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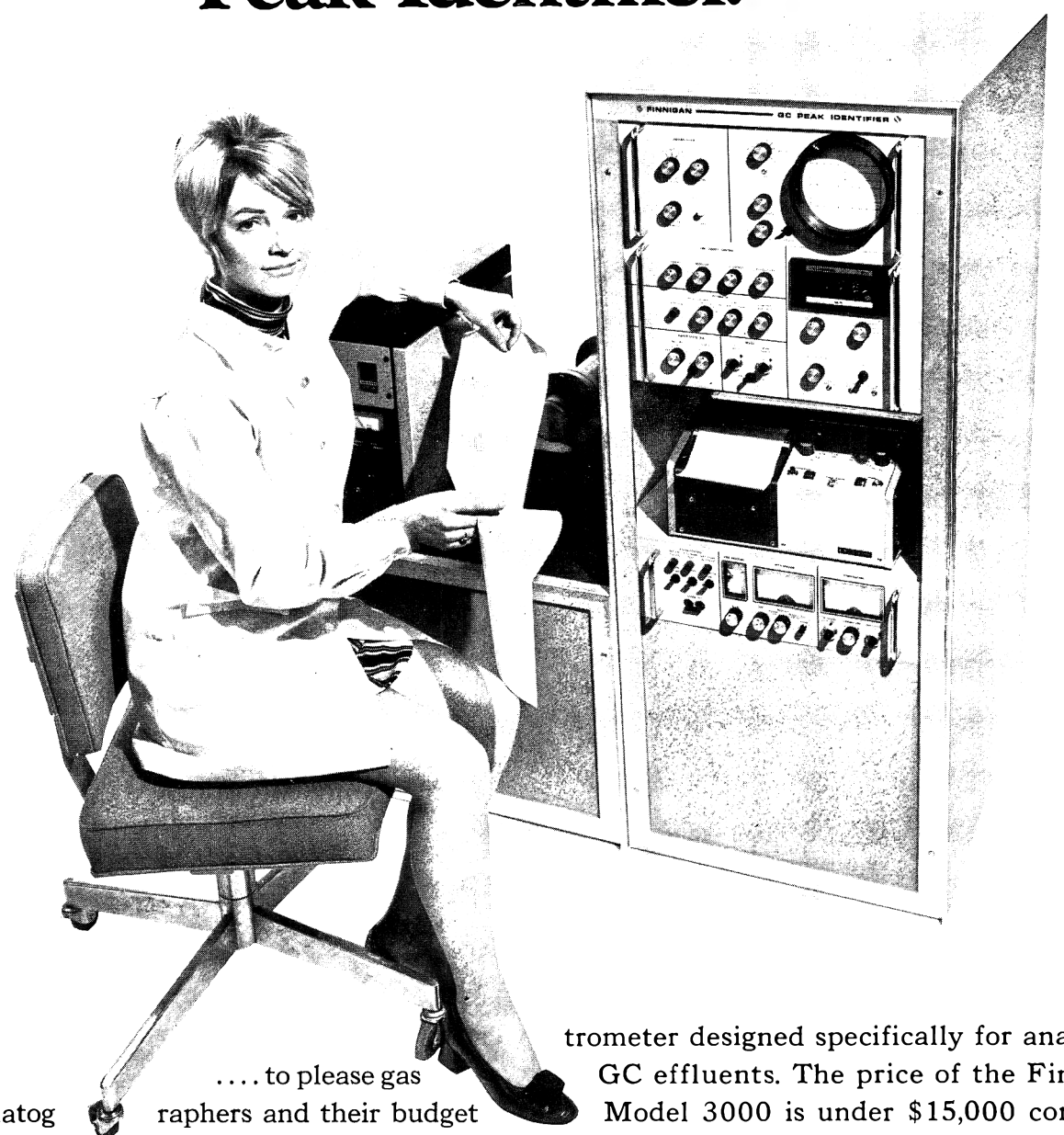

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Memo: WHERE ARE WE?

□ AMONG THE Institute members I believe it is more or less common knowledge that we are having problems with the publication of research papers. We have more papers to publish than we can provide journal pages for them. The situation is being compounded by rising publication costs.

The Executive Committee of the Institute has declared a policy of publishing *all* worthy papers offered for publication. Such policy recognizes that publications are valuable means for:

(a) Giving outlet for those who produce the information

(b) Providing access for those who use the information

and **(c)** Recording advances.

As a scientific and technological organization, the IFT renders a valuable service through its publication of research findings.

But a policy to publish *all* worthy papers received could demand more than is possible with the financial resources of the Institute. Hence, the carrying out of this policy requires that additional support for the publications be sought. This can mean that some changes will be needed.

Those most involved are being asked to help support publication costs. Authors of papers are asked to pay part of the cost in a page-charge. With this issue the *Journal of Food Science* is a subscription journal, even to regular Institute members, and hence, readers also help in supporting costs.

Advertising in the *Journal of Food Science* is being accepted. Soon a less costly printing process will be employed. Other cost reduction measures are being studied.

Other changes are possibilities. One that has much to recommend it, is to publish all research papers received by the Institute in a single research journal and not to divide them between *Food Technology* and the *Journal of Food Science* as is now done.

A recent readership survey on *Food Technology* tells much about the wants and dislikes of the readers of that journal. These expressions are of interest and provide valuable guidance for action. They are not always, however, in accord with what is possible in terms of the resources available. Some recognition of what is feasible and adjustment to this feasibility must be a part of our thinking for today and the future.

Ours is an organization of diverse membership. Many interests and needs exist. Practically all benefit from publication of research findings. While today we are in a jam in our efforts to publish these findings, our determination to find a way is strong.

We trust you are with us in this.

Walter M. Urbain
Scientific Editor

Letters *to the Scientific Editor*

Letter to the Editor

October 6, 1969

Dear Sir:

In a recent research note, Caldwell et al. (1969) stated that in contrast to previous findings by other workers and myself (Chajuss et al., 1962b, Gawronski et al., 1967) they did not observe a decrease in total sulfhydryl groups content in excised chicken breast muscle during post mortem aging. Caldwell et al. explained that the differences in the observations are probably due to different methods of testing for thiol groups.

This is indeed the case. What Caldwell et al. observed was definitely not the total actual thiol groups present in the muscle in any moment but only some of the available thiol groups.

In order to estimate correctly the total actual thiol groups, care should be given first to prevent any possible oxidation of the thiol groups during analysis (i.e., use of sonic oscillator chamber under nitrogen instead of Waring blender), secondly and most important to use an unoxidative denaturing agent such as 8*M* urea, or similar denaturant, to unmask all the inner enclosed thiol groups. Without the use of urea, or similar denaturant, only some of the thiol groups are available for analysis and these probably are not involved to any great extent in establishing the strained protein network within the muscle.

My observations have indicated a vast difference between the number of reactive and available thiols in muscle homogenates in water and muscle homogenates in 8*M* urea. While such denaturation is not necessary with few soluble proteins, it is definitely needed in the muscle proteins. The use of urea not only changes the gross physical structure of the muscle homogenate, but it mainly causes an unfolding of the structure of the proteins by disruption of hydrogen bonds thus revealing the enclosed thiol groups making them available for analysis.

Muscle homogenates are highly complicated mixtures wherein interchange reactions between various sulfhydryl and disulfide groups might continuously take place. Thus a method which could cause oxidation and conversion of certain thiols (non-protein SH) to disulfides should not, prima facie, be used to measure SH groups (total or protein) in a system such as muscle wherein the various types of thiols exist. Such measurement and differentiation is meaningless and misleading especially also as rapid oxidation of the thiol groups could have occurred under the condition of the experiment (see Jocelyn et al., 1962).

The importance of SH-SS reactions and their contribution to tenderness is indeed clearly evident (Chajuss et al., 1962a).

DANIEL CHAJUSS
Hayes (ASHDOD) Limited
Ashdod, Israel

REFERENCES:

Caldwell, K.A. and Lineweaver, H. 1969. Sulfhydryl content of excised chicken breast muscle during postmortem aging *J. Food Sci.* **34,** 290.

Chajuss, D. and Spencer, J.V. 1962a. The effect of oxidizing and reducing aging media on the tenderness of excised chicken muscle. *J. Food Sci.* **27,** 303.

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Gawronski, T.H., Spencer, J.V. and Uubols, M.H. 1967. Changes in sulfhydryl and disulfide content of chicken muscle and the effect of N-ethylmaleimide. *J. Agr. Food Chem.* **14,** 781.

Jocelyn, P.C. 1962. The effect of glutathione on protein sulfhydryl groups in rat-liver homogenates. *Biochem. J.* **85,** 480.

Letter to the Editor

October 24, 1969

Dear Sir:

In reply to the letter of Dr. Chajuss, I offer the following:

Chajuss makes two main assertions: **(1)** that experimental conditions were not controlled to exclude air and that, therefore, autoxidation occurred during experimental analyses and that the method is inapplicable for study of oxidation of thiol groups and **(2)** that proteins were not denatured and were therefore not accessible to the sulfhydryl reagent.

In response to these:

1. Experiments were indeed carried out under carefully controlled conditions that would preclude oxidation as effectively as the procedure used by Chajuss:

a) Samples were maintained at −196°C until analyzed.

b) Homogenates were prepared from the still frozen, pulverized tissue. Air was excluded from the blender by specially constructed baffles during dispersal of the tissue in water at 0°C.

c) Homogenates were maintained at 0°C and analyzed very soon after preparation. The –SH content was shown to be quite stable under these conditions.

d) Jocelyn does not state or indicate that his method permits unintended oxidation to occur. He states "Non-protein SH and total SH determined in this way are both proportional to homogenate concentration when this is varied, provided that an ice-cold homogenate is used and the reagents are added immediately after sampling. Any delay leads to a loss of non-protein SH. This is shown in Table 1, which illustrates the effect of a delay (15 min) between addition of the disulphide for the total SH estimation and addition of the disulphide for the nonprotein SH estimation to different samples of the same homogenate. Recoveries of GSH added to the homogenate and estimated as non-protein SH are very poor whereas recoveries as total SH are satisfactory, though this estimation is completed much later. This difference is probably due to the rapid oxidation of GSH by the homogenate (Fig. 5), which is arrested as soon as the disulphide is added."

e) In our laboratory, we did not conduct experimental work until we established satisfactory recoveries and reproducibility in preliminary trials. During these trial runs, we observed that **(1)** the total and non-protein-SH content of homogenates remained unchanged when homogenates were maintained at 0°C during the working day and **(2)** if homogenates were frozen overnight, re-homogenized to facilitate sampling and subsequently analyzed, the –SH content was still unchanged.

f) Jocelyn applied the analytical procedure in much the same manner as we—to measure total and non-pro-

tein sulfhydryl content of tissue homogenates as a function of holding time—with the exceptions that he used **(1)** liver rather than muscle **(2)** time periods of 0–1 hrs rather than 0–6 hrs and **(3)** incubations at 37°C rather than aging (i.e., "incubations") at 0°C.

2. We know of no evidence to support Dr. Chajuss' contention that critical sulfhydryl groups are not analyzed by the procedure reported in our paper. The fact that urea or other denaturants alter the tertiary structure of proteins and expose sulfhydryl groups buried within that structure does not mean that such groups would be unreactive to all chemical agents. The reactivity of these groups will vary depending upon the reagent used, its concentration, the time course of reaction and other experimental parameters. Jocelyn reports that both protein and nonprotein sulfhydryls react with the Ellman reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) without prior denaturation of proteins. Jocelyn reports that total -SH values for undenatured proteins as determined by his method are in agreement with the totals found for denatured proteins determined by other methods. "With bovine serum albumin there appears to be nearly complete reaction with the available SH despite the fact that these groups are not reactive enough to be oxidized even by ferricyanide (Kolthoff & Anastasi, 1958)." *Reference:* Jocelyn, C.P. (1962). The effect of glutathione on protein sulfhydryl groups in rat-liver homogenates. *Biochem. J.* **85**, 480.

As we have had no opportunity to evaluate either the data obtained or methods used by Dr. Chajuss, we are unable to comment on his results referred to in his letter. The analytical method which we used does not cause unwanted oxidations to occur and analytical samples were adequately protected from autoxidation.

K. A. CALDWELL
Albany, California

Letter to the Editor

Dear Sir:

I wish to make a correction to the paper "A Comparison of Techniques for Determining the Fat Content of Ground Beef" by D. R. Bellis, J. L. Secrist and M. J. Linsky, *J. Food Sci.* **32,** 521 (1967). The following changes are in order:

Page 253: The formulas for the curves in Figure 2a, 2b and 2c, representing the best fit of the experimental data in Table 1, were generated by our computer (GE 225). The matrixes obtained for the fourth order polynomials are as follows:

Figure 2a	**Figure 2b**
−0.0010660514	−0.0016480609
0.0859905	0.13676634
−2.5007673	−4.1224982
31.909498	54.432125
−138.28668	−253.90246

Figure 2c

−0.0021054943
0.17792401
−5.4803314
73.831641
−355.41612

In an effort to simplify the formulas for publication, the values were rounded as seen in Figure 2 of the paper. The usable nature of the plotted curves was not affected in any way. However, to reconstruct the curves from the rounded values will produce curves which are something less than accurate. Likewise, the variance and standard error values in Table 3 are affected. Therefore, we present the following matrixes for Figure 2a, 2b and 2c which contain values rounded to the maximum extent and yet will reproduce curves closely approximating those of best fit found in this paper. The matrixes follow:

Figure 2a	**Figure 2b**
−0.001066	−0.0016481
0.086	0.13677
−2.501	−4.1225
31.91	54.432
−138.3	−253.9

Figure 2c

−0.0021055
0.1779
−5.48
73.83
−355.3

When using these rounded values in the fourth order polynomial formulas, the following variance and standard error values are obtained:

	Ground		
	Commercial	Laboratory No. 2	Laboratory No. 3
Fourth Order			
Variance	0.170	0.086	0.177
Standard Error	0.498	0.340	0.508

DEXTER R. BELLIS

ABSTRACTS:
IN THIS ISSUE

PECTIN METHYL ESTERASE ACTIVITY IN SOUTHERN PEAS (*Vigna sinensis*). J. L. COLLINS. *J. Food Sci.* **35,** 1–4 (1970)—The optimum salt level for maximum PME atcivity was 0.25 *M*. The optimum pH depended upon the variety of peas. The Purple Hull Pink Eye variety required a pH of 8.5 (Princess Ann, pH 7.5 to 8). Maximum activity occurred at 50°–60°C. The Q_{10} was 1.35. The more immature the peas, the higher the activity. Freezing caused an increase in the observed activity. Rinsing removed a significant amount of PME from the peas. PME activity of the slurries prepared from frozen peas continued to increase up to 3–4 hr.

ROLE OF CUTICLE IN SPOILAGE OF CHICKEN EGGS. D. V. VADEHRA, R. C. BAKER & H. B. NAYLOR. *J. Food Sci.* **35,** 5–6 (1970)—The cuticle, a mucilaginous layer on the egg shell, was found to play an important role in preventing spoilage of eggs. Eggs collected from the uterus (devoid of cuticle) spoiled at a much faster rate than the normally laid eggs. The protection afforded by cuticle lasts at least up to 96 hr post lay.

CHICKEN MYOFIBRIL FRAGMENTATION IN RELATION TO FACTORS INFLUENCING TENDERNESS. R. N. SAYRE. *J. Food Sci.* **35,** 7–10 (1970)—Susceptibility of chicken pectoralis major myofibrils to mechanical fragmentation was investigated after various periods of aging, as an index of tenderness. Treatments were used which accelerated, retarded or prevented post-mortem glycolysis. Fragmentation, with breaks always beside the Z line, was measurd by microscopic examination of homogenized muscle. The fragmentation pattern of glycolysing muscle showed a general correspondence to tenderness changes but could not be used as an accurate index of tenderness. Muscle in which glycolysis had been prevented exhibited changes in fragmentation but no change in tenderness.

FLAVOR COMPONENTS IN COGNAC. J. SCHAEFER & R. TIMMER. *J. Food Sci.* **35,** 10–12 (1970)—Flavor components of a genuine cognac were identified by means of CGLC/MS. The complex extract was fractionated on a packed column preceding further separation on a capillary column. The identification of fatty acids, phenolic acids and some of the carbonyl compounds was achieved by other chromatographic methods. 81 components, of which 24 have not previously been reported in cognac and other grape brandies, were identified.

THE EFFECTS OF FREEZING ON THE SURVIVAL OF SALMONELLA AND E. Coli IN PACIFIC OYSTERS. R. DIGIROLAMO, J. LISTON & J. MATCHES. *J. Food Sci.* **35,** 13–16 (1970)—Salmonellae showed 1.0 to 1.5 log reduction in count as a result of freezing and were more sensitive than *E. coli*, which showed a 0.5 log decrease. The number of salmonellae declined steadily during frozen storage, but the decline in *E. coli* was quite erratic and unpredictable during the first seven days. When salmonellae and *E. coli* were suspended in shellfish homogenate which was then frozen, held in the frozen state and tested for survivors, it was found that proportionately more organisms survived than in the whole oysters. The bacteria died off more rapidly when held in homogenates at 32°F than at −30°F. These results suggest that it would be dangerous to apply bacterial standards derived for unfrozen oysters to the frozen product or to use *E. coli* counts on frozen samples as an index of the quality of the unfrozen oysters.

FREE AMINO ACIDS AND OTHER NITROGENOUS FRACTIONS IN WINE GRAPES. W. M. KLIEWER. *J. Food Sci.* **35,** 17–21 (1970)—The concentrations of eight free amino acids, total nitrogen, amino-acid-fraction nitrogen, and nonamino-acid-fraction nitrogen in the juices of 26 red- and 23 white-wine varieties of grapes was determined at early and late stages of fruit maturity. Arginine and proline were the most prominent amino acids, followed in order by α-alanine, glutamic acid, and γ-aminobutyric acid. The amino acid fraction and nonamino acid fraction nitrogen in the juices ranged from 53 to 76% and from 23 to 56% of the total Kjeldahl nitrogen, respectively. The eight determined amino acids accounted for 29 to 72% of the total nitrogen and 47 to 96% of the amino acid fraction nitrogen. Arginine contributed the most nitrogen of the amino acids, accounting for 6 to 44% of the total nitrogen in the juices of the various fruits.

PASTEURIZATION OF PACIFIC OYSTERS BY RADIATION. POST-MORTEM CHANGES IN NUCLEOTIDES DURING STORAGE AT 0–2°C. E. J. GUARDIA & A. M. DOLLAR. *J. Food Sci.* **35,** 22–25 (1970—The concentration of nucleotides was lower in the adductor muscle of the oyster (1.64 μmoles/g) than in the remaining dark tissues of the oyster (2.75 μmoles/g), and the concentