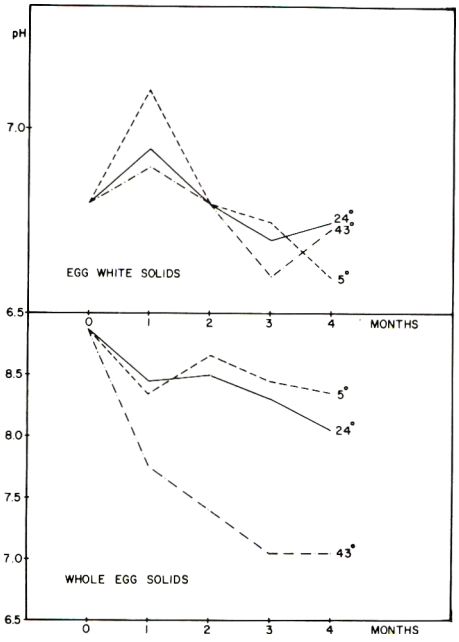

Fig. 1—Changes in pH with storage at different temperatures (3% suspensions).

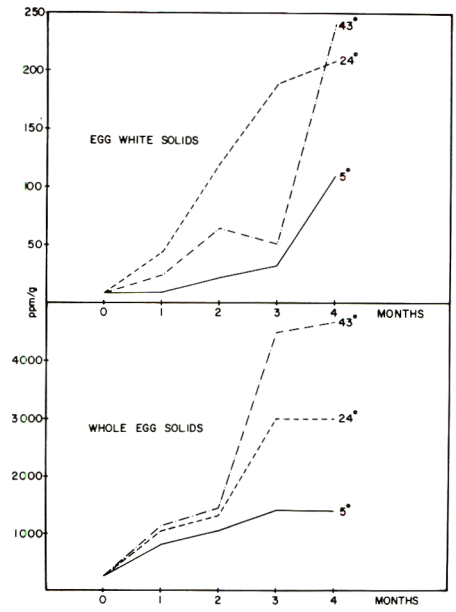

Fig. 2—Changes in total carbonyl content (ppm/g) with storage at different temperatures.

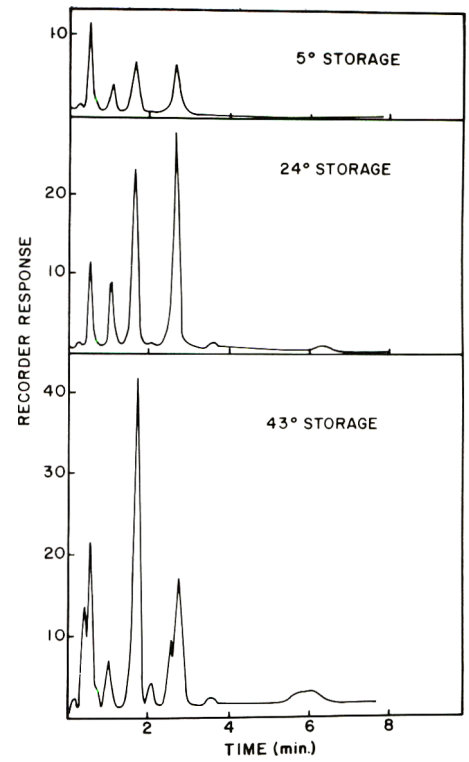

Fig. 3—GLC headspace profiles of whole egg solids stored for 4 months.

Cake preparation

The standard cake formula consisted of 216g cake flour, 275g sugar, 10g baking powder, 7.6g salt, 154g liquid shortening, 14g nonfat dry milk, 370 ml water and 85g whole egg solids for yellow layer cakes or 56g egg white solids for white cakes. The dry ingredients were sifted into a 12-qt bowl of a Hobart A-120 mixer. The liquid shortening and water were added and the batter mixed for 2 min at speed 3 followed by 3 min at speed 2 using a wire whip. 400g batter were poured in 8 in. paper-lined cake pans and baked at 375°F for 20–23 min. The cakes were evaluated approximately 1 hr after baking. External and internal cake characteristics such as volume (15), crust color (5), symmetry (10), character of crust (5), grain (15), crumb color (10), aroma (10), taste (20) and texture (10) were scored. (The number in parenthesis indicates the maximum number of points for each characteristic.)

RESULTS & DISCUSSION

THE MOISTURE CONTENT of dry egg samples has been found to have a significant effect on storage stability (Pearce et al., 1946) and, therefore, has been included in this investigation. Forsythe (1964) reported moisture contents of 14% max for albumen and 5% max for whole egg solids.

Our samples were below these values as they arrived and decreased in moisture with time and temperature of storage (Table 1). A storage temperature of 5°C resulted in the least loss of moisture while storage at 43°C resulted in maximum moisture loss of whole egg solids as well as egg white solids. The changes in pH of 3% suspensions of egg white solids increased slightly after 1 month storage at each storage temperature but then decreased with time. The final pH value after 4 months' storage was approximately the same for all samples and only slightly lower than the original pH. 3% suspensions of whole egg solids underwent more drastic pH changes with time indicating changes in chemical composition. The pH of whole egg solid samples decreased with time. The changes at 5°C of storage were slight while the pH changes at 43°C were considerable (Fig. 1).

Total carbonyl content

Egg white solids. The total carbonyl content (ppm/g) of egg white solids in-

Table 1—Effect of time and temperature on moisture content of dry egg products

	Moisture of egg white solids (%)			Moisture of whole egg solids (%)		
	Storage temperature			Storage temperature		
Time (months)	5°C	24°C	43°C	5°C	24°C	43°C
0	10.2	10.2	10.2	4.8	4.8	4.8
2	9.4	5.4	4.1	3.7	3.0	3.0
3	7.3	5.2	3.8	3.7	3.0	2.0
4	7.1	4.9	3.3	3.9	3.3	2.4

Table 2—Effect of storage time on cake quality and total carbonyl content

	Whole egg solids[a]			Egg white solids[a]		
Storage time (months)	Yellow cake specific vol.	Cake score	Total carbonyl content (ppm/g)	White cake specific vol.	Cake score	Total carbonyl content (ppm/g)
0	3.85	88	250	3.49	92	8
1	3.79	81	1160	3.43	88	44
2	3.44	75	1320	3.45	80	105
3	2.96	70	3000	2.91	72	190
4	2.98	69	3000	2.86	71	210

[a]Samples stored at 24°C.

creased gradually with time at each temperature of storage (Fig. 2). At 5°C storage the changes in total carbonyl content were slight for the first 3 months. During the fourth month the total carbonyl value more than tripled. The total carbonyl value of egg white solids stored at 24°C increased more rapidly with time than that of the sample stored at 5°C. After 4 months, storage at 24°C produced approximately twice the total carbonyl value as storage at 5°C. Storage at 43°C produced the highest final total carbonyl value of all samples.

Whole egg solids. The initial and final total carbonyl values of whole egg solids were considerably higher than those of egg white solids at each time and temperature of storage. Storage at 5°C produced the lowest final total carbonyl value while storage at 43°C produced the highest value (Fig. 2).

It was possible to correlate total carbonyl value and baking performance of the egg white solids and whole egg solids. The higher carbonyl values after extended storage times coincided with decreased specific cake volumes, a more open and nonuniform grain, and a harsher texture. Our results indicated that for satisfactory baking performance the total carbonyl value should not exceed 100 ppm/g in egg white solids or 1200 ppm/g in whole egg solids. Table 2 shows the decrease in specific volume and in total cake score as the total carbonyl content of the egg products increased.

Headspace profiles

Whole egg solids. Headspace profiles of whole egg solids stored for 4 months at 5, 24 and 43°C are shown in Figure 3. Total GLC peak area increased with time and temperature of storage and was indicative of the baking performance of whole egg solids. Yellow layer cakes which were baked from the stored whole egg solids at the same time the samples were analyzed by gas-liquid chromatography are shown in Figure 4. Cake No. 1 was baked from whole egg solids stored at 5°C. The sample stored at 24°C was used in Cake No. 2 and that stored at 43°C was used to bake Cake No. 3. With our formula and procedure a total GLC peak area of 1.7 cm^2 produced a very good cake (Cake No. 1) while an area of 5.8 cm^2 produced a poor cake (Cake No. 3 in Fig. 4). Critical total peak areas will have to be established for each cake formula and procedure.

Egg white solids. Headspace profiles of egg white solids stored for up to 4 months at 5, 24 and 43°C permitted the same conclusion. Total GLC peak area increased with time and temperature of storage and was indicative of the baking performance of the egg white solids in white cakes and angel food cakes.

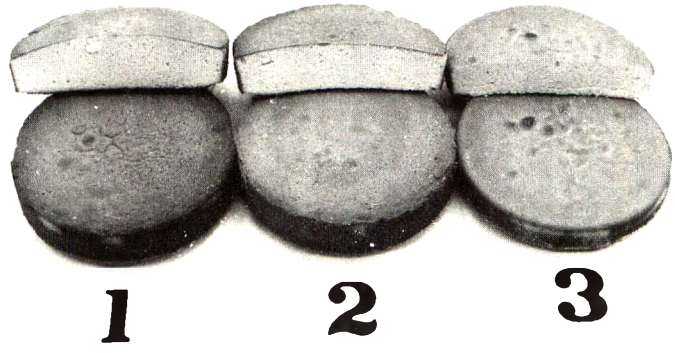

Fig. 4–Yellow cakes baked with whole egg solids stored for 4 months at 5°C (Cake No. 1), 24°C (Cake No. 2) and 43°C (Cake No. 3).

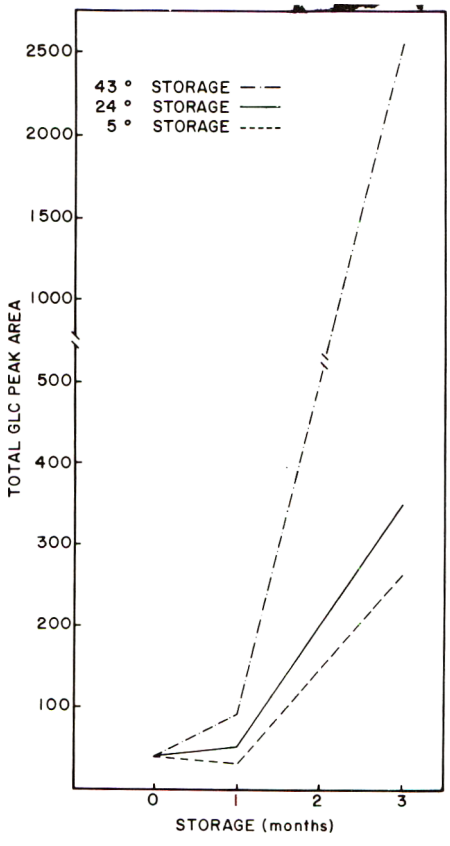

Fig. 5–Effect of time and temperature on the fatty acid content of egg white solids.

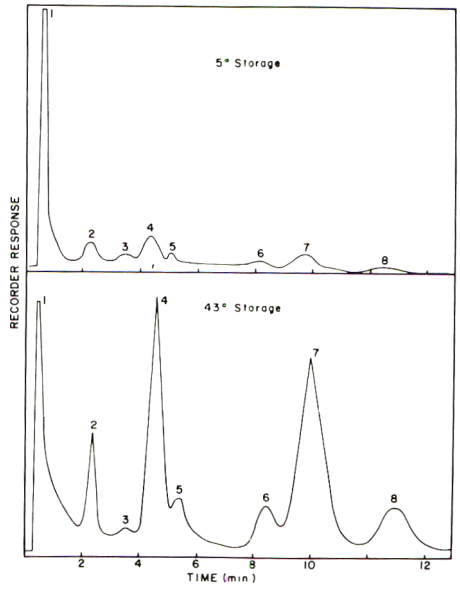

Fig. 6–Separation of methyl esters of fatty acids in egg white solids stored for 4 months. Identification of peaks: 1–solvent, 2–myristic, 3–pentadecanoic, 4–palmitic, 5–palmitoleic, 6–stearic, 7–oleic and 8–linoleic acid.

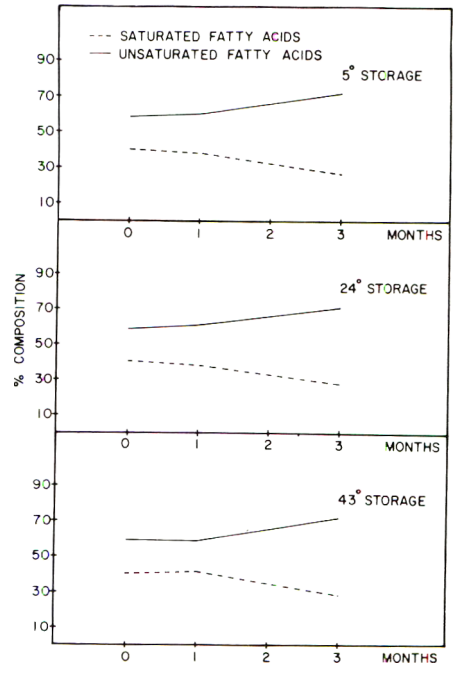

Fig. 7–Changes in saturated vs. unsaturated fatty acid composition with storage of whole egg solids.

Table 3—Change in fatty acid composition of whole egg solids during storage

Fatty acid	Storage (months) 0 (%)	1 (%)	3 (%)
	5°C Storage		
C-14	0.6	0.7	0.5
C-15	0.4	0.3	0.3
C-16	28.3	27.6	14.6
C-16:1	4.1	3.7	0.5
C-18	7.0	6.3	10.3
C-18:1	41.2	37.7	60.1
C-18:2	14.2	19.8	11.4
C-20	1.0	1.7	1.4
C-22	3.2	2.2	0.9
	24°C Storage		
C-14	0.6	0.4	0.4
C-15	0.4	0.1	0.2
C-16	28.3	26.4	19.8
C-16:1	4.1	5.1	4.9
C-18	7.0	8.9	6.3
C-18:1	41.2	43.2	59.4
C-18:2	14.2	13.3	7.6
C-20	1.0	0.8	0.7
C-22	3.2	1.8	0.7
	43°C Storage		
C-14	0.6	0.6	0.3
C-15	0.4	0.1	0.1
C-16	28.3	26.0	19.8
C-16:1	4.1	4.8	4.0
C-18	7.0	11.1	6.0
C-18:1	41.2	42.7	60.2
C-18:2	14.2	10.8	7.8
C-20	1.0	2.1	0.7
C-22	3.2	1.8	1.1

Fatty acid composition

Egg white solids. Egg white solids are reported to be free of fat while whole egg solids should not contain more than 40% fat (Forsythe, 1964). Traces of fatty acids were found in egg white solids at the start of this study. Measuring total GLC peak areas of methylesters of these fatty acids after different lengths of storage indicated that fatty acid content increased slightly but steadily when egg white solids were stored at 5 or 24°C for up to 3 months (Fig. 5). A very rapid increase in fatty acid content was observed when the product was stored at 43°C. After 3 months' storage, the fatty acid content of the sample stored at 43°C was approximately eight times as high as that stored at 5°C. The samples stored at 5 and 24°C produced good and fair quality white cakes, respectively, after 3 months at those temperatures. The 43°C sample made a cake of poor quality.

The following fatty acids were tentatively identified in all stored samples of egg white solids: myristic, pentadecanoic, palmitic, palmitoleic, oleic and linoleic acid. Oleic was the major component in egg white solids at each temperature and after each time of storage. A separation of methylesters of fatty acids in egg white solids is presented in Figure 6.

Whole egg solids. The changes in fatty acid composition of whole egg solids during storage at 5, 24 and 43°C are reported in Table 3. Besides the fatty acids found in egg white solids, arachic acid and behemic acid were tentatively identified in whole egg solids.

Several definite trends are noticeable from Table 3. At each time and temperature of storage oleic was found to be the major component of the mixture of fatty acids. Palmitic acid, linoleic acid and behemic acid decreased while the percentage of oleic acid increased with time of storage. After 3 months' storage, oleic acid made up approximately 60% of the mixture of fatty acids in samples at each temperature of storage. One would expect that the percentage of unsaturated fatty acids in the total mixture decreases with increasing storage time and that was found to be true for fatty acids of higher unsaturation (linoleic acid). However, it is interesting to note that the percentage of unsaturated fatty acids in whole egg solids actually increased due to the preponderance of oleic acid while the percentage of saturated fatty acids decreased. This was found to be true regardless of the temperature of storage and has been illustrated in Figure 7. Although the total amount of fatty acids extractable from the samples stored at 43°C was considerably higher than that of the 5°C whole egg solid samples, the total composition of the fatty acid mixture of the samples stored at 5, 24 and 43°C varied very little.

CONCLUSION

GLC EQUIPMENT has been introduced into many food laboratories during the last decade as a research tool. It might also be possible to use the equipment for the quality control of food ingredients as demonstrated.

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RETARDATION OF OXIDATIVE COLOR CHANGES IN RAW GROUND BEEF

SUMMARY—Ascorbic acid plus either BHA or PG effectively retarded both lipid and pigment oxidation in raw ground beef for up to 8 days of refrigerator storage in oxygen-permeable film. Both trained and consumer-type panels judged the additive-treated meat to be significantly better than controls. Percent metmyoglobin was found to be a useful objective measurement of color in raw beef, but total pigment concentration did not provide an adequate evaluation of color. Treatment with these additives did not appear to mask bacterial spoilage.

INTRODUCTION

THE COLOR of raw meat is a primary factor used by the consumer to judge meat quality. In order to reveal the fresh red color (oxymyoglobin), meat is commonly covered with an oxygen permeable wrapping in the display counter. However, this type of wrapping renders meat susceptible to metmyoglobin formation (brown discoloration) and rancidity during refrigerated storage (Landrock and Wallace, 1955; Greene, 1969). When meat color changes from bright red to brown, the meat loses much of its appeal to the consumer and is not purchased. Since meat packers wish to use central packaging, suitable methods of reducing nonmicrobial changes are needed to avoid economic loss.

Many studies, in both foods and in model systems, provide evidence that lipid and pigment oxidation (metmyoglobin formation) are closely interrelated. Ferric pigments are catalysts for lipid oxidation (Younathan and Watts, 1960; Brown et al., 1963). The free radical products of lipid oxidation are known to damage pigments (Haurowitz et al., 1941). Researchers have reported a positive correlation ($r_s = 0.73$) between metmyoglobin accumulation and lipid oxidation in raw meat (Hutchins et al., 1967), but it has not been established whether oxidation of the pigment precedes and causes lipid oxidation or whether pigments are damaged in the presence of oxidizing lipids. Greene (1969) attempted to retard rancid odor formation in refrigerated ground raw beef with the common lipid antioxidants propyl gallate (PG) and butylated hydroxyanisole (BHA). Not only were lipid oxidation and rancid odor formation retarded in treated samples, but these samples retained their red color somewhat longer than controls. Low concentrations of the common reducing agent, ascorbic acid (AH_2), have a color preservative effect, although higher levels often accelerate pigment oxidation and destruction (Watts and Lehmann, 1952; Rickert et al., 1957). A combination of additives may be extremely effective in preserving both pigments and lipids.

The objective of this work was to study the effects of antioxidants and ascorbate alone and in combination toward prolonging the shelf-life of refrigerated raw ground beef. Meat pigments and the effect lipid oxidation has on their color are of paramount importance to the packer, as is the relationship between objective color measurements and acceptability of meat to consumers; rancidity is a secondary problem which can be easily remedied if phenolic antioxidants are approved for use in fresh meat.

EXPERIMENTAL

FRESH BEEF eye of round or top round (U.S. Choice) of unknown history was purchased at a local supermarket. These cuts were removed from the intact hindquarter shortly before sample preparation. The initial pH was between 5.6–5.7. The meat was trimmed of external fat and excess marbling or connective tissue and ground twice in a stainless steel Hobart meat grinder (Hobart Co. Model 4812). Immediately after grinding, the meat was mixed thoroughly and aliquots taken for addition of the various additives. PG and BHA were used at a concentration of 0.01%; AH_2 at 0.05%. Amounts of reagents used in combination were additive. PG and AH_2 were dissolved in glass-distilled water, but BHA was dissolved in propylene glycol (propylene glycol itself was found to have no effect on color or flavor properties of the meat). Since microbiological spoilage can contribute to color change, 30 ppm chlortetracycline (CTC) was added in most variables to prevent interference from this source during storage. Hutchins et al. (1967) determined that CTC does not interfere with metmyoglobin reducing activity or oxygen consumption in raw ground beef. 80g portions from each aliquot were wrapped in highly oxygen-permeable "Prime Wrap" by Goodyear (950 ml/100 sq in./hr) after being shaped in a hamburger press to obtain uniform surface area. The meat patties were immediately stored at the test temperature of 3–5°C.

Analyses

Analyses run on samples initially and after several days of storage included:

Objective color measurement. Percentage of meat pigments as metmyoglobin (MetMb) was determined by reflectance spectrophotometry (Stewart et al., 1965) using an Hitachi Perkin-Elmer 139 spectrophotometer.

Objective lipid oxidation measurement. Milligrams of malonaldehyde per 1000g meat (TBA number) was determined by the method of Tarladgis et al. (1960).

Determination of total pigment content. Milligrams of total pigment per g meat were determined by a water extraction method developed by Rickansrud and Henrickson (1967).

Subjective judgments of rancidity in raw and cooked meat and of raw meat color. A trained panel was used to evaluate rancidity and color on an intensity scale of 6 (no detectable rancidity, very good color) to 1 (very strong rancid odor; very badly discolored). This technique has been described by Tarladgis et al. (1959). In addition to the trained panel, 12–36 freshman home economics students were asked to evaluate the color of meat samples after 8-days storage. The students were not asked to make a purchase-reject decision on each sample, but merely to indicate which samples they would purchase or reject in a self-service supermarket situation. All correlations were calculated using the Spearman rank-order correlation coefficient (r_s) (Siegel, 1956).

RESULTS & DISCUSSION

REPRESENTATIVE data for eye of round are in Table 1. All antioxidant-treated samples had TBA numbers of less than 0.5, indicating that lipid oxidation was prevented. In samples with only BHA or PG treatment, pigment oxidation was greatly reduced (approximately 50% over controls), but it was not prevented. When only AH_2 was used, both pigment and lipid oxidation were slowed. Ascorbic acid may have been able to function as a MetMb reductant until lipid oxidation free radicals became numerous and either destroyed the AH_2 activity or overpowered AH_2. Lipid oxidation in these samples may have been retarded by maintaining the pigment largely in the ferrous state.

When the combination of antioxidant and AH_2 was used, pigment oxidation was greatly retarded throughout the 8 days of storage (11% MetMb vs. 82% for control; 16% and 26% vs. 77%). Usually the effects of antioxidant and AH_2 together were greater than those of either additive alone. To determine if phenolic antioxidants were protecting AH_2 from destruction by free radicals from lipid oxidation, samples containing PG + AH_2, BHA + AH_2 alone were assayed for AH_2 loss over a period of 10 days (Loeffler

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Table 1—Effect of additives on pigment and lipid oxidation in refrigerated raw ground beef eye of round stored 8 days

Variable	MetMb %	Color score[a]	TBA number[d]	Total pigment mg/g sample	Total pigment Loss, %
Control-initial	11	–	1.2	3.19	0
Control	89	1.3	11.9	2.54	20.4
PG	47	3.6[b]	0.6	2.78	12.7
AH_2	50	3.3[b]	5.4	2.75	14.1
Control-initial	14	–	0.9	3.04	0
Control	82	1.2	10.0	2.40	21.1
PG	41	4.1[b]	0.3	2.56	15.7
AH_2	46	3.9[b]	2.8	2.67	12.2
PG + AH_2	11	5.2[b]	0.2	2.73	10.0
Control-initial	0	–	0.3	3.22	0
Control	77	–	8.9	2.57	20.2
BHA	38	3.9	0.3	2.77	13.6
AH_2	28	4.4	2.7	2.80	14.3
BHA + AH_2	16	5.3[c]	0.3	2.99	7.3
PG + AH_2	26	4.9[c]	0.4	2.89	10.5

[a]Color scores: 6 (very good color) to 1 (very badly discolored).
[b]Significant > control.
[c]Significant > BHA.
[d]Higher initial TBA numbers reflect time lapse between grinding of meat and completion of sample preparation.

Table 2—Color acceptability of refrigerated raw ground beef to a consumer panel after 8 days storage[a,b]

MetMb %	Purchase %	Reject %	Variable
11	100	0	PG + AH_2
16	100	0	BHA + AH_2
26	100	0	PG + AH_2
26	94	0	PG + AH_2
28	94	0	AH_2
32	75	8	BHA + AH_2
34	100	0	BHA + AH_2
41	48	4	PG
46	50	4	AH_2
47	55	0	PG
50	74	3	AH_2
57	17	42	BHA
59	0	100	BHA + PG
60	19	50	PG + AH_2
89	0	100	Control

[a]Data are taken from five experiments.
[b]Judges were not asked to make a purchase-reject decision on each sample, but merely to indicate which samples they would purchase or reject in a self-service supermarket situation.

and Ponting, 1942). All samples lost 60–80% of the AH_2 within 6 days. Phenolic antioxidants were not protecting AH_2. The effectiveness of the combination cannot be readily explained except as synergism.

In relating lipid and pigment oxidation to actual pigment loss, it can be seen (Table 1) that amount of pigment loss is dependent upon amount of pigment oxidation. A highly significant correlation ($r_s = 0.72$; significant at 0.0005 level) was found between % MetMb and % total pigment loss. Less overall pigment loss and less ferric pigment were found in samples containing BHA or PG plus AH_2 than in samples treated with BHA or PG alone. Ferric pigment formation and total pigment loss were greatest in control and AH_2 samples where lipid oxidation was apparent. If denaturation of globin had been the primary target of lipid oxidation free radicals, it should have been observed in the total pigment determination where a water extraction is made. Precipitation of denatured proteins or turbidity in extracts would be logical expectations, but they were not found. This suggests that lipid oxidation free radicals act by first oxidizing the heme to MetMb rather than by initially damaging the globin.

Figure 1 is a graphic representation of the relationship between color scores and % MetMb. The area enclosed within the dotted lines shows that the larger proportion of samples considered by trained judges to have a good or very good color contained between 0–40% MetMb. In Table 2, which shows acceptability to the consumer panel, the line of rejection again appears to be between 30–40% MetMb. The relationship between % MetMb and color scores (Fig. 1) is highly significant at the 0.0005 level ($r_s = 0.73$). This indicates that measurement of % MetMb by the method of Stewart et al. (1965) is a useful criterion for meat color acceptability.

No apparent relationship exists between total pigment concentration and degree of discoloration as measured by color scores. Samples with color scores below 4 (slightly discolored) had more than 45% MetMb regardless of pigment concentration. This suggests that the relative amount of ferric pigments rather than pigment concentration is the decisive factor in color acceptability.

Raw and subsequently cooked samples were rated for rancid odor by the trained panel. Results were in general agreement with previously reported findings, i.e., antioxidant-treated samples rated higher than untreated controls; sensory scores correlated significantly with TBA numbers ($r_s = 0.43$ for raw, significant at 0.005 level; $r_s = 0.59$ for cooked, significant at 0.0005 level).

In order to recommend use of these additives in fresh meat on a commercial basis (although use of phenolic antioxidants in food is generally under attack), it is important to know whether or not the additives will obscure bacterial change. Bacterial counts on each variable (Service Lab., CSU Microbiology Dept.) were included in several experiments. Samples with and without antioxidants and AH_2 and each of these variables with and without CTC were included. In general, spoiled samples were noticeably brown or green whether or not they contained the antioxidant and AH_2. Thus, it appears likely that the consumer would be able to detect spoiled meat even though the color and flavor would be protected during its unspoiled shelf life. Further studies of this type should be carried out in conjunction with use of these additives. A significant finding is the length of time required for bacterial spoilage to become

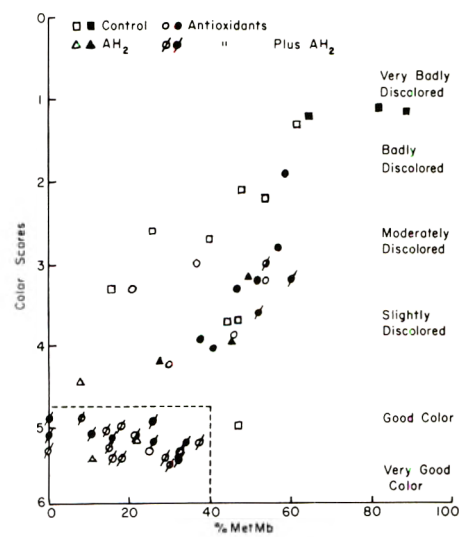

Fig. 1—Relationship of % MetMb to color scores. Unshaded symbols represent determinations at 3 days of storage; shaded at 8 days. ($r_s = 0.73$, significant at 0.0005 level.)

apparent. Meat ground to order at the supermarket contained 3–10 times the microbial flora initially found in meat ground in the laboratory. Shelf life of laboratory-ground meat was considerably longer than that of supermarket-ground beef (6 days vs. 3 days). Therefore, sanitation at the place of cutting and packaging meats play a more important role in shelf life than may be realized. (This topic is well reviewed by Brody, 1970.) CTC did not influence lipid oxidation, but it did retard pigment oxidation resulting from bacterial action.

CONCLUSIONS

THIS STUDY clearly indicates that a combination of PG or BHA and AH_2 is effective in prolonging the shelf life of refrigerated ground beef. Treated meat was judged to be significantly better in color than controls by both trained and consumer-type panels. When MetMb levels were greater than 40%, judges down graded or rejected samples for purchase. Use of these additives does not appear to mask bacterial spoilage.

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DISCOLORATION OF SAUERKRAUT PROBABLY CAUSED BY A LEUCOANTHOCYANIDIN

SUMMARY—A series of experiments was conducted to determine what kinds of pigments bring about discoloration of sauerkraut. UV light altered the color of potential discolored sauerkraut but had no effect on normal or discolored sauerkraut. Spectrophotograms of HCl extracts of potential discolored and discolored sauerkraut peaked at 540 nm whereas normal sauerkraut had an extinction at 540 nm but no peak. A specific profile for discolored sauerkraut was determined via thin-layer chromatography on silica gel. The compound causing the discoloration is apparently a leucoanthocyanidin.

INTRODUCTION

IN SOME YEARS, the discoloration of sauerkraut causes considerable economical losses for the Dutch industry (Steinbuch, 1970). Staden (1963) found an uncolored yeast that is very often present in discolored sauerkraut and identified it as *Saccharomyces exiguus*; Steinbuch (1965, 1969) corroborated these findings.

Steinbuch and Staden (1964) assumed that undesirable microorganisms produced enzymes which, in the presence of oxygen, converted substrates of cabbage into discolored material. When we tried to obtain discolored sauerkraut by incubating with the liquid from a pink batch it was not always possible to obtain a discolored product.

Pederson and Albury (1969) reported that the general use of potash fertilizer for cultivation of cabbage lessened the production of discolored sauerkraut. Steinbuch (1970) remarked about the importance of manganese, among other factors, for an increased growth of lactic acid bacteria. This rapid lactic acid fermentation retards the development of undesired yeasts and thus no discoloration appears.

In this paper we are referring to the discoloration which varies from a pink-purple to a dirty pink or gray. When the vat is opened the sauerkraut presents an abnormal odor but color appears to be normal; however, after exposure to air (1–48 hr) it begins to discolor. The entire batch of sauerkraut may discolor when removed from the vat. Consequently we are not considering the discoloration brought about by pink yeasts (Fred and Peterson, 1922; Pederson and Kelly, 1938) or inappropriate packing or storage (Steinbuch, 1968).

So far as we know, there is no clear explanation for the mechanism which leads to the production of discolored sauerkraut; accordingly we decided to try to determine what kinds of pigments bring about the discoloration of sauerkraut. This paper reports results of the first part of a biochemical study on the pink discoloration of sauerkraut.

MATERIALS & METHODS

Sauerkraut

Discolored. Batches of pink sauerkraut were obtained from the industry when such a batch occurred.

Potential discolored. Refers to that portion of a batch of pink sauerkraut (inside the vat) that had not become discolored as it was not exposed to light.

Normal (good quality). This was either purchased or prepared by the Department of Technology of our Institute.

We did not know whether any of these substrates were prepared from one variety of cabbage or several. Usually the industry prepares sauerkraut from various varieties. Later our Department of Technology furnished a batch of pink and normal sauerkraut from the same variety of cabbage.

Lyophilized. Both normal and discolored sauerkraut were homogenized in a Waring Blendor for several minutes at 5°C. The homogenate was either lyophilized directly or frozen at −20°C and lyophilized when possible.

Batches of fresh sauerkraut which were impossible to lyophilize were kept at −20°C but not homogenized.

UV light treatment

Sauerkraut (normal, potential discolored and discolored) was exposed at room temperature, for 30–60 min at a distance of 30–40 cm from the UV light source (3500 Å: Philips TL 20W/08; 2537 Å: Philips TL 30W).

Characterization of the discolored sauerkraut

This proof is based on the test developed by Nicolaisen and Pieper (1956) but modified by the determination of spectrophotograms to objectivize the result. All spectrophotograms presented in this article were determined with the aid of a Bausch and Lomb spectrophotometer, model Spectronic 505, band-width 5Å.

Fresh pink (or potential discolored) sauerkraut (wet weight) was suspended in 12N HCl (1:4 w/v). After 30 min the suspension was filtered and the spectra of the liquid phase determined with respect to 12N HCl (see Fig. 1). The cuvettes used were organic resistent and hermetically sealed to avoid damage to the spectrophotometer by vapors of the conc HCl. A discolored batch of sauerkraut which did not present the spectra of Figure 1 was discarded. As control, the same procedure was followed but using normal sauerkraut.

Stability of pigment vs. temperature

Sauerkraut (discolored and normal) was suspended in 12N HCl (1:4 w/v). After 30 min the suspension was filtered and aliquots of the liquid phase (ca. 5 ml) heated for 45 min at 40, 50, 60, 70 and 80°C. Spectrophotograms of the aliquots were determined at room temperature

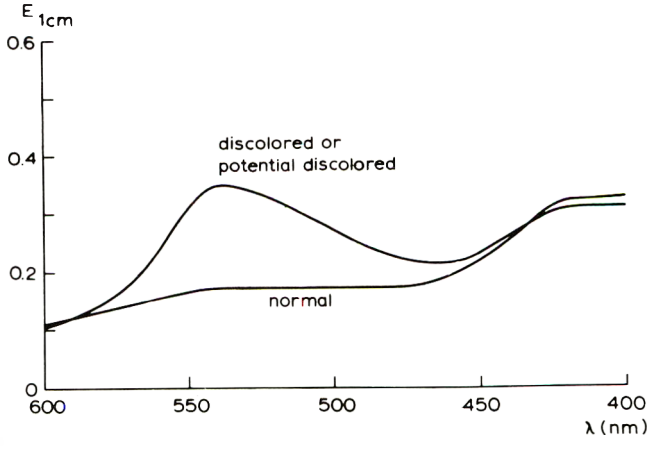

Fig. 1—Spectrophotograms of HCl extracts of normal, discolored or potential discolored sauerkraut.

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Table 1–Extraction of pigment from lyophilized sauerkraut with various solvents

Solvent[a]	Dielectric constant at 20°C[b]	Peak with max at 540–550 nm[c]	
		Discolored	Normal
n-Pentane	1.844	–	–
Petroleum ether (40–60)		–	–
Carbon tetrachloride	2.238	–	–
Benzene	2.284	–	–
Diethyl ether	4.335	–	–
Chloroform	4.806	+	–
Ethyl acetate	6.02	–	–
Acetic acid	6.15	+	–
Dichloromethane	9.08	+	–
Pyridine	12.3	+	–
Propanol-1	20.1	++	–
Acetone	20.7	++	–
Ethanol	24.30	++	–
Methanol	33.62	++	–
Distilled water	80.37	++	–

[a]All solvents are A.R. Merck.
[b]Data from Chemical Rubber Co. (1970–71).
[c]Data deduced from Figure 3. (+) slight peak; (++) clear peak; (–) no peak.

Table 2–Extraction of pigment from wet sauerkraut with various polar solvents

Solvent	Color of extract after mixing at room temp with 12N HCl (1:1 v/v)	
	Discolored sauerkraut	Normal sauerkraut
Propanol-1	intense red	pale rose
Ethanol	intense red	pale rose
Methanol	intense red	pale rose
Distilled water	intense red	pale rose

Table 3–Effect of UV light (2537 Å or 3500 Å) on various kinds of sauerkraut

Sauerkraut	Color after treatment
Normal	no change
Potential discolored	becomes pink
Discolored	no change

with respect to 12N HCl. The spectra of heated and unheated 12N HCl samples are identical.

Extraction of pigment

1.50g portions of lyophilized discolored sauerkraut (as well as normal) were suspended in darkness (at room temperature) in 30 ml of various solvents ranging from nonpolar to polar (Table 1) (Neher, 1958) and reordered according to the dielectric constants mentioned by Karrer (1950). After one night, suspensions were filtered through fluted filter paper, and the liquid phase (4 ml) evaporated to dryness with the aid of a rota-evaporator. 4 ml of 12N HCl was added. If turbidity arose, the suspension was centrifuged at 30,000 × G for ca. 1 hr. The spectra of the supernatants were determined. All spectrophotograms were determined with respect to an appropriate blank, i.e., using the same solvent and procedure as previously mentioned but without lyophilized sauerkraut.

In another experiment, wet sauerkraut (discolored and normal) was extracted with the polar solvents mentioned in Table 2. After filtration, the respective liquid phases were mixed with 12N HCl (1:1 v/v) and the color registered visually.

Ethanolic extract

Either discolored or normal lyophilized sauerkraut (ca. 20g) was suspended in (ca. 200 ml) absolute ethanol (A. R. Merck) and shaken mechanically for 16 hr. The ethanol was decanted and the sediment suspended in another portion (150 ml) of absolute ethanol. After shaking for 3 hr, the liquid phase of the suspension was decanted and the sediment again extracted as previously mentioned. The three portions (ca. 500 ml) of ethanol were collected and filtered through filter paper. The filtrate was concentrated with the aid of a rota-evaporator at 30°C to a volume of ca. 3 ml. The concentration process brought about a precipitate which was removed by centrifuging at 700 × G for 5 min at room temperature. The supernatant (ca. 2.5 ml) was kept in a centrifuge tube and the sediment washed four times with absolute ethanol by centrifuging for 5 min at 700 × G (room temperature) and resuspending the sediment in absolute ethanol.

The supernatant from the four washings (total ca. 6 ml) was added to the 2.5 ml of extract and concentrated in a rota-evaporator at 30°C. The concentration process again brought about a precipitate which was removed by centrifuging at 700 × G for 15 min at room temperature. The supernatant (ca. 1.5 ml) constitutes the ethanolic extract. In this work it was important that the ethanol extract from discolored sauerkraut contained a leucoanthocyanidin.

We preferred absolute ethanol to methanol as ethanol extracted less salt from the sauerkraut than the methanol. When employing methanol, tailing due to salt was obtained in the chromatograms.

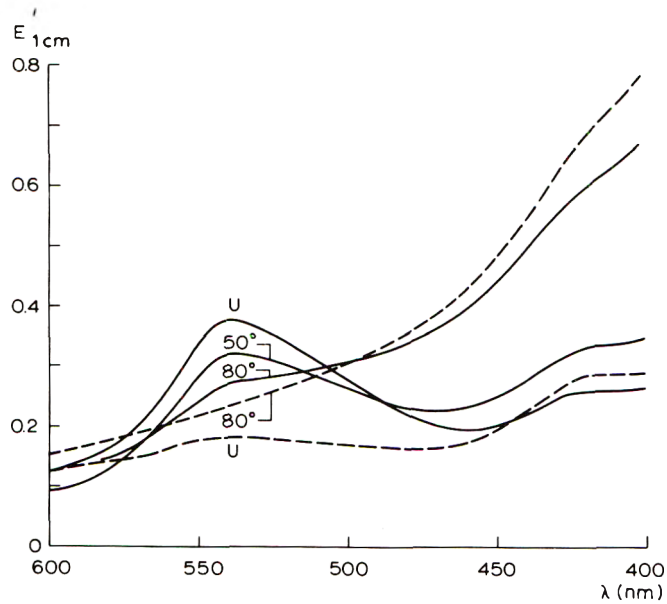

Fig. 2–Spectrophotograms of HCl extracts of discolored (——) and normal (– –) sauerkraut heated for 45 min at various temperatures: (U) unheated; (50°) heated at 50°C; (80°) heated at 80°C.

Thin-layer chromatography

Ethanolic extract from discolored sauerkraut (as well as from normal) was applied on preparative plates (20 × 20 cm) on silica gel (DO, Camag), coating thickness 1 mm. They were prepared according to Graveland (1970). The solvent used was a mixture of chloroform:methanol:acetic acid (65:20:4 v/v). We found acetic acid better than water as it avoids dissociation of compounds; this is also advised

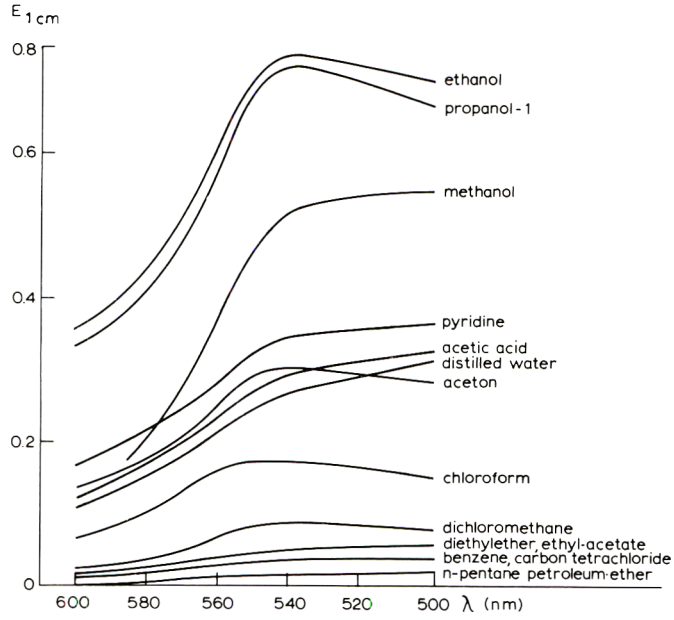

Fig. 3—Spectrophotograms of various solvent extracts of pigment from lyophilized discolored sauerkraut. Different portions of lyophilized sauerkraut (discolored and normal) were extracted with the solvents indicated. After solvent removal, HCl was added and spectra determined with respect to an appropriate blank (see text).

by Hathway (1969). Leucoanthocyanidin was detected by spraying the chromatograms with an ethanolic solution of 20% p-toluenesulfonic acid (PTS) (A. R. Merck) and leaving it for 3–5 min at ca. 103°C. We found that a concentration of 20% PTS (Stahl, 1967) was better than 3% (Roux, 1957). The reaction of leucoanthocyanidin with the PTS reagent gave an intense red color.

RESULTS

RESULTS of experiments with various kinds of sauerkraut (normal, potential discolored and pink) under UV light (Table 3) show that only potential discolored sauerkraut becomes pink, whereas the color of normal and discolored sauerkraut is not altered.

The spectrophotograms (Fig. 1) of HCl extracts of potential discolored as well as of discolored sauerkraut possess a λ max at 540 nm, i.e., there is a clear peak, while the spectrophotogram of the normal sauerkraut has an extinction at 540 nm but not a peak. Therefore, we characterized the substrate with the aid of the whole spectrophotogram and not with the extinction value at 540 nm.

In addition, we found that batches of sauerkraut which became pink under effect of UV light (potential discolored, Table 3) also gave a positive result with the HCl test (λ max at 540 nm, Fig. 1).

In Figure 2 are presented the spectrophotograms pertaining to HCl extracts of normal and discolored sauerkraut heated at various temperatures. For a clearer presentation, only spectrophotograms of the extracts heated at 50 and 80°C are included. We observe that in the case of discolored sauerkraut, the higher the temperature the lower the peak at 540 nm (region 520–550 nm) whereas the extinction values in the region of 400–500 nm increase. In the case of normal sauerkraut the picture is different, with extinction values increasing in both mentioned regions.

The observations made with the spectrophotograms of the heated HCl extracts of discolored sauerkraut with respect to the unheated extract allowed us to assume that the main component is a leucoanthocyanidin (Ranganna and Govindarajan, 1966).

Data pertaining to the extraction of pigment from pink sauerkraut with various solvents (ranging from nonpolar to polar) are given in Table 1. Figure 3 records the spectra leading to the composition of Table 1. The spectra of normal sauerkraut were omitted because they have no peak at 540 nm.

From Table 1 we see that the pigment of discolored sauerkraut is soluble in polar solvents; therefore it is a polar compound. It is interesting to note that ethyl acetate does not extract the pigment from the lyophilized discolored sauerkraut; however, when the leucoanthocyanidin has already been extracted with another solvent, it can then be extracted from an aqueous solution by means of ethyl acetate. This indicates that the leucoanthocyanidin "in situ" is bound in some way and ethyl acetate cannot extract it, suggesting that the leucoanthocyanidin is probably a flavan-3,4-diol (Clark-Lewis, 1962).

Results of the chromatograms of the ethanolic extracts of discolored and normal sauerkraut (both prepared with the same variety of cabbage) are presented in Figure 4, which is a representative photo of various experiments. Moreover, the chromatograms of discolored and normal sauerkraut that were not prepared with one variety of cabbage are the same as those of discolored and normal sauerkraut prepared with the same variety of cabbage.

The color of the band marked with the letter L (Fig. 4) is bright red after treating the chromatogram with PTS. This is the only way to detect this band as under UV and visible light it is invisible. This band (R_f 0.46) appears only in the chromatogram of discolored sauerkraut.

DISCUSSION

TO DETERMINE whether a batch of sauerkraut would or would not become pink, our Department of Technology would leave the samples under natural light which was filtered by the glass of the roof. If a batch was potential discolored it became pink after 1–2 days. The same batch exposed to UV light (3500 Å or 2537 Å) became pink after 30–60 min. These results indicate that the effect of light is important. The appearance of the pink color is probably catalyzed by a photochemical reaction. This is specific for potential discolored sauerkraut as normal and discolored sauerkraut are not affected (Table 3). This indicates that the fermentation process (enzymes involved ?) of cabbage leading to the formation of sauerkraut leaves the substrate in such a condition that light can bring about the pink color.

Furthermore, we found a close correlation between the UV test and the characterization of the substrate by the HCl test (Fig. 1). We may say that the peak at 540 nm developed by the treatment of the substrate (potential discolored and discolored) with HCl at room temperature is due to the formation of an oxonium-type structure (Blessin et al., 1963), which would pertain to an anthocyanidin arising from a leucoanthocyanidin. As this occurs at room temperature (treatment with cold HCl) the leucoanthocyanidin is in a "pseudo-base" form. The observation that the spectrophotogram of the HCl extract from normal sauerkraut presents a slight extinction value at 540 nm but not a peak, indicates that the precursor is present in the cabbage but that some change must occur to obtain an oxonium-type structure. This is supported by our experience that after treating an ethanolic extract of shredded cabbage with conc HCl, the extract becomes red with a λ

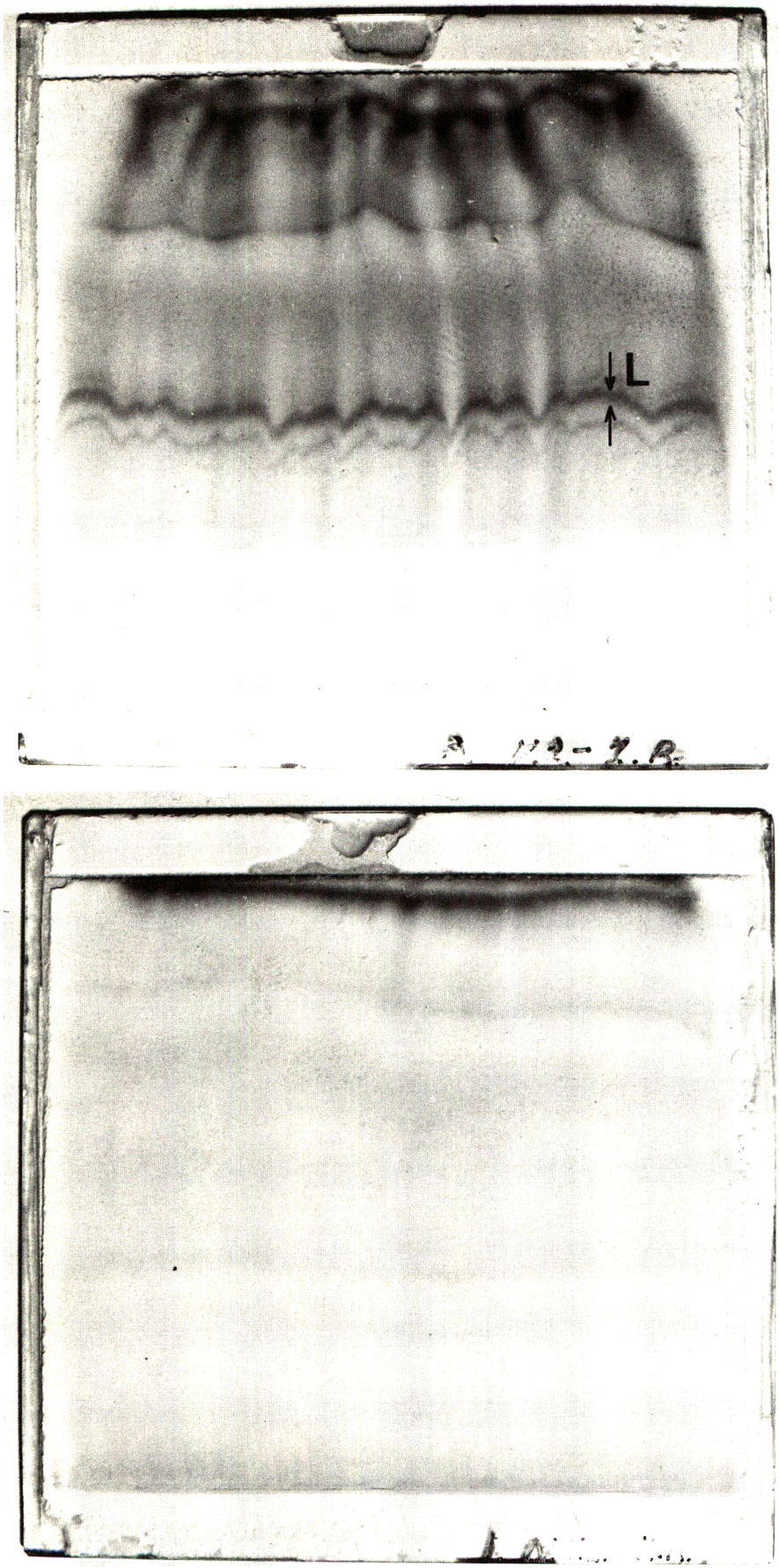

Fig. 4–Photo of chromatograms of discolored (top) and normal (bottom) sauerkraut after treatment with PTS. Band L is bright red and appears only in the chromatogram of discolored sauerkraut.

max at 540 nm, as well as by the work of Ranganna and Govindarajan (1966) who found leucoanthocyanin in cabbage.

Considering that: (1) the pigment of pink sauerkraut is rather polar; (2) the ethanol extract of discolored sauerkraut becomes red after treatment with conc HCl at room temperature (similar to the experience of Blessin et al., 1964); (3) that when the HCl extract of discolored sauerkraut is heated the peak of the spectrophotogram at 540 nm decreases while the extinction values in the region of 400–500 nm increase (similar to results of Ranganna and Govindarajan, 1966); and (4) that when HCl extract is brought to pH 3 the color changes from red to yellow, permit us to conclude that the pink discoloration is brought about mainly by a leucoanthocyanidin.

Once we knew that a leucoanthocyanidin was important in the pink discoloration, we started to screen for this kind of compound and also tried to obtain (via chromatography) a profile for pink and normal (control) sauerkraut.

We also screened for anthocyanins and anthocyanidins originating from anthocyanins according to Harborne (1959, 1967) and slightly for phenolic acids according to Ribéreau-Gayon (1968). However, we obtained a qualitative difference between the profiles of pink and normal sauerkraut only with leucoanthocyanidins (Fig. 4).

The chromatograms of Figure 4 present profiles of pink and normal sauerkraut, both prepared with the same variety of cabbage, to avoid the probability that some differences would be due to varietal differences. It is interesting to note that the profile of pink sauerkraut obtained with one variety of cabbage is the same as the profile of pink sauerkraut obtained from industry (a mixture of varieties). The same occurs with the chromatogram of normal sauerkraut prepared with one variety of cabbage and normal sauerkraut prepared with various varieties. This means that the profile of Figure 4 is specific for pink sauerkraut. The typical band of this profile is the one which becomes bright red after treatment with PTS (band L of Fig. 4).

We purified to some extent the compound contained in the previously mentioned band. We did this via thin-layer chromatography on silica gel, successively: (a) twice with chloroform:methanol:acetic acid (65:12:4 v/v); (b) once with chloroform:methanol:acetic acid (65:20:4 v/v); and (c) with chloroform:propanol-1 (65:20 v/v). This compound when checked on cellulose plates (A. R. Merck, Art. 5716/0025) using sec. butanol:acetic acid:water (4:1:5 v/v) as solvent showed two impurities which were detected under UV light: one spot was yellow the other blue. This com-

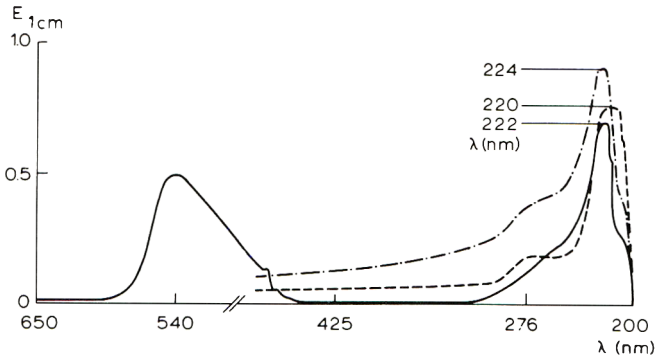

Fig. 5—Spectrophotograms of the partly purified compound from band L chromatograms of discolored sauerkraut. (——) in ethanol; (——) in 12N HCl; and (—·—·—) in 0.1N NaOH.

pound (not yet chromatographically pure) in absolute ethanol, 12N HCl and 0.1N NaOH showed the spectrophotograms recorded in Figure 5. We see that the peak with λ max at ca. 222 nm remains in the three solvents; in addition there is no subsidiary peak above 300 nm, indicating a possible flavan-3,4-diol structure (Harborne, 1964). The slight peak at ca. 280 nm in NaOH and ethanol would represent the polyphenol structure which contains no carbonyl conjugation as mentioned by Blessin et al. (1963), or it could be a consequence of the impurities. The 12N HCl solvent, in addition to the two peaks in the UV region, presents a clear peak at 540 nm. This is typical of an oxonium-type structure pertaining to an anthocyanidin which in this case arises from a leucoanthocyanidin in the pseudo-base form. This demonstrates that the compound purified by us to some extent is bringing about the typical spectra of the HCl extract pertaining to discolored sauerkraut (Fig. 1). The content of the "pure" compound is ca. 1 mg/1 kg of wet discolored sauerkraut, i.e., 1 ppm. The purification of this leucoanthocyanidin is being completed.

Before concluding, we would like to point out that proofs were done in order to assure that the main substance of band L (Fig. 4) was not an indole compound reacting with PTS (Prabhakar, 1965).

The alcohol of the ethanolic (95%) extract of discolored sauerkraut, as well as of normal, was removed under pressure. The remaining water solution was extracted four times with pentane, five times with diethyl ether and five times with ethyl acetate. The first fraction of each solvent was concentrated, chromatographed and treated with PTS as previously described under "Materials & Methods." Only the ethyl acetate fraction of discolored sauerkraut presented the typical band L. If indole compounds were present, they should appear in the chromatogram of diethyl ether extracts. Moreover the R_f values (0.9–1.0) of indole, 3-indoleacetic acid, 3-indolealdehyde and 3-indoleacetonitrile were different from the compound of band L (R_f 0.46).

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EVALUATION OF FROZEN BOILED PEANUTS AS AFFECTED BY COOK TIME, COOL TIME AND SALT CONCENTRATIONS

SUMMARY—Frozen boiled peanuts were evaluated in terms of the variables, cooking time, cooling time and salt concentration. Cooling was accomplished in the same brine used for cooking. The three variables were evaluated in a triple-cube experimental design, and the data were analyzed on the Mississippi State University computer. Titratable salt below 2.3% in the finished product was generally considered too low. Salt concentrations lower than 4 oz per gallon of water in the cooking and cooling brine always resulted in peanuts too low in salt. 8 oz of salt per gallon of water resulted in salt content in the finished product that was too low, when either the cooking or cooling time was short. 10 oz of salt per gallon of water resulted in a satisfactory salt content over a cooking time range of from 20–30 min with a cooling time of 13 hr. Cooking time of 15 min or less resulted in peanuts which were too firm, based on shear press data. Peanuts cooked 20–25 min were usually judged by the taste panel to have satisfactory texture, although taste panel results were not significantly correlated with the independent variables. The best boiled frozen product resulted when peanuts were cooked 10–30 min at 212°F in brine containing 6–14 oz of table salt per gallon of water and were cooled 9–29 hr in the same brine. Samples were packaged in 1.5-mil polyethylene, vacuum-sealed pouches and stored at −20°F. No detectable degeneration of the product resulted from holding the frozen boiled peanuts in storage 8 months. The most time-consuming step in processing was cooling to get salt absorption. Future research will be aimed at reducing cooling times to practical commercial limits.

INTRODUCTION

IN 1933, August Chevalier wrote, "The problem of the origin of the peanut has made floods of ink flow" (Higgens, 1951). This excellent food crop probably originated in the Gran Caco area of Paraguay and was distributed throughout the tropical regions of the Americas at a very early date. There is no proof that the peanut was present in the United States in the pre-Columbian era. Available evidence indicates that some types were imported from Africa, Spain, Mexico and Central America.

The peanut is an excellent source of oils, protein and some carbohydrates. 1g of a typical boiled peanut yields 3.8 calories as compared to 2.3 for beef, 3.6 for whole wheat, 2.6 for white bread and 4.0 for cane sugar (Anonymous, 1946). Peanut oil is of excellent quality, containing up to 25–35% linoleic acid.

Proteins of the peanut contain all of the essential amino acids, but some are present in less than optimum amounts. The nutritive value of peanut protein is limited by its content of lysine and methionine and it is marginal in tryptophan and threonine. The amino acid content of peanut protein makes it particularly well suited for combination with dry skim milk protein (Milner, 1962).

The carbohydrate content of peanuts is about 20% and includes starch, sucrose, cellulose and some pectic substances. Peanuts are an excellent source of thiamine, riboflavin, nicotinic acid and vitamin E; however, they are almost devoid of vitamins A, C and D.

The literature reveals many studies concerning the storage and packaging of fresh or cured peanuts (Cobb et al., 1969; Davis, 1966; Mitchell and Malphrus, 1968; Schenk, 1959; Woodroof, 1966), the preparation of flakes (Mitchell and Malphrus, 1968), roasting, frying and the manufacture of peanut butter and peanut oil (Thomas et al., 1968). Ramamuriti et al. (1964) described preparation of a cheese-like product from peanut protein and Milner (1962) has considered peanuts as a protein resource in international feeding programs.

Willich et al. (1954a,b) evaluated the stability of peanut oils to oxidative rancidity and the effect of processing and storage on vitamin A added to peanut butter.

The literature, however, yields little information concerning the factors affecting boiling of peanuts in the shell and subsequent freezing of the boiled product.

MATERIALS & METHODS

NINE LEVELS of cooking time from 5 through 45 min at 5-min intervals were studied. Nine cooling variables, ranging from 1 through 33 hr, at 4-hr intervals, also were evaluated. Salt concentration in the cooking and cooling brine ranged from 0–16 oz per gallon of water at 2-oz increments. These variables constituted nine levels of cooking time, nine levels of cooling time and nine levels of salt concentration, as shown in the triple-cube design in Table 1

Table 1—Triple-cube experimental design showing relationship of the independent variables of cooking time, cooling time and salt concentration in the cooking and cooling water. Values in the cells represent salt concentration in ounces of sodium chloride per gallon of water

Cooking time (min)	Cooling time (hr) 1	5	9	13	17	21	25	29	33
	Salt concentration (oz per gal)								
5					8				
		2						2	
10		14						14	
			4				4		
15			12				12		
				6		6			
20				10		10			
					0				
					8				
25	8				16				8
				6		6			
30				10		10			
			4				4		
35			12				12		
		2						2	
40		14						14	
45					8				

[1] Dept. of Horticulture. [2] Dept. of Agronomy.

(Trammel, 1957). Numbers in the cells of the table represent salt concentration in ounces per gallon of water.

Peanuts of the Valencia variety were grown on the Agronomy farms at Mississippi State University. They were planted on May 27 and harvested on October 1, 1969. The peanuts were held at 32°F and were processed within 48 hr from harvest.

The peanuts were cooked at 212°F for the indicated times and cooled in the cooking solution. 1,200g of peanuts to each gallon of brine was the ratio used in cooking and cooling each lot. The peanuts were washed, cooked, cooled, vacuum-packaged in polyethylene pouches, frozen and stored at −20°F until analyzed. They were processed in October, 1969, and analyzed in June, 1970. 82 lb of peanuts were utilized in processing the 31 experimental variables.

After 8 months in storage, the frozen peanuts were thawed, shelled and evaluated for salt content by titration, flavor by taste panel, tenderness by shear press and color in terms of Hunter "L," "a" and "b" values.

Taste panel values were the average of scores assigned by 10 panel members for each sample. The peanuts were heated to about 85°F and served to the panel members in the shell. The 10 panel members rated the coded samples on a 1 to 9 hedonic scale, with 1 representing peanuts "liked extremely" and 9 indicating peanuts "disliked extremely." Panel members were instructed to indicate the reason they liked or disliked the product, giving particular attention to salt concentration and firmness.

All the other values were the average of two determinations for each treatment combination. Shear press values were obtained on the Model S-2HE Shear Press equipped with a 1,000-lb ring with the selector set on the 100 range. A 30-g sample of peanuts was used in making each shear determination, and all values were expressed as pounds of pressure required to shear the sample.

Salt was determined by the official method of the AOAC for soluble chlorine (AOAC, 1965).

The data were analyzed using a regression program developed at Mississippi State University. Regression coefficients, correlation coefficients and analysis of variance for the regression were determined for six dependent variables and six computed variables as related to the three independent variables.

Table 2—Relationship between the three independent variables of cooking time, salt concentration and cooling time and the six dependent variables of taste panel, percent salt, Hunter "L," "a" and "b" and shear values

Independent variables			Dependent variables					
Cooking time (min)	Salt conc (oz/gal)	Cooling time (hr)	Taste panel scores[a]	Percent salt by titration	Shear press (lb/force)	Average Hunter values "L"	"a"	"b"
45	8	17	5.0	3.82	293	32.1	1.9	10.3
40	14	29	5.6	6.64	520	36.9	1.7	12.3
40	14	5	6.0	6.32	493	37.2	1.7	12.9
40	2	29	4.2	0.90	235	30.0	1.3	9.2
40	2	5	4.8	0.58	220	35.0	2.3	10.4
35	12	25	5.2	4.12	270	32.1	2.8	10.5
35	12	9	6.3	3.60	275	35.5	2.1	11.3
35	4	25	6.3	1.48	220	32.0	2.6	10.0
35	4	9	4.4	1.12	200	31.3	4.2	10.3
30	10	21	6.0	4.08	370	29.3	3.1	10.5
30	10	13	3.7	3.32	340	37.7	2.9	12.9
30	6	21	5.6	3.34	333	34.0	2.7	10.6
30	6	13	5.0	3.10	383	32.9	2.3	10.9
25	16	17	4.0	3.68	348	36.7	2.8	13.2
25	8	33	2.9	2.04	323	33.9	1.9	10.9
25	8	17	2.9	2.28	363	32.8	0.1	10.6
25	8	1	3.8	1.40	393	36.3	3.1	12.5
25	0	17	7.6	0.64	293	39.2	0.6	11.7
20	10	21	3.8	3.08	383	34.6	2.3	11.7
20	10	13	3.8	2.36	370	39.0	2.5	12.3
20	6	21	3.6	2.28	358	31.9	1.9	10.6
20	6	13	3.1	1.66	320	36.0	1.7	11.8
15	12	25	4.5	2.50	333	33.7	2.1	12.2
15	12	9	5.6	2.86	405	31.9	1.9	10.4
15	4	25	5.2	1.28	325	36.5	0.9	10.4
15	4	9	4.1	0.90	333	39.3	2.0	11.7
10	14	29	3.5	2.42	395	36.0	1.0	11.3
10	14	5	4.4	2.20	425	38.4	1.3	12.3
10	2	29	6.4	1.30	350	38.3	1.5	10.8
10	2	5	4.5	0.88	395	32.8	2.0	10.0
5	8	17	5.2	1.08	395	38.9	2.1	11.5

[a]Low numbers indicate good peanuts (1 = excellent, 10 = poor).

RESULTS & DISCUSSION

RELATIONSHIPS among the three independent variables of cooking time, salt concentration and cooling time, and the dependent variables of taste panel values, percent titratable salt, Hunter "L," "a" and "b" values, and shear values are shown in Table 2.

Salt concentrations of 6 oz or less per gallon of water resulted in concentrations of salt in the peanuts that were too low to be acceptable, unless the cooking time was 20 min or longer and the cooling time longer than 13 hr. Peanuts cooked for 30 min in a solution containing 6 oz of table salt per gallon were higher in finished product salt, 3.34 and 3.10%, as compared to samples boiled 20 min, which contained 2.28 and 1.66% salt. The 20-min sample with 2.28% salt was cooled 21 hr; the sample with 1.66% salt was cooled 13 hr (Table 2).

A titratable salt in the finished product of 2.28% or less was generally considered too low. Concentrations of 4 oz of salt per gallon of water in the cooking and cooling brines always resulted in a concentration of 1.48% salt or less, regardless of cooking or cooling time (Table 2). 8 oz of salt per gallon in the brine resulted in unacceptable salt content when either the cooking or cooling times were short. The data indicated that 6–14 oz of table salt per gallon of water produced peanuts with generally acceptable salt levels over a range of from 10–30 min cooking and 9–29 hr cooling time. Peanuts cooked for 20 min in boiling brine containing 10 oz of salt per gallon and cooled in the brine for 21 hr had a salt content of 3.08% by weight, and were rated good in terms of both salt and texture. Finished product salt above 3.80 was generally considered too high. Salt concentrations between 8 and 16 oz per gallon of cooking solution resulted in high salt content in the peanuts when both long cooking and cooling times were used (Table 2). For example, a sample cooked 30 min in a solution containing 10 oz of salt per gallon and cooled 21 hr had a salt content of 4.08%, whereas a sample cooked the same length of time at the same concentration and cooled 13 hr had a salt content of 3.32% (Table 2).

The shear press data suggested an interaction between cooking time and salt concentration and tenderness, although this relationship was not close (R = 0.342). A trend was noted that high salt and long cooking time resulted in higher shear values. A 40-min cook at 14 oz of salt per gallon of water resulted in the highest shear values, 493 and 520. By contrast, a cook of 45 min in a brine containing 8 oz of salt per gallon resulted in a shear value of 293. The average brine concentration for the 15 lots having the lowest shear value was 6.2 oz of salt per

gallon of water, as compared to an average salt concentration of 11.2 for the 15 lots with the highest shear values (Table 2). It is reasonable to expect that longer exposure times at higher salt concentrations would result in cell plasmolysis and a tendency toward toughness.

The analysis of variance of the regression for the independent and computed variables versus salt content in the finished product is presented in Table 3. The variance ratio of 21.275** was significant at the 1% level of probability. The evaluation showed that 90% of the variance was due to regression (R = 0.901). Interaction of cooking time and salt concentration was found to be the most important factor as regards final salt content of the peanuts.

Analysis of variance for the regression for the independent and computed variables versus the shear press values is shown in Table 3. The variance ratio of 3.599** was significant at the 1% level of probability. 60% of the variance was shown to be due to regression (R = 0.607). Analysis for the standardized partial regression coefficients showed that cook time was the most important contributor to tenderness as measured by shear press. The interaction of cook time and salt concentration in the brine was the second most important factor influencing tenderness.

The analysis of variance for regression for the independent and computed variables versus the Hunter "b" values is shown in Table 3. The data showed that 58% of the variation was due to regression (R = 0.581). The variance ratio of 3.237* was significant at the 5% level of probability. The standardized partial regression coefficient of −1.143 indicated that the cook time was the most important factor affecting the color of the boiled peanuts; however, salt concentration also was a significant factor.

The analysis of variance for the regression for the independent and computed variables versus Hunter "a," Hunter "L" and panel scores showed these relationships to be nonsignificant (Table 3).

Table 3–Analysis of variance for regression for the independent and computed variables as related to the cut-out salt concentration, shear press values, Hunter "L," "a" and "b" values and taste panel scores for frozen boiled peanuts

Variables	R value	Degrees of freedom	Variance ratio-regression
Percent cut-out salt vs. independent and calc. variables	0.901	9	21.275**
Shear press values vs. independent and calc. variables	0.607	9	3.599**
Hunter "b" vs. independent and calc. variables	0.581	9	3.237*
Hunter "L" vs. independent and calc. variables	0.397	9	1.537 ns
Hunter "a" vs. independent and calc. variables	0.351	9	1.264 ns
Taste panel scores vs. independent and calc. variables	0.410	9	1.618 ns

*Significant at 5% level of probability.
**Significant at 1% level of probability.
ns–Nonsignificant.

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EFFECTS OF A CRYOGENIC AND THREE HOME FREEZING METHODS ON SELECTED CHARACTERISTICS OF PORK LOIN CHOPS

SUMMARY—Thirteen pairs of pork loin chops from each of four carcasses were assigned randomly to 13 treatments to study effects of freezing by liquid nitrogen vapor and by three home methods, and effects of 1 and 4 wk of storage in three types of home freezers. Regardless of freezing method, cooking losses (total, volatile and dripping) were higher ($P < 0.01$) and water-holding capacity of the LD muscle lower ($P < 0.05$) for frozen than for fresh chops. Acid number, flavor scores and over-all acceptability scores were higher ($P < 0.05$, $P < 0.01$, $P < 0.01$) for fresh than for frozen chops. Both immediately after freezing and after 1 and 4 wk of storage, liquid nitrogen freezing produced chops superior in appearance to home-frozen chops. Total and dripping cooking losses were greater ($P < 0.05$ and 0.01), and total moisture of the LD lower ($P < 0.05$) for liquid nitrogen-frozen than for home-frozen chops. Free fatty acid was higher ($P < 0.05$) in liquid nitrogen-frozen than in home-frozen chops. Tenderness and over-all acceptability scores were higher ($P < 0.05$) for chops stored in a one-door refrigerator-freezer than for those stored in a two-door refrigerator-freezer or an upright household freezer. Regardless of freezing method or storage conditions, volatile cooking losses and free fatty acids increased ($P < 0.05$), and over-all acceptability and tenderness scores decreased ($P < 0.01$) between 1 and 4 wk of storage. The interaction between storage conditions and storage time resulted in greater ($P < 0.05$) increase in free fatty acids between 1 and 4 wk for chops stored in a one-door refrigerator-freezer than for those stored in the two other types of home freezers. Flavor and over-all acceptability scores decreased ($P < 0.05$) between 1 and 4 wk for chops stored in either refrigerator-freezer combination, but did not decrease during 4 wk of storage for chops in the upright household freezer.

INTRODUCTION

ADVANCES in cryogenic freezing technology have improved quality in many food products (Aref, 1967). Many early researchers investigated the effects of freezing on the quality of meat, stressing the value of rapid freezing rates and low storage temperatures. Hankins and Hiner (1938) reported increased tenderness in steaks frozen at −23 and −40°C compared with those frozen at −7°C. Brady et al. (1942) found that the smallest total cooking losses in steaks resulted from "quick" freezing at −26°C and cooking from the frozen state compared with those frozen at −18°C or thawed before cooking. Dubois et al. (1940) reported that pork stored at −12 and −9°C became rancid in 4 months. Modern commercial cryogenic freezing processes are much more rapid than the freezing rates used in those studies.

Gray (1967) claimed advantages of little loss of moisture, good color and absence of dehydration with liquid nitrogen-frozen hamburger patties. When frozen lamb chops were thawed before cooking, Lind et al. (1971) reported significantly higher tenderness scores for chops frozen in moving air at −29°C and by liquid nitrogen vapor at −107°C than for those frozen in still air at −18°C. However, when the lamb chops were cooked from the frozen state, tenderness scores and Warner-Bratzler shear values did not differ significantly for chops frozen by the three methods.

Some consumers purchase frozen meat from supermarkets as well as purchase fresh meat and freeze it at home. Williams (1966) found that about 80% of all red meat purchased in supermarkets is frozen at home. Another study (Anonymous, 1967) indicated that 94% of the consumers interviewed froze some fresh meat at home. Kansas Agricultural Experiment Station workers (1969) found that about 61% of the consumers interviewed sometimes bought frozen meat, and that over 90% froze meat after taking it home. Weidenhamer et al. (1969) reported that 87% of their respondents sometimes froze fresh meat at home, but few homemakers were interested in purchasing previously frozen raw meat. Research is needed to evaluate the relative effects of cryogenic freezing and freezing by methods available in the home on the acceptability of red meats. This study was designed to investigate the effects of freezing by a commercial liquid nitrogen process and in three types of home freezers on selected characteristics of pork loin chops stored in the three types of home freezers for 1 and 4 wk.

EXPERIMENTAL

PORK LOINS from each of four animals were removed posterior to the 11th thoracic vertebra and anterior to the 6th lumbar vertebra and cut into 13 pairs of chops 1 in. thick, numbered 1 to 13 from the anterior to the posterior end of the loin. A pair consisted of one chop from the left loin and one from the same region of the right loin. Pairs from each animal were randomly assigned to 13 treatments. One pair from each animal was held unfrozen and cooked fresh to provide a basis for studying the effects of the freezing-storage treatment combinations. Each of the remaining 12 pairs was processed by a commercial liquid nitrogen-freezing procedure or by one of three home-freezing procedures. All frozen chops were stored for 1 or 4 wk in each of the three types of home freezers.

For home-freezing treatments, six pairs of chops per animal were packaged in styrofoam trays with an overwrap of polyvinyl film 0.005-in. thick to simulate retail market wrapping. Home treatments employed three household freezers for which temperature fluctuation ranges and average temperatures were determined in preliminary work. These were: H_1, the freezing compartment of a one-door refrigerator-freezer with a temperature range of −10 to −8°C and an average temperature of −9°C; H_2, the freezing section of a two-door refrigerator-freezer with a temperature range of −22 to −16°C and an average temperature of −20°C; and H_3, an upright household freezer with a temperature range of −28 to −14°C and an average temperature of −22°C.

Six pairs of chops per animal were packaged in Dupont Iolon skin-tight film with gas-permeability values ranging from 46.5–77.5 cc per sq m per 24 hr. They were frozen in a simulated liquid nitrogen freezer. Freezing required 8–9 min using the following temperature program: −18°C, 0.5 min; −46°C, 0.5 min; −73°C, 1 min; −101°C, 1 min; −129°C, 1 min; temper 1 min with no more liquid nitrogen vapor entering the cabinet. The time in each step was taken when the cabinet reached a given temperature.

One chop from each pair was selected randomly for sensory evaluation of the longissimus dorsi (LD) muscle and the matching chop was used for objective measurement of the LD muscle. Back fat from both chops was used to measure free fatty acids.

Data for each measurement used to evaluate the chops were analyzed by analysis of variance for a randomized complete block design in which carcasses (animals) were blocks. Data for cooking time of frozen chops were analyzed separately to determine differences among the freezing and storage treatment combinations.

Two pairs of fresh chops were cooked and evaluated at each of two experimental periods, and four pairs of frozen chops were cooked and evaluated at each of 12 experimental periods. Fresh chops were held in the original package in

Table 1–Means and F-values for objective and subjective measurements of fresh and frozen pork loin chops

Measurement	Fresh	Frozen	F-value
Cooking time (min)	36	51	60.00**
Cooking time (%)			
Total	15.71	21.31	29.90**
Volatile	10.07	13.39	13.50**
Dripping	5.26	7.58	23.56**
Total moisture (%)	66.5	65.9	<1
Water-holding capacity[a]	0.71	0.75	6.91*
Juiciness score[b]	5.3	4.5	2.50
Shear value (lb/1.3-cm core)	6.5	6.8	<1
Tenderness score[b]	5.2	4.8	2.42
Oleic Acid (%)	0.72	0.54	20.27**
Acid number[c]	1.02	0.79	7.32*
Flavor score[b]	5.5	4.7	9.22**
Over-all acceptability score[b]	5.4	4.5	11.68**

*P < 0.05. **P < 0.01.
[a]1.0–(expressible-liquid index); the larger the value, the greater the amount of liquid expressed.
[b]7 (extremely desirable, juicy or tender) to 1 (extremely undesirable, dry or tough).
[c]mg KOH per g fat.

Table 2–Means and F-values for objective and subjective measurements of liquid nitrogen- and home-frozen pork loin chops

Measurement	Liquid nitrogen-frozen	Home-frozen	F-value
Cooking losses (%)			
Total	21.92	20.71	4.52*
Volatile	13.50	13.27	<1
Dripping	8.07	7.08	13.92**
Total moisture (%)	65.5	66.4	4.26*
Water-holding capacity[a]	0.75	0.75	<1
Juiciness score[b]	4.4	4.7	2.39
Shear value (lb/1.3-cm core)	6.9	6.6	1.55
Tenderness score[b]	4.8	4.8	<1
Oleic Acid (%)	0.56	0.52	6.36*
Acid number[c]	0.84	0.74	5.04*
Flavor score[b]	4.8	4.6	1.93
Over-all acceptability score[b]	4.5	4.5	<1

*P < 0.05. **P < 0.01.
[a]1.0–(expressible-liquid index); the larger the value, the greater the amount of liquid expressed.
[b]7 (extremely juicy, tender or desirable) to 1 (extremely undesirable, dry or tough).
[c]mg KOH per g fat.

a refrigerator at 5°C for 48 hr before cooking; frozen chops were cooked from the frozen state. Each pair of chops was placed on a 4-in.-high wire rack set in a shallow pan and cooked in a rotary hearth oven at 176°C to an internal temperature of 75°C measured in one chop of each pair. Cooking time was the minutes required to reach 75°C. Time for frozen chops to reach 5°C also was recorded to compare with fresh chops with an initial internal temperature of 5°C.

Cooking losses (total, volatile and dripping) were calculated as the percentage of the initial fresh or frozen weight of a pair of chops.

Two 1.3-cm cores from lateral and medial positions in the LD muscle were sheared once on the Warner-Bratzler shearing apparatus with a 11.4-kg dynamometer.

Percentage total moisture was determined by drying duplicate 10-g samples of ground LD muscle in a C.W. Brabender Semiautomatic Rapid Moisture Tester for 60 min at 121°C.

Water-holding capacity (WHC) was measured by the method described by Miller and Harrison (1965) on 0.3-g samples from the center of the cores used for Warner-Bratzler shear values. Eight samples (two per chop) were measured at one time. This is a press method in which the expressible-liquid index is calculated as a ratio of the area of pressed muscle to the area of expressed liquid. Unity arbitrarily is assumed as the maximum expressible-liquid index for any particular sample of meat and the relative WHC expressed as: 1.0-(expressible-liquid index). The expressible-liquid index is inversely related to the amount of liquid expressed from the sample; therefore, the larger the value for WHC, the more liquid expressed.

The outside fat covering on the chops was ground and extracted by the method of Watts and Peng (1947). Free fatty acids were measured by titrating an aliquot of the fat extracts in neutral alcohol. Results were expressed as percentage oleic acid and acid number (mg KOH per g extracted fat).

The LD muscle was cut into 1.3-cm cubes for sensory evaluation. Cubes were placed in the top of a 0.5-liter enamel double boiler and set over hot water. The entire system was held at a constant low heat (35 ± 1°C) on a General Electric hot tray until all panelists had judged the samples (no longer than 30 min).

A five-member laboratory panel selected cubes at random and evaluated them for flavor, juiciness, tenderness and over-all acceptability. Flavor and over-all acceptability were evaluated on a desirability scale of 7 (extremely desirable) to 1 (extremely undesirable). Juiciness and tenderness were scored on an intensity scale of 7 (extremely juicy or tender) to 1 (extremely dry or tough). Each panelist standardized his tenderness scores by the number of chews required to masticate the cube completely.

RESULTS & DISCUSSION

THROUGHOUT the discussion, H_1 refers to a one-door refrigerator-freezer; H_2, a two-door refrigerator-freezer; and H_3, an upright household freezer.

Appearance of frozen chops

All chops frozen by liquid nitrogen appeared much like fresh chops and had a desirable color. No "bleaching" or cracking was observed in any chop, as in earlier work reported by the Kansas Agricultural Experiment Station (1969). During storage, liquid nitrogen-frozen chops stored in H_1 darkened and developed a grayish color; those in H_2 also darkened, but to a lesser degree; whereas, those in H_3 maintained their original light color. The color of liquid nitrogen-frozen chops was superior to that of home-frozen chops after freezing and after 1 or 4 wk of storage in any freezer. Among home-frozen chops, those frozen in H_1 were darker immediately after freezing than those frozen in the two other types of home freezers. Chops frozen in H_2 and H_3 were light and had good color. During storage, chops in both H_1 and H_2 darkened some, whereas chops in H_3 retained good color. Occasionally, spots of dehydration or frosting, or both, were observed on both liquid nitrogen-frozen and home-frozen chops, but neither dehydration nor frosting was extensive.

Cooking time and losses

Mean total cooking time for fresh chops was 36 min and for frozen chops was 51 min (Table 1), significantly ($P < 0.01$) longer (about one-third) for cooking from the frozen state than for fresh chops. This is consistent with reports of one-third to one-half more time needed to cook frozen meat than to cook fresh or thawed meat (Lind et al., 1971; Lowe et al., 1952; Vail et al., 1943). Time required to reach 5°C was significantly less ($P < 0.05$) for chops stored in H_1 (14 min) than for those stored in H_2 or H_3 (16 min).

Total, volatile and dripping losses were higher ($P < 0.01$) for frozen than for fresh chops (Table 1). The method of freezing also affected cooking losses (Table 2). Total and dripping losses were higher ($P < 0.05$ and $P < 0.01$) for chops frozen by liquid nitrogen than for chops frozen by home methods. Lind et al. (1971) reported higher cooking losses for lamb rib chops frozen by liquid nitrogen vapor at −107°C than for those frozen in moving air at −29°C or in still air at −18°C.

Total and volatile losses increased with storage from 1 to 4 wk, but dripping losses decreased. Differences in cooking losses between storage periods were significant ($P < 0.05$) only for volatile losses (Table 3).

Total moisture, water-holding capacity and juiciness

Total moisture of the LD was not affected significantly by freezing per se (Table 1), storage time (Table 3) or

Table 3—Means and F-values for objective and subjective measurements of pork loin chops after 1 and 4 wk of storage

Measurement	1 wk	4 wk	F-value
Cooking losses (%)			
Total	20.75	21.88	3.94
Volatile	12.72	14.06	7.01*
Dripping	7.60	5.57	<1
Total moisture (%)	66.4	65.6	4.02
Water-holding capacity[a]	0.74	0.76	3.64
Juiciness score[b]	4.7	4.3	2.83
Shear value (lb/1.3-cm core)	6.6	7.0	3.05
Tenderness score[b]	5.0	4.5	10.05**
Oleic Acid (%)	0.52	0.56	4.67*
Acid number[c]	0.74	0.84	4.05
Flavor score[b]	4.9	4.6	<1
Over-all acceptability[b]	4.8	4.2	11.61**

*P < 0.05. **P < 0.01.
[a]1.0−(expressible-liquid index); the larger the value, the greater the amount of liquid expressed.
[b]7 (extremely juicy, tender or desirable) to 1 (extremely undesirable, dry or tough).
[c]mg KOH per g fat.

Table 4—Means, F-values and LSD's for objective and subjective measurements of pork loin chops stored in three types of home freezers

Measurement	Storage conditions H_1	H_2	H_3	F-value	LSD[a]
Cooking losses (%)					
Total	21.36	20.90	21.98	<1	–
Votatile	13.52	13.11	13.54	<1	–
Dripping	7.44	7.48	7.83	<1	–
Total moisture (%)	65.6	66.1	66.3	1.16	–
Water-holding capacity[b]	0.76	0.74	0.75	<1	–
Juiciness score[c]	4.6	4.7	4.4	<1	–
Shear value (lb/1.3-cm core)	6.8	6.8	6.7	<1	–
Tenderness score[c]	5.3 *	4.5	4.8	3.86*	0.4
Oleic Acid (%)	0.57	0.51	0.55	1.98	–
Acid number[d]	0.86	0.72	0.78	2.13	–
Flavor score[c]	5.2	4.5	4.6	<1	–
Over-all acceptability score[c]	4.8 *	4.3	4.4	5.86*	0.4

*P < 0.05.
[a]LSD, Least significant difference at 5% level.
[b]1.0−(expressible-liquid index); the larger the value, the greater the amount of liquid expressed.
[c]7 (extremely juicy, tender or desirable to 1 (extremely undesirable, dry or tough).
[d]mg KOH per g fat.
H_1, one-door refrigerator-freezer. H_2, two-door refrigerator-freezer. H_3, upright household freezer.

storage conditions (Table 4), but was higher ($P < 0.05$) for home-frozen chops than for liquid nitrogen-frozen chops (Table 2). More ($P < 0.05$) liquid was retained (water-holding capacity, Table 1) by LD muscle from fresh than from frozen chops, but water-holding capacity was not affected significantly by method of freezing (Table 2), storage time (Table 3) or storage conditions (Table 4). Taste panel evaluation indicated fresh chops were slightly more juicy than frozen meat, and home-frozen chops were slightly juicier than liquid nitrogen-frozen chops. Juiciness decreased, but not significantly, between 1 and 4 wk of storage. Juiciness scores were not affected significantly by storage conditions (Table 4).

Tenderness

Tenderness, as measured by Warner-Bratzler shear values, was not affected significantly by freezing per se, method of freezing, storage time or storage conditions (Tables 1, 2, 3, 4). Taste panel scores for tenderness were affected significantly only by storage time and conditions (Table 1, 2, 3, 4). Tenderness scores decreased ($P < 0.01$) between 1 and 4 wk of storage, and mean scores were higher ($P < 0.05$) for chops stored in H_1 than for those stored in H_2 or H_3.

Free fatty acid, flavor and over-all acceptability

Both percentage oleic acid and acid number (mg KOH/g fat) are measurements of hydrolytic decomposition of fat around the LD muscle. Although calculations for both values were made with the same titration data, higher F-values were obtained for free fatty acid when reported as percentage oleic acid than when reported as acid number. However, both methods of reporting free fatty acids indicated the same trends (Tables 1, 2, 3, 4). Free fatty acid was higher in fat on fresh chops (percentage oleic acid, $P < 0.01$; acid number, $P < 0.05$) than for frozen chops (Table 1). Values for both measurements were higher ($P < 0.05$) for liquid nitrogen-frozen chops than for home-frozen chops. Taste panel scores for flavor of the LD muscle were higher ($P < 0.01$) for fresh than for frozen chops (Table 1), and approximately the same for liquid nitrogen- and home-frozen chops (Table 2).

Percentage oleic acid values increased ($P < 0.05$) between 1 and 4 wk of storage, acid numbers also increased during storage, but not significantly. Flavor scores did not change significantly between 1 and 4 wk of storage (Table 3). Neither free fatty acid in the fat around the LD muscle nor flavor scores for LD muscle were affected significantly by storage conditions (Table 4).

The interaction between storage conditions and storage time (Table 5) was significant for free fatty acids, flavor and over-all acceptability scores. Free fatty acids increased significantly ($P < 0.05$) between 1 and 4 wk of storage for chops stored in H_1; increased slightly for those in H_2 and decreased slightly for chops in H_3. Mean flavor and over-all acceptability scores decreased ($P < 0.05$) between 1 and 4 wk for chops stored in H_1 and H_2, but remained about the same for those stored in H_3.

Hall et al. (1961) reported that samples of pork fat with the highest free fatty acid did not always receive the lowest flavor scores. For fresh pork fat, the highest flavor scores often were given for samples with the highest free fatty acid values. After 24 wk of freezer storage, samples with free fatty acid levels in the same range as in the fresh samples were given lower flavor scores. Those researchers pointed out that the direct effect of free fatty acids on flavor and aroma would depend on the type of acids liberated as a result of a treatment. In this study, the highest free fatty acids in external fat and highest flavor scores for LD muscle were for fresh chops held in the refrigerator 48 hr before cooking and evaluating; whereas, an increase in free

Table 5–Mean values for percentage oleic acid, acid number, flavor and over-all acceptability scores attributable to the interaction of storage conditions and time

Storage conditions and time	Oleic acid (%)	Acid number[a]	Flavor score[b]	Over-all acceptability[b]
H_1				
1 wk	0.50	0.70	5.4	5.3
4 wk	0.64 *	0.99 *	4.9 *	4.4 *
H_2				
1 wk	0.50	0.70	4.8	4.6
4 wk	0.51	0.76	4.1 *	3.9 *
H_3				
1 wk	0.57	0.80	4.4	4.3
4 wk	0.53	0.75	4.6	4.4
LSD[c]	0.07	0.16	0.5	0.5

*$P < 0.05$.
[a]mg KOH per g fat.
[b]Range, 7 (extremely desirable) to 1 (extremely undesirable).
[c]LSD, Least significant difference at 5% level.
H_1, one-door refrigerator-freezer. H_2, two-door refrigerator-freezer. H_3, upright household freezer.

fatty acid between 1 and 4 wk of freezer storage was accompanied by a slight decrease in flavor scores for the LD muscle (Table 3). Shrewsbury et al. (1942) pointed out that development of free fatty acid does not necessarily parallel development of oxidative rancidity; it usually accompanies an oxidative reaction, and indicates rancidification.

Each source of variation that affected flavor or tenderness of chops significantly also significantly affected over-all acceptability. Over-all acceptability scores were higher ($P < 0.01$) for fresh chops than for frozen chops (Table 1), but did not differ significantly between home- and liquid nitrogen-frozen chops (Table 2). Acceptability scores decreased ($P < 0.01$) between 1 and 4 wk of storage (Table 3). The type of storage affected over-all acceptability (Table 4); scores for chops stored in H_1 were higher ($P < 0.05$) than for those stored in H_2 and H_3. Over-all acceptability was affected ($P < 0.05$) by the interaction between storage conditions and time (Table 5). Mean scores for chops stored in H_1 and H_2 decreased ($P < 0.05$) between 1 and 4 wk of storage, but were approximately the same for chops stored in H_3.

Liquid nitrogen freezing resulted in superior appearance, but the cooked product from home freezing was comparable in quality. All frozen chops were stored in home freezers at −18°C for 4 wk and at −9°C for 1 wk without serious loss of quality.

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MICROBIOLOGY OF BEEF SHELL FROZEN WITH LIQUID NITROGEN

SUMMARY—The effect of freezing primal cuts of beef with liquid nitrogen on the subsequent microbiological quality of their retail cuts was studied. Cryovac-packaged loins were shell frozen by spraying with liquid nitrogen and held for 2 or 3 days at room temperature (25–27°C) in insulated styrofoam boxes. Frozen packaged loins were also aged at 2°C for 21 days and at 22°C for 3 days before being cut into steaks. Steaks from each treated loin and steaks from fresh loins (controls) were packaged and stored in a display case at about 5°C. Examination was made of the loins and packaged steaks during storage to determine total anaerobes, fluorescent Pseudomonas, *coliforms, enterococci,* Clostridium perfringens, *and incidence of* Salmonellae *and coagulase-positive staphylococci. Initial contamination on steaks increased with loins aged for a long time at low temperatures. Aging of loins at 22°C for 3 days promoted multiplication of* C. perfringens *and resulted in the steaks having the highest occurrence of coagulase positive* Staphylococcus. *Recovery of* Salmonella *from the steaks was more closely related to the source and level of contamination of the fresh meat than to time and temperature of holding of the wholesale cuts. These findings have application to shell freezing of beef with liquid nitrogen for air transport and to possible commercial aging practices.*

INTRODUCTION

THE USE OF liquid nitrogen as a refrigerant is a fairly recent development in the food industry for preserving foods (Reynolds and Wagner, 1964; Breyer et al., 1966; Trauberman, 1966; Gray, 1967). The high freezing rate provided by the low temperature of the liquid nitrogen can reduce tissue damage (Meryman, 1956). As a consequence, there could be less shrinkage, drip loss, and color loss characteristic of defrosted foods, resulting in improved quality (Bengtsson, 1967).

Increased amounts of perishable, high value foods may be moved by air in jumbo jet airliners in the future; meat is such a commodity (Walrath and Konicek, 1967; Gill, 1969). Liquid nitrogen sprays have been successfully used for preserving perishable cargoes during transport (Dixon and Robe, 1966).

Interest in the use of cryogenic processing of meats for air transport stimulated the present study. This work was undertaken to investigate the effect of shell freezing primal cuts of beef in liquid nitrogen on subsequent bacteriological quality of the retail cuts prepared from the beef. Consideration was also given to delays and holding conditions that the product might encounter during distribution. Previous work (Rey et al., 1970) was aimed at studying changes in microorganisms on carcasses subjected to elevated temperature for rapid aging; this aspect was also considered for beef frozen with liquid nitrogen in the present work.

MATERIALS & METHODS

BEEF LOINS from different processing plants were divided into three wholesale cuts in the University Meat Laboratory; each cut weighed about 7 lb. For measuring the initial quality of the fresh meat, one piece from each loin was separated at random, cut into steaks and stored under conditions similar to those in a retail store. The other two pieces from each loin were distributed for different treatments. These cuts were vacuum packaged in Cryovac bags (Cryovac Div., W. R. Grace and Co., Duncan, S.C.). Air was removed from the bags with a Cryovac machine to obtain a vacuum of 8–10 in.

A spray method was used for freezing with liquid nitrogen in a Heath freezer tunnel, 15 ft

Table 1—Bacteriological procedures employed

Quantitative determinations[a]	Growth media	Plating technique	Incubation	Confirmatory tests
Total aerobes	Trypticase soy agar (BBL)[b]	Pour plates	15°C, 5 days	
Fluorescent *Pseudomonas*	Medium B of King et al., 1954	Surface plating	15°C, 6 days	Fluorescence under UV light
Enterococci	KF Streptococcus medium (Difco)[c]	Pour plates	37°C, 2 days	Growth: 45°C, 10°C, 6.5% NaCl, pH 9.6. Reduction of 0.1% methylene blue (Kenner et al., 1960)
Coliforms	Violet red bile agar (Difco)[c]	Pour plates (overlayed)	37°C, 24 hr	Levine EMB agar (Difco)[c]
Clostridium perfringens	SPS agar (Angelotti et al., 1962)	Anaerobic pouches (Bladel and Greenberg, 1965)	37°C, 24 hr	Motility and H_2S (Angelotti et al., 1962)
Qualitative determinations[d]	**Enrichment**	**Isolation**		**Confirmatory tests**
Salmonella	Procedure for meats (Galton et al., 1968)	BGS agar (Galton et al., 1968)		TSI and agglutination (Galton et al., 1968)
Coagulase positive *Staphylococcus*	Broth medium of Wilson et al., 1959	Staph 110 medium with egg yolk (Herman and Morelli, 1960)		Tube coagulase test

[a]A swab technique was used for sampling 10 cm² of surface.
[b]BBL Division of BioQuest, Cockeysville, Md.
[c]Difco Laboratories, Detroit, Mich.
[d]A swab technique was used for sampling 60–80 cm² of surface.

Table 2–Experimental design for treatments applied to the meat

	Wholesale cuts						Packaged steaks (Retail conditions)
			Time of holding frozen meat at room temp		Aging		
Conditions simulated	Cryovac-packaged	Liquid nitrogen freezing	2 days	3 days	2°C 21 days	22°C 3 days	5°C 7 days
Fresh controls							X
Short trip with immediate distribution	X	X	X				X
Short trip with subsequent holding	X	X	X		X		X
Long trip with immediate distribution	X	X		X			X
Long trip with subsequent holding	X	X		X	X		X
High temperature aging	X					X	X
Low temperature aging	X				X		X

Table 3–Analysis of variance of bacteria recovered from steaks

		Mean squares				
Source of variation	d.f.	Total aerobes	Fluorescent Pseudomonas	Enter-cocci	Coli-forms	Clostridium perfringens
(A) Treatments of the wholesale cuts	8	105.127[a]	134.233[a]	116.593[a]	216.161[a]	33.873[a]
(B) Storage time of the steaks under retail conditions	4	310.005[a]	377.283[a]	0.627	108.398[a]	2.579[a]
(A) × (B)	32	3.658[a]	5.554[a]	0.707	8.432[a]	0.863[a]
Error	405	1.039	2.244	0.639	2.483	0.446

[a]Significant at 5% level.

long (Union Carbide Corp., Chicago, Ill.). Before every operation, the tunnel was precooled to −170°C; each freezing cycle consisted of 6 min for precooling and spraying. These operations resulted in shell freezing of the loins so that an ice crust about ¼ in. thick was formed on the surface. Styrofoam boxes (2 × 1½ × 2¼ ft and 2½ in. thick), precooled with liquid nitrogen, were used for storing frozen loins.

Short and long periods of aging were combined respectively with high and low temperature. Short aging involved holding of the boxed Cryovac-packaged loins in a chamber at 22°C (± 1.5°) for 72 hr. Long time aging consisted of storage of the samples in a cooler at 2°C (± 1.5°) for 21 days.

Steaks approximately ¾ in. thick were cut from every primal cut immediately after arrival of the meat for the fresh controls, or at the end of each of the storage times for the aged samples. The steaks were individually placed in 6 × 5½ in. plastic trays and wrapped with 195 MSAD-80 cellophane (E. I. DuPont de Nemours and Co., Inc., Wilmington, Del.). Packages were stored in a display case at 5°C (± 1.5°) under soft white fluorescent lights to simulate retail holding conditions for the meat. Samples were analyzed at intervals to determine the progress of spoilage and incidence or numbers of potential food poisoning organisms during storage.

Procedures used for bacteriological analyses are summarized in Table 1.

Records were kept of: (1) the temperature of the environment of the insulated boxes in which the frozen wholesale cuts were stored; (2) the internal temperature of the frozen samples; and (3) the ambient temperature of the room where the insulated boxes containing the frozen meat were located for holding. An automatic recorder (Honeywell Potentiometer, Ft. Washington, Pa.) and properly calibrated copper-constantan thermocouples were used for temperature measurements.

Treatments given to the meat were designed to study effects that various conditions of handling of the wholesale cuts would have on the microbiological quality of the meat reaching the consumer. A summary of the treatments is presented in Table 2.

Analysis of variance described by Snedecor (1965) was performed for all determinations. A modification of the test of Tuckey given by Snedecor (1965) for all comparisons among means was applied to all qualitative determinations. The frequency of occurrence of coagulase positive *Staphylococcus* was evaluated by a Chi-square test.

RESULTS & DISCUSSION

FIGURE 1 shows that the minimum temperature observed at the center of the loins was −22.5°C, recorded 3 hr after shell freezing. After that time, the temperature of the loins increased fairly rapidly and then gradually, maintaining an almost constant difference with the temperature of the box atmosphere. The normal temperature variations in the storage room did not seem to influence the temperature of environment of the boxes or of the meat. With storage in insulated boxes, the meat temperature was maintained below 5°C for 72 hr.

An analysis of variance (Table 3) indicated that the treatments applied to the loins had a significant effect on the contamination of steaks subsequently cut from the loins. Tests for comparisons among means demonstrated that the differences observed in initial contamination of steaks increased with increases in time involved in the treatments. All freezing and holding treatments for loins produced greater initial loads on steaks than did freshly cut loins.

A regression line relating treatments of loins to subsequent initial loads on steaks at the time they were cut from the loins, and their keeping time is shown in Figure 2. This again points out that, in general, the longer the time involved in treatment,

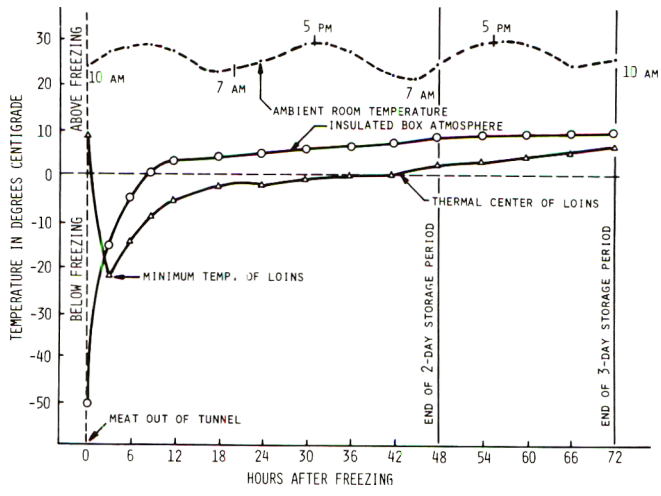

Fig. 1–Changes in temperature of environment and shell-frozen loins during storage in insulated boxes.

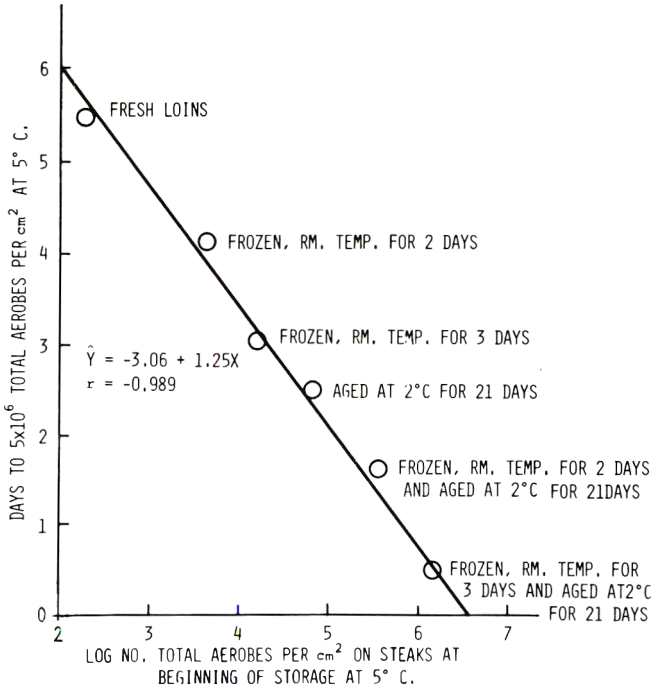

Fig. 2—Relation of treatment of loins to initial contamination and keeping time of steaks during storage at 5° C.

the greater the initial bacterial loads on steaks and the more rapidly the meat became spoiled. Lowest numbers of total aerobes present initially on steaks corresponded to fresh loins. Effects of freezing and holding appeared to be additive. The influence of aging of loins at 2°C for 21 days carried over to the steaks during later storage at 5°C. Aging of frozen loins resulted in shorter keeping time for retail cuts than obtained when the frozen loins were not aged.

Growth curves for various types of bacteria on steaks from fresh loins, loins frozen with liquid nitrogen and held for 2 or 3 days, and loins aged at elevated temperature are shown in Figure 3. The analysis of variance indicated that these treatments produced significant differences in numbers of fluorescent *Pseudomonas*, coliforms, enterococci and *Clostridium perfringens*, recovered from steaks during subsequent storage at 5°C. Aging of loins at 22°C resulted in greatest numbers of all types of bacteria on the steaks. This treatment also produced somewhat greater initial loads of coliforms than fluorescent *Pseudomonas* on steaks, but the latter type of organisms formed a greater proportion of the aerobic flora during later storage. Regardless of treatment of the loins, counts of fluorescing organisms increased, more closely approximating those of total aerobes than did the other types. Coliforms were not always detected on fresh meat, and enterococci were recovered even less frequently. During storage of steaks from aged loins, levels of enterococci were quite constant. Counts for *C. perfringens*, similar to those for enterococci, indicated that aging of wholesale cuts at 22°C for 3 days promoted multiplication of the organism. However, storage of the steaks at 5°C significantly reduced the numbers of *C. perfringens* during the 7-day period. *C. perfringens* and enterococci were present in such low numbers on fresh or frozen unaged beef that they are not shown here.

Salmonellae were recovered from only 14 of 470 steak samples, with an incidence of about 3%. Samples yielding *Salmonellae* most frequently during holding at 5°C corresponded to the sets of fresh steaks with the highest incidence of enterococci and coliforms as initial contaminants. However, no *Salmonellae* were recovered from steaks prepared from fresh beef loins. Incidence of the potential pathogen was influenced more by degree of contamination of the loins than by subsequent treatments given to the loins. There was more difference in recoveries of *Salmonellae* between different lots of loins than between treatments within the lots.

As may be expected, coagulase positive staphylococci were recovered from steaks more frequently than *Salmonellae* were. Incidence of staphylococci ranged from about 60% for steaks from loins held at high temperature for a short time to about 5% from steaks prepared from loins kept at 2°C for 21 days. For treatments involving low temperature, including liquid nitrogen freezing, staphylococci were recovered most frequently from steaks prepared from loins held for the shortest time. During storage of the retail cuts at 5°C, incidence of staphylococci also decreased, as the storage period progressed. Differences were significant as noted in Table 4.

From the results presented here, the following conclusions were made: (1) Shell freezing with liquid nitrogen may be used to advantage without undue health hazard for transportation or hold-

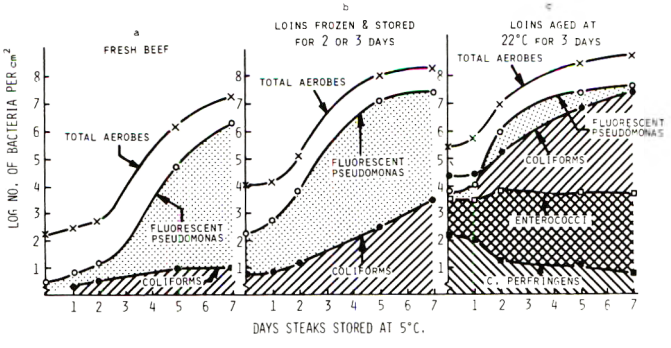

Fig. 3—Effect of treatment of loins on growth of various bacteria on steaks stored at 5° C.

Table 4—Tests for significance of occurrence of coagulase positive staphylococci on steaks

Variables tested	d.f.	Chi-square	Level of significance (%)
Treatment of loins	6	82.389	0.5
Storage of steaks at 5°C for 7 days	4	15.203	0.5
Loins after freezing and holding at room temp for 2 or 3 days			
vs. fresh loins	1	4.759	5.0
vs. aged[a] loins	1	27.310	0.5
Fresh vs. aged[a] loins	1	9.452	0.5
Aging at 2°C for 21 days vs. aging at 22°C for 3 days	1	36.966	0.5

[a]Low-temperature-long time aging (2°C for 21 days).

ing of wholesale beef cuts for 1–3 days with no further refrigeration, but with insulated packaging, when environmental temperatures are not excessively high; (2) Prolonged aging of fresh wholesale cuts at 2°C for 21 days does not increase probabilities of occurrence of *Salmonellae, C. perfringens*, or coagulase positive staphylococci on the subsequently prepared retail cuts but does proportionately decrease their shelf life because of development of spoilage types of psychrophilic bacteria; (3) Aging of wholesale cuts at 22°C for 3 days promotes growth of *C. perfringens* and increases the probability of incidence of coagulase positive staphylococci on the retail cuts. It also results in retail cuts with a high initial contamination, thus accelerating spoilage; and (4) Neither aging at 22°C for 3 days nor aging at 2°C for 21 days provides desirable keeping quality for shell-frozen meat which is kept in insulated containers for 2 or 3 days, similar to conditions described. Liquid nitrogen freezing of beef loins subsequently held in insulated containers at room temperature for 2 or 3 days does not retard spoilage of retail cuts if the loins are aged for a long period (21 days) before cutting.

Studies are continuing on the effects of fluctuating temperatures, such as might be encountered during diurnal variations in transport and holding of meats shell frozen with liquid nitrogen.

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COMPARISON OF TEXTURE OF CARROTS FROZEN BY AIRBLAST, FOOD FREEZANT-12 AND NITROGEN VAPOR

SUMMARY—Carrot sticks were frozen by immersion in Food Freezant-12, in vapor from liquid nitrogen, in a fluidized bed tunnel and on trays. Those frozen in Food Freezant-12 were the firmest and most crisp. Texture was related to time needed to freeze the carrots. Either a puncture-testing device or shear press can be used to test for firmness.

INTRODUCTION

RECENT INNOVATIONS have resulted in more rapid freezing of foods, along with improvement in their texture. The effect of rapid freezing of green beans on texture has been demonstrated by Wolford and Brown (1965). They showed that rapid freezing in liquid nitrogen resulted in less tissue damage and consequently better texture than found in conventionally frozen beans. Brown (1967) showed tissue damage during freezing was related to speed of freezing. When the rate was changed during freezing, the part frozen most slowly showed the most tissue damage.

In exploratory experiments on freezing vegetables by immersion in liquid nitrogen or in Food Freezant-12 (dichlorodifluoromethane, or F-12), we observed that carrot sticks frozen by immersion in either medium retained much firmer, crisper texture and, after thawing, were suitable as a snack item to be eaten out of hand.

The work reported here compares carrot sticks frozen by immersion in F-12 or by exposure to vaporized liquid nitrogen as in a commercial liquid nitrogen freezer with those frozen by airblast on a tray with −16°F air blowing over the product or in a fluidized bed tunnel at −32°F.

EXPERIMENTAL

FRESHLY HARVESTED Royal Chatenay variety carrots of 2- to 3-in. diameter were washed, peeled and cut into 4-in. sections. The sections were cut into 4-in. julienne strips of 3/8-in.-square cross section. Most of the carrots were steam blanched at 205°F for 2.5 min. Raw, unblanched carrots also were frozen.

The freezing methods used were: 1) Tray freezing (TF), in which the carrot sticks were spread in a single layer on a wire-mesh tray put directly under the circulating fans in a room at −10 to −16°F with air velocity at 840 ft/min. 2) Individual quick freezing (IQF), in which the carrots moved on a wire-mesh belt through a tunnel in which refrigerated air at −20 to −30°F was forced upward through the product at a rate that kept the product in a fluidized condition. Air velocity in the tunnel was measured at 1,600 lineal feet per minute. The measurement was made with no load in the tunnel. However, the engineer who designed the tunnel estimated 800 ft/min velocity with the belt loaded. 3) Nitrogen vapor (N_2), in which trayloads of carrot sticks were placed in a chamber into which liquid nitrogen was vaporized. The chamber was designed to simulate the tunnels in which freezing with vaporized liquid nitrogen is done commercially. The starting temperature in the freezing chamber was −30°F and dropped to −130°F during the freezing process. It was not possible to measure the wind velocity in the chamber at the time we had use of it. However, the lender of the equipment estimated gas movement roughly equal to a 15-mile-per-hour wind. 4) F-12 freezing, accomplished by lowering the carrots in a closed basket into liquid Food Freezant-12 at −22°F. When the freezant came in contact with the product a rapid boiling action took place. 2.5 lb of F-12 were boiled off for every pound of food frozen. The heat removed from the product provided only heat of F-12 vaporization, and the temperature stayed at the F-12 boiling point.

When N_2 and F-12 lots were frozen, the center temperature of a carrot stick in the batch was followed by means of a thermocouple inserted into the center of the carrot stick. The thermocouple wires led to a potentiometer calibrated to read temperatures in degrees Fahrenheit. When the temperature approached 0°F, the basket was removed from the freezant. Test runs were made using the thermocouple in carrots as they were frozen by TF or IQF to determine the speed of travel of the fluidized bed belt for IQF and the minimum time of retention under the blower for lowering the temperature of TF carrots to 0°F.

The frozen carrots were sealed in laminated aluminum-clad bags and stored at −10°F. When thawing for textural measurements, the packages of frozen carrots were immersed in warm running water at 100–110°F until thawed. Thawing was accomplished in 1 hr. When thawing for drip testing, frozen carrots were weighed into funnels located so the drippings from thawing would be caught in graduated cylinders for measurement. Testing of frozen carrots was done after 3–6 months of 10°F storage.

Characteristics of thawed carrots

Flexibility of thawed carrots was determined by holding a carrot stick by one end to see if it remained erect, or by bending the stick until it snapped or coiled without breaking. Coiling is the term we used to denote that the carrot stick could be bent through 360 degrees or more without breaking.

Loss of fluid from thawed carrots was determined by allowing 100g of carrots to thaw in a funnel draining into a graduated cylinder. The drippings were caught and measured. The carrot pieces were also coiled so that any liquid squeezed from them in the process would be collected in a graduated cylinder for measurement.

Puncture resistance

A Ballauf Puncture Tester equipped with a 0.311-in.-diameter plunger was used to test puncture resistance. Thawed carrot sticks were placed in a block containing a groove slightly larger in cross section than that of the carrot stick. The plunger was pushed against the carrot until the tissue was ruptured and the plunger snapped through the carrot stick. The puncturing pressure was read on five punctures in each of 20 carrot sticks per sample, with sampling as available material permitted.

Shear values

Shear values were determined by using six carrot sticks arranged at right angles to the direction of the slots in the standard cell of the L.E.E.-Kramer Shear Press. Triplicate readings were made of each sample, unless the available sample was insufficient to allow three readings. The 1,000-lb scale with 3,000-lb ring was used for thawed carrots.

RESULTS

Freezing times

The time required for the carrot sticks to reach zero center temperature varied from 1 min in F-12 experiments to about 12 min when tray freezing was used (Fig. 1). The fluidized bed freezing was accomplished in 6 min. The temperature in N_2 vapor-frozen carrots was lowered to +9°F in 6.5 min, with the temperature falling rapidly at that time. Center temperature readings during freezing are presented in Figure 1.

Thawed carrot characteristics

Figure 2 shows thawed F-12 and IQF carrot sticks. The former retained enough rigidity to stand upright with very little bending, while the airblast-frozen carrot sagged. This illustrates that correspondingly little structural damage was done to the tissue rapidly frozen in F-12, whereas considerable change occurred in the carrot sticks frozen in a period six times as long as that required to freeze in F-12.

F-12-frozen carrot sticks, after thawing, could not be bent double without breaking. More slowly frozen samples could, after thawing, be twisted through 360 degrees or more without breaking (Fig. 3).

Another indication that less tissue

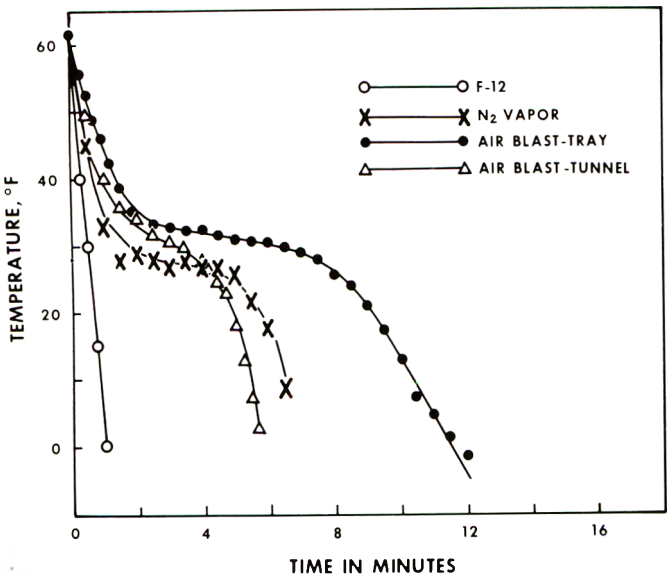

Fig. 1—Center temperature of carrot sticks during freezing operation.

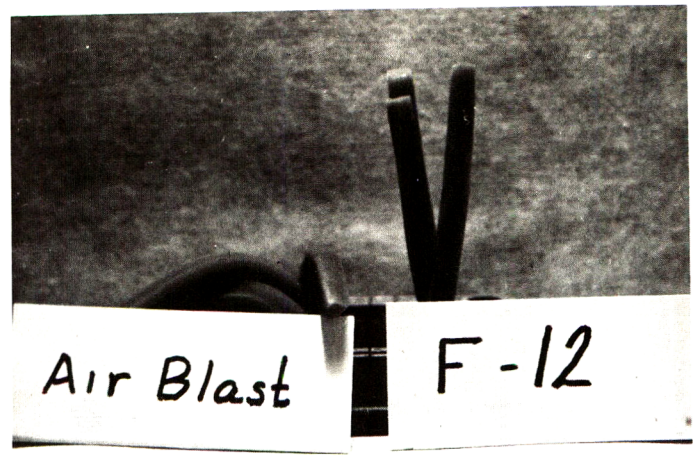

Fig. 2—Thawed carrot sticks showing loss of rigidity in air-blast-frozen and retention of rigidity in F-12-frozen carrots.

damage was done by freezing in 1 min than in 6 min was the amount of liquid lost during thawing and bending or coiling the thawed carrots. When blanched carrots were tested, a drip loss of 1–2% during thawing was found, regardless of freezing methods. However, when bent or coiled, considerable difference was recorded (Fig. 4). Liquid loss during bending of F-12-frozen sticks was in the order of 1%. With all other freezing treatments, many times this amount of liquid was lost during bending or coiling the thawed sticks. F-12-frozen unblanched carrots behaved much differently during thawing than did any of the blanched samples. As the unblanched F-12-frozen sticks thawed, drops of liquid oozed from the carrots. Roughly ten times the amount of drip loss came from unblanched than from the blanched F-12 samples. The unblanched F-12-frozen carrots maintained their rigidity, however, and bending them until they snapped produced very little more fluid loss. A surface bleeding was noted when unblanched tray-frozen carrots were thawed. The drip loss was greater for this treatment than for blanched tray-frozen carrots. Unblanched tray-frozen carrots lost more fluid when coiled (they did not break when bent double) than did the unblanched F-12-frozen carrots, which broke before being bent double. Lack of turgidity and fluid loss from the more slowly frozen carrot sticks indicate greater tissue damage during freezing than occurred in F-12-frozen carrots.

Textural measurements

The quantitative measurement of textural properties as determined by shear press or puncture test showed highest readings in F-12-frozen carrots. Puncture values of carrot sticks frozen by the various treatments are shown in Table 1. The data presented in Table 1 show the mean and standard error of the mean of puncture values of 40–80 carrot sticks, or of 200–400 puncture readings per freezing treatment. When analyzed by Tukey's procedure for multiple comparison (1953), all possible comparisons were significantly different at the 0.01 level.

Shear press data for the same lots of carrot sticks as were tested for the puncture tests summarized in Table 1 are presented in Table 2. These data represent means and mean standard of error for at least nine shear press determinations of each treatment, with the exception of the blanched TF sample, for

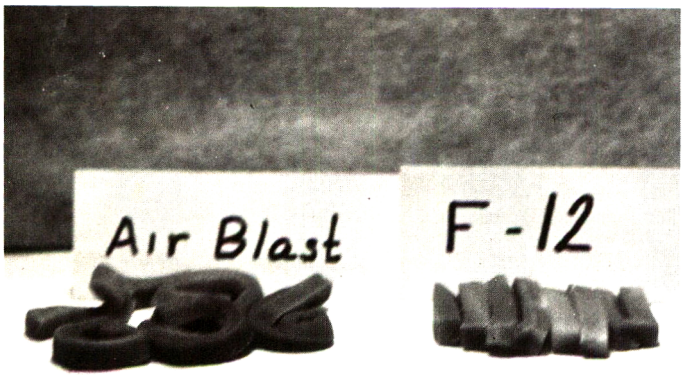

Fig. 3—Coiling of carrot sticks. F-12-frozen carrots broke when bent double; air-blast-frozen sticks could be coiled without breaking.

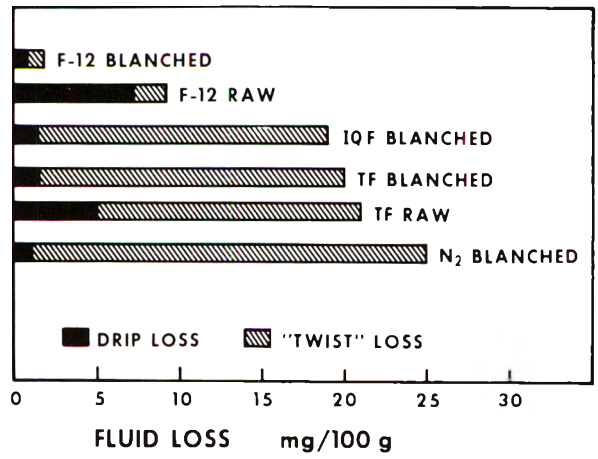

Fig. 4—Fluid loss from carrots during thawing and twisting or coiling.

which sufficient TF carrot sticks were available for only four readings. Significant differences (0.05 level) were found between the F-12 and IQF, and between IQF and N_2F (0.01 level), but not between N_2F and TF values when the four freezing treatments of blanched carrots were tested. Shear values of unblanched carrot sticks were significantly different at the .01 level from their blanched counterparts. Tukey's procedure was also used in comparing shear data.

When tasting thawed sticks, the crispness of the F-12-frozen samples made them the predominantly favored ones. Blanched F-12-frozen carrot sticks were preferred to unblanched, even though the unblanched had a little more snap when chewed. The unblanched samples had an unnaturally sweet flavor. Also, the liquid which oozed from the surface made the unblanched sticks unpleasant to handle when eating them out of hand. The carrot sticks frozen by airblast, whether by tunnel or by tray, and by nitrogen vapor were less satisfactory for eating out of hand, as they lacked the crispness of the F-12-frozen carrots.

Table 1—Effect of freezing method on puncture values of thawed carrot sticks

Freezing method	Puncture values	
	Blanched	Raw
F-12	20.88 ± 0.33	24.84 ± 0.38
IQF	17.69 ± 0.31	
N_2F	15.12 ± 0.29	
TF	13.08 ± 0.31	18.98 ± 0.30

Table 2—Effect of freezing method on shear value of thawed carrot sticks

Freezing method	Shear value	
	Blanched	Raw
F-12	805 ± 57	1033 ± 63
IQF	605 ± 60	
N_2F	334 ± 24	
TF	300 ± 10	644 ± 95

DISCUSSION

OF THE FREEZING METHODS used in this study, F-12 freezing was clearly the most impressive in producing frozen carrots retaining much of the textural quality of fresh carrots. Retention of firm texture is a function of speed of freezing. Earlier experience in this laboratory with liquid nitrogen immersion freezing produced carrot sticks which maintained firm, crunchy textural characteristics after thawing. This LN freezing was accomplished in less than a minute. With liquid nitrogen immersion freezing, however, the danger of cracking or shattering of the product is great unless the operation is carefully controlled. Freezing with nitrogen vapor eliminated the great temperature differences, but slowed freezing to the point where there was little advantage over airblast freezing. This must be balanced against the elimination of shattered product. By controlled spray application of liquid nitrogen to the product, as is done in some other commercial applications of liquid nitrogen to food freezing, splitting may be minimized without slowing the freezing time to the point where there is no textural advantage over airblast freezing. Spray-freezing equipment was not available to us at the time this work was done.

Both methods for determining the numerical values of firmness of the product were useful. The Ballauf Puncture Tester showed differences in carrot sticks which agreed with other observations of the product. The L.E.E.-Kramer Shear Press data also agreed with the observation that shorter freezing times produce firmer products. The shear press can give usable data in a shorter time. The pressure tester requires a steady hand and replicated readings. In the manner in which the two instruments were used, 20 carrot sticks, each punched five times, were found roughly to equal a triplicated reading of the shear press. There was considerable variation in data from both instruments. It must be considered, however, that there is variation in the carrot sticks depending on the amount of core tissue in the individual piece of carrot. Core tissue, giving lower readings than the outer portion of the carrots, was eliminated whenever possible, but much of the product being tested had some core tissue in each stick and this also varied within the individual carrot stick.

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STABILIZATION OF ANTHOCYANINS IN FROZEN TART CHERRIES BY BLANCHING

*SUMMARY—Unpitted red tart cherries (*Prunus cerasus *L. cv. Montmorency) were blanched in steam (100°C) for 0, 30, 45 and 60 sec, then frozen at −20°C. The anthocyanin color of the fruit was determined periodically during frozen storage for 3 months in one experiment and 10 months in another. When the cherries were not allowed to thaw before the analysis, no color loss due to anthocyanin destruction was observed in either the blanched or unblanched cherries. When they were thawed at room temperature (22°C) in single layer for 2 and 4 hr, the unblanched cherries lost 14 and 25% anthocyanin color, respectively; cherries subjected to 45- or 60-sec blanching showed no significant color loss. When the cherries were disintegrated in a Waring Blendor for up to 30 min, the unblanched cherries lost considerable color (70%) after 30 min under oxygen or air, but those blanched for 45 or 60 sec suffered no color loss. Some anthocyanin destruction was observed in the 30-sec blanch lot. Blending under oxygen was slightly more deleterious to the color than blending under air. Blending under nitrogen minimized the color loss but did not eliminate it. Blanching resulted in a 4–7.5% loss of weight.*

INTRODUCTION

THE RED COLOR of Montmorency tart cherries is due to several anthocyanin pigments (Dekazos, 1970). These pigments are rather unstable and susceptible to enzymatic degradation (Huang, 1955; van Buren et al., 1959; Peng and Markakis, 1963; Grommeck and Markakis, 1964). A process which inhibits or inactivates the enzymes responsible for the anthocyanin degradation might be effective in preventing the color loss due to enzyme-anthocyanin interactions. Also, other enzyme-catalyzed deteriorative changes (e.g., browning) may be controlled by such a process.

Blanching of vegetables before storage in the frozen state is an established industrial practice. Blanching before freezing has also been recommended for some fruits (Bauernfeind et al., 1947; Cruess and Seagrave-Smith, 1946; Lee, 1944; Sorber, 1943), mainly apple slices.

Large quantities of red tart cherries are frozen for subsequent processing. If the frozen fruit is heated quickly after removal from the freezer at temperatures sufficiently high to inactivate the enzymes, the color may be preserved. Slow thawing is a frequent occurrence, however, and comminution of the unheated fruit for the preparation of infant foods is also a common practice. In this study, steam blanching before freezing was explored as a possible way of minimizing post-freezing color loss in Montmorency cherries.

MATERIALS & METHODS

THE RED TART cherries (*Prunus cerasus* L. cv. Montmorency) were obtained from Western Michigan. They were received at the laboratory within 2 hr after harvest. Upon arrival at the laboratory the fruit was soaked for 4 hr in vats containing cold (7–10°C) running water, sorted to remove unsound cherries, placed in single layer on perforated stainless steel blanching trays of known tare and weighed. Three periods of blanching in 100°C steam were used, 30, 45 and 60 sec. There was also an unblanched control lot. Immediately after blanching, the trays with the fruit were placed in a −20°C freezer with low-velocity air movement. 16 hr later the fruit and the trays were reweighed, the cherries were transferred into 30-lb cans and returned to the freezer. Two cans for each blanching period were held in frozen storage. The storage lasted 300 days for the crop harvested in 1968. The experiment was repeated in 1969 and the storage that time lasted 100 days.

Total anthocyanin content of the fruit was determined periodically during storage by a procedure which combined the methods of Sondheimer and Kertesz (1948) and Fuleki and Francis (1968). Randomly picked cherries were quickly pitted by hand over a weighed beaker to obtain a 50-g sample of still frozen tissue. 100 ml of extracting solvent composed of 85 ml of 95% ethanol and 15 ml of 1.5 N HCl was added to the sample, the mixture boiled 1 min and then disintegrated in a Waring Blendor for 2 min at high speed. The blended mixture was transferred to a beaker quantitatively by using another 25 ml of extracting solvent and stored overnight in the refrigerator. The following morning the mixture was filtered through sharkskin paper under suction, the residue washed with another 50 ml of extracting solvent and the combined filtrate made up to 250 ml by adding extracting solvent.

3 1-ml aliquots of the above extract were

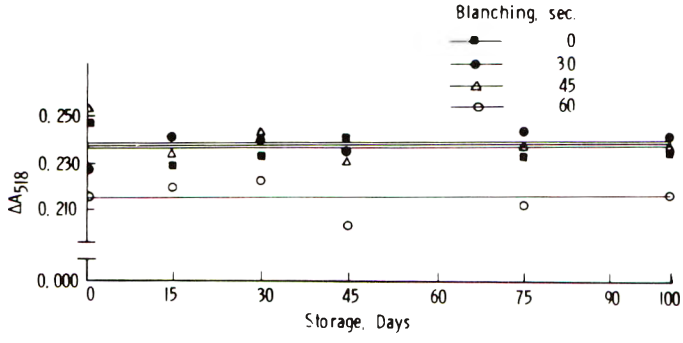

Fig. 1—Differential absorbance values, ΔA_{518} (see Table 1), of Montmorency cherries steam blanched for various periods and periodically analyzed during 100 days of frozen storage (average of triplicate readings).

Table 1—Differential absorbance values, ΔA_{518}[a], of Montmorency cherries steam blanched for four different periods and periodically analyzed during 300 days of frozen storage. (Average of triplicate readings)

Storage time (days)	Blanching time (sec)			
	0	30	45	60
0	0.293	0.306	0.330	0.300
15	0.323	0.305	0.312	0.333
30	0.275	0.319	0.333	0.316
45	0.292	0.305	0.298	0.295
60	0.297	0.306	0.311	0.313
75	0.314	0.304	0.318	0.307
135	0.283	0.303	0.323	0.323
150	0.318	0.300	0.315	0.327
180	0.276	0.316	0.293	0.335
240	0.303	0.309	0.318	0.310
300	0.295	0.317	0.335	0.335
Average	0.297	0.308	0.317	0.318

[a] ΔA_{518} = absorbance at 518 nm of fruit extract adjusted to pH 1.0 minus absorbance at 518 nm of same extract adjusted to pH 4.5.

Table 2—Effect of blanching on the anthocyanin retention in red tart cherries during thawing at 22°C

Thawing time (hr)	Blanching time (sec)			
	0	30	45	60
	Percent anthocyanin retention			
0	100	100	100	100
2	86	97	100	100
4	75	92	98	100

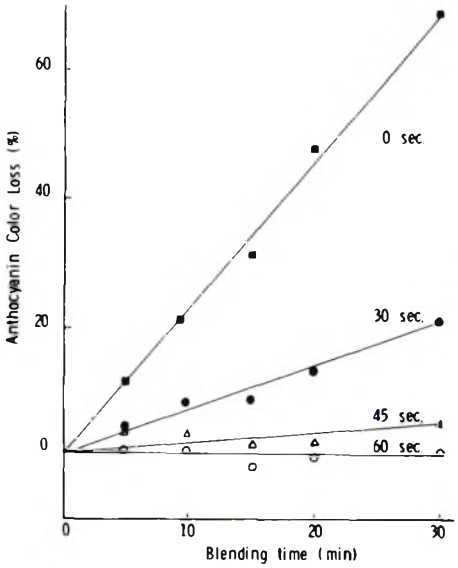

Fig. 2–Anthocyanin color loss in red tart cherries steam blanched for 0, 30, 45 and 60 sec, frozen and thawed in a Waring Blendor under oxygen.

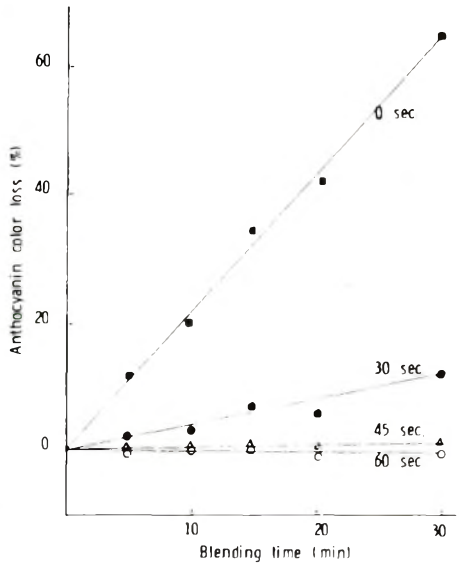

Fig. 3–Anthocyanin color loss in red tart cherries steam blanched for 0, 30, 45 and 60 sec, frozen and thawed in a Waring Blendor under air.

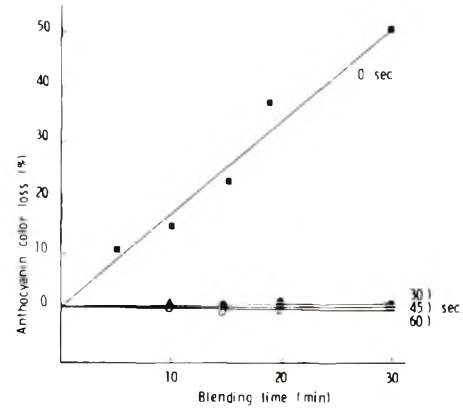

Fig. 4–Anthocyanin color loss in red tart cherries steam blanched for 0, 30, 45 and 60 sec, frozen and thawed in a Waring Blendor under nitrogen.

each diluted with 9 ml of 0.05 M KCl –0.13 M HCl buffer, pH 1.0. Similarly, another 3 1-ml aliquots of extract were diluted with 4 ml 0.16 M sodium acetate –.25 M HCl buffer, pH 4.5. The buffered extracts were held in the dark for 1 hr before their absorbance was measured in a Beckman DU spectrophotometer at 518 nm, the absorption maximum for the cherry extract, against a water blank. The difference between the absorbance of the sample buffered at pH 1.0 and half (dilution correction) the absorbance of the sample buffered at pH 4.5 was taken as a measure of the total anthocyanin content of the fruit.

In the rapid oxidation test, 100g pitted cherry sample was mixed with 100 ml distilled water in a Waring Blendor at high speed for 2 min. After 3 additional min of blending at low speed, a 20-ml aliquot was removed from the mixture and added to 20 ml of extracting solvent. Similar aliquots were removed and mixed with extracting solvent after 10, 15, 20 and 30 min of total blending time. Subsequent extraction, filtration, mixing with buffers and absorbance measurement were performed as previously described.

In the thawing experiment the cherries were allowed to thaw for 2 and 4 hr in single layer at room temperature (22°C) before analysis with the procedure described for frozen samples.

RESULTS & DISCUSSION

LOSS OF WEIGHT resulting from the blanching and the following 16-hr storage in the freezer was 0.6% for the control, 4.6% for the 30-sec blanch lot, 6.3% for the 45-sec blanch lot and 8.1% for the 60-sec blanch lot. Some of this loss is certainly due to dripping and some may be caused by the higher evaporation from the heated fruit. Quicker freezing, such as the high-velocity cold blast, could reduce the weight loss.

The anthocyanin content of the red tart cherries during the 300-day storage period is presented in Table 1. Since the average deviation of the anthocyanin determination method is ± 5%, the differences observed either among periods of storage for the same treatment or among treatments for the same period appear insignificant. The data on the 100-day storage experiment are illustrated in Figure 1 and again indicate no significant differences either among storage periods or among treatments for the 0-, 30- and 45-sec blanch periods; the 60-sec data are consistently lower, but the mean difference between this treatment and the average of the other three is only 10% and it could be due to excessive drip. Therefore, it can be concluded that steam blanching did not result in better anthocyanin retention in frozen red tart cherries which were not allowed to thaw before pigment was determined.

Visual observation also revealed no color loss, browning or scald storage.

Rapid oxidation tests on the frozen cherries of the 1968 crop were conducted on the second, 20th and 40th wk of storage. Results of all three tests were very similar and are summarized in Figures 2, 3 and 4. The data represent percentage loss of color due to anthocyanin during 30 min of blending.

Unblanched cherries subjected to mixing with air or oxygen while they were being blended suffered severe losses in anthocyanin color (65 to 70% after 30 min of blending). The losses were smaller (50–55% at 30 min) when the blending was done under nitrogen. The 30-sec blanch decreased the loss considerably (15–25% at 30 min) for the air and oxygen blending and to a negligible level for blending under nitrogen. The 45-sec blanch reduced to an insignificant level the loss of color under nitrogen and air; a small loss was observed in the oxygen blending. The 60-sec blanch prevented the loss of color under all conditions of blending tried.

Results of the thawing tests are shown in Table 2. They indicate that unblanched cherries lose anthocyanin color during slow thawing and that steam blanching for 45 or 60 sec can prevent anthocyanin discoloration for 4 hr of thawing at room temperature.

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A Research Note

INFLUENCE OF EXTRACTING AGENTS DURING THE COLORIMETRIC DETERMINATION OF PECTIC SUBSTANCES

INTRODUCTION

THE CARBAZOLE reaction developed by Dische (1950) for the study of polyuronides was used by McComb and McCready (1952) for the determination of pectic substances in plant material. Considerable use of this procedure has been made since that time in studies involving the characterization of pectic substance in fruits and vegetables. Such work usually involved the successive extraction of an alcohol-washed sample with water, a sequestering agent such as ammonium oxalate or versene (trade name for ethylenediaminetetracetic acid tetrasodium salt) and finally, extraction under very alkaline conditions (Rouse and Atkins, 1955) or a treatment with versene at pH 11.5 followed by digestion with pectinase at pH 5.0–5.5 (McCready and McComb, 1952).

A modification of the uronic acid-carbazole procedure proposed by Bitter and Muir (1962) resulted in increased sensitivity, improved stability of the color and less interference by chlorides and oxidants.

The versene soluble or oxalate soluble fraction is often only a very small percentage of the total pectic substances present in a fruit (Shewfelt, 1965) and, for this reason, a sample of such an extract cannot be diluted with water before proceeding with the colorimetric determination. In such instances, relatively large quantities of the sequestering agent are present during color development and it may be far in excess of the 200 μg of versene which gave no interference when present in 15 ml of reaction mixture (McComb and McCready, 1952).

This report deals with the effect of versene concentration on color development when using the carbazole reaction.

EXPERIMENTAL

Reagents

(a) 0.025M sodium tetraborate · $10H_2O$ (ACS cert.) in conc. sulfuric acid (ACS reagent).
(b) 0.125% carbazole (Eastman Organic Chemicals) in absolute methanol (ACS spec).
(c) 0.1% α-D-galacturonic acid monohydrate (Sigma Chemical Co.).
(d) 0.5% versene [sodium (tetra) ethylenediamine tetraacetate; technical grade].
(e) 0.5% sodium (di) ethylenediamine tetraacetate; analytical reagent.
(f) 0.5% ammonium oxalate (ACS spec).

Procedure

The procedure described by Bitter and Muir (1962) was used with minor modifications. Tubes containing the sulfuric acid reagent were covered with parafilm and stored at −30°C until needed. Further, after adding the sample and also after heating, tubes were cooled in an ice bath.

Absorption spectra were obtained using a Shimadzu Model MPS-50L recording spectrophotometer and a distilled water blank. Optical density readings at 530 nm were made with a Bausch and Lomb spectronic 20 colorimeter using all the reagents with 1 ml water as a blank.

RESULTS & DISCUSSION

IN FIGURE 1 absorption spectra are presented using the carbazole procedure with samples containing versene, ammonium oxalate and combinations of these reagents with galacturonic acid monohydrate. From this it is clear that the presence of 2.5 mg ammonium oxalate in the reaction mixture had no effect on the absorption spectrum obtained with galacturonic acid. However, the same concentration of versene gave a purplish-blue color with high general absorption throughout the visible range. The effect of versene concentration on the determination of galacturonic acid is given in Table 1.

With increasing versene concentration, the optical density at 530 nm increased with versene samples and versene samples containing a fixed amount of galacturonic acid. In fact, 20 μg anhydrogalacturonic acid in the presence of 500 μg versene gave a value of 22 μg. In the presence of 100 μg versene this was reduced to 20.35, which was still an error of close to 2%. On the other hand, ammonium oxalate in concentrations of 0–10 mg per sample did not have an effect on the optical density at 530 nm and its presence did not affect the readings obtained with galacturonic acid.

It is possible to correct for the effect of versene by subtracting the absorbance reading at 420 nm from the reading obtained at 530 nm (see Fig. 1). Yet, careful control of heating and cooling

Table 1—Effect of versene and sodium (di) ethylenediamine tetraacetate (AR) concentrations on determination of anhydrogalacturonic acid (AHA)

	Versene		Sodium (di) ethylenediamine tetraacetate (AR)	
μg in sample	Absorbance	AGA equivalent μg	Absorbance	AGA equivalent μg
1000	.073	4.6	.032	2.0
500	.035	2.0	.016	1.0
250	.014	0.9	.008	0.5
100	.007	0.35	.003	0.2
50	.002	0.18	–	–

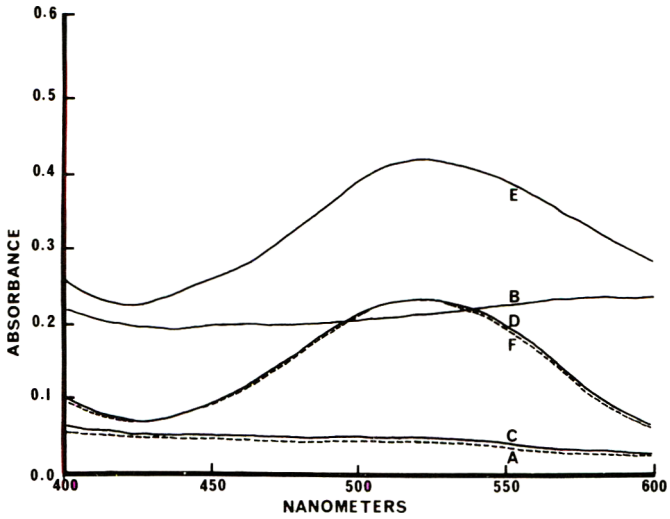

Fig. 1–Color produced with carbazole reagent (distilled water blank). (A) H_2O; (B) 2.5 mg versene; (C) 2.5 mg ammonium oxalate; (D) 14 µg anhydrogalacturonic acid; (E) 2.5 mg versene + 14 µg anhydrogalacturonic acid; and (F) 2.5 mg ammonium oxalate + 14 µg anhydrogalacturonic acid.

would be necessary if such a procedure is used. The final 15-min heating time gives maximum color development with galacturonic acid and variations in heating time between 12 and 18 min do not affect absorbance materially. However, color intensity and absorbance with versene increased markedly with increasing heating times. Absorbance values, using ca 4 mg versene were 0.118, 0.150 and 0.210, respectively, for final heating times of 12, 15 and 18 min.

As shown in Table 1, interference was about half of that obtained with versene when analytical grade sodium (di) ethylenediamine tetraacetate was used. Whether interference with the latter was due to impurities still left in the reagent or to interference by the reagent itself is not clear. However, it is evident from the data that 0.5% versene extraction of pectic materials can only be used in cases where the concentration of galacturonic acid is high enough to allow dilution of the versene to very low levels. If this is not feasible, procedures should rather be standardized using an alternative extracting agent such as ammonium oxalate.

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A Research Note

CELLULASE IN DATES AND ITS ROLE IN FRUIT SOFTENING

INTRODUCTION

IT IS generally believed that softening of fruit during ripening is related to alterations in the pectic substances through the action of pectic enzymes. This appears to be the case in dates, since we showed not only that mature dates possess considerably higher polygalacturonase activity but also demonstrated there is a close correlation between polygalacturonase activity and fruit softening (Hasegawa et al., 1969). It has been suggested also that cellulase, in addition to pectic enzymes, might contribute to softening of fruit (Dickinson and McCollum, 1964; Maclachlan and Perrault, 1964; Hall and Mullins, 1965). In this report we show that cellulase is present in Deglet Noor dates and that its activity increases dramatically during ripening, and we discuss the possible role of cellulase in the softening process.

EXPERIMENTAL

DATES (*Phoenix dactylifera* L. var. Deglet Noor) used in this study were the same samples used previously for polygalacturonase (Hasegawa et al., 1969) and invertase (Hasegawa and Smolensky, 1970) analyses. Na-carboxymethyl cellulose (DP = 1000, DS = 0.7) used as a substrate for cellulase assay was purchased from Hercules Incorporated, Wilmington, Delaware.

Six dates were pitted and ground for 2 min in a Waring Blendor with 100 ml of 4% NaCl solution containing 1g of presoaked insoluble polyvinylpyrrolidone. The mixture was adjusted to pH 7, placed in an ice bath for 10 min and then centrifuged at 20,000 *g* for 15 min. The residue was washed with 100 ml of the same solution and the mixture centrifuged. The washing was repeated. A 50-ml portion of the pooled extracts was dialyzed against running distilled water at 2°C for 6 hr. To the dialysate was added 0.1 M $CaCl_2$ solution sufficient to precipitate pectic substances which interfere with subsequent cellulase assays. The precipitate formed was removed by centrifugation, and the supernatant was dialyzed again against cold distilled water and used as the enzyme source.

Routinely, enzymic reaction mixtures consisted of 2 ml of the extract, 7 ml of 0.25% CM cellulose and 1.0 ml of 0.4 M acetate buffer at pH 5. A 4-ml portion of the mixture was withdrawn, incubated at 30°C and changes in viscosity measured with an Ostwald viscometer. Boiled extracts were used as control. Figure 1 shows the typical curves for the action of the extract and boiled extract on the changes in viscosity of CM cellulose solution. There was a linear relation between $1/\eta$ sp. (reciprocal of the specific viscosity) and reaction time at least within 18 hr of incubation under the conditions used. Thus, the activity was measured within this period of incubation. One unit of enzyme was defined as the amount that caused the change in viscosity of 1 centipoise per hr under the above conditions.

RESULTS & DISCUSSION

DATE CELLULASE was relatively heat stable. The activity was tested by exposing the enzyme extract at pH 7 to different temperatures for 5 min and measuring subsequent activity under the standard conditions. A treatment at 40°C had no effect on activity, a 60°C treatment caused 27% loss, whereas an 80°C treatment caused an 84% loss when compared to a control held at 0°C.

Table 1 shows cellulase activity of dates at various stages during maturation and ripening. Mature dates possess considerable cellulase activity; immature dates show little to none. The range of cellulase activity values obtained at a given maturity stage was widely spread. Technical difficulties involved in the preparative procedures used for enzyme extraction might be responsible to some extent for this wide range of values, but these values are not unusual, as indicated by results of other investigations (Dickinson and McCollum, 1964).

The activity was absent at the green stage and began to develop during the period that the fruit matured from the green to the early red stage. The sharp increase in activity occurred during the period when the fruit ripened from the early red to the late red stage and the activity was fairly similar at the 50 ~ 100% soft stage. Differences in the

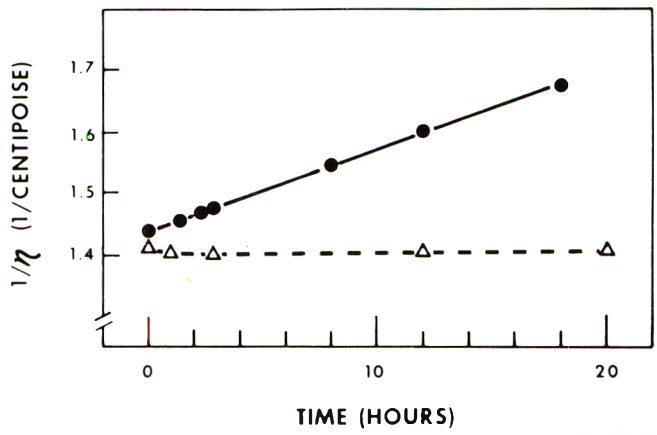

Fig. 1—Effect of date cellulase on viscosity of carboxymethyl cellulose solution. Boiled extract —△— and extract —●—. The assay was carried out under the standard conditions by using 23-m units of enzyme. The incubation was carried out at 30°C.

Table 1—Cellulase activity in Deglet Noor dates at different stages of maturity

Maturity	Fresh wt (g/date)	Sample[a] 1	Activity (m units/date) 2	3	4	5	Average
Green	12.7	0	0	0	0	0	0
Early red	12.3	8.28	0	0	4.58	0	2.57
Late red	14.0	65.4	29.7	150.0	191.0	215.0	130.2
50 ~ 100% Soft	12.5	64.3	293.0	256.0	60.0	–	134.7

[a]Each sample represents an extract obtained from six dates selected for uniformity of size.

activity between the late red stage and the 50 ~ 100% soft stage were not statistically significant.

This dramatic onset of cellulase activity beginning at the early red stage parallels the cases of polygalacturonase (Hasegawa et al., 1969) and soluble invertase (Hasegawa and Smolensky, 1970). The onset of these hydrolytic enzymes and sharp increases in their activities during the early stages of ripening are a typical phenomenon occurring in climacteric fruits (Pratt and Goeschl, 1969). These changes coupled with changes in color, chemical composition including carbohydrates (Rygg, 1946; Coggins and Knapp, 1969), cell wall polymers (Coggins and Knapp, 1969) and proteins (Hasegawa et al., 1969), and cell structures (Coggins et al., 1967) support the previous suggestion that dates are a climacteric fruit (Hasegawa and Smolensky, 1970).

More important, the onset and development of these hydrolytic enzymes appear to be closely related to softening of dates. Evidence of such a relation has been demonstrated previously by Maier and Metzler (1961), who observed the importance of sugar hydrolysis on the textural quality of dates and used the extent of invertase-catalyzed sucrose hydrolysis as an index of hydrolytic enzyme activity. Hasegawa et al. (1969) demonstrated by using samples obtained from a single fruit that the development of polygalacturonase and softening occurred simultaneously in dates.

The anatomical studies of Long (1943) and Coggins et al. (1967) showed the occurrence of considerable cell wall dissolution during the preharvest softening of dates. This dissolution of cell walls may be due to the action of hydrolytic enzymes or it may occur by means of some other mechanisms. Recently, Coggins and Knapp (1969) made a comprehensive study of chemical changes associated with fruit development and ripening. They showed that a sudden increase in breakdown products of cell wall polymers occurred during the late red stage, suggesting that hydrolytic enzymes play a part in the cell wall dissolution during the softening process. Since we observed the development of cellulase activity at this stage, it is reasonable to assume that cellulase is involved in the ripening process of dates.

In pears (Jermyn and Isherwood, 1956) and peaches (Sterling, 1961), as contrasted with dates, cellulose micelles undergo a very limited degradation during the ripening period. Presumably, this limited degradation of cellulose cannot contribute greatly to the dramatic softening during ripening. Hobson (1968) suggested also that cellulase is not a principal factor controlling the softening of tomato fruit during ripening. The greater apparent involvement of cellulase activity in date softening as compared with these other fruits might be because dates undergo a much more extensive tissue breakdown.

Presently, it is very difficult to surmise how closely the in vitro cellulase activity, determined by using CM cellulose as a substrate, correlates with the in vivo activity of native cellulase, since its substrate specificity is not yet known. However, it seems likely that the cellulase acts in conjunction with other hydrolytic enzymes to cause softening during ripening of dates.

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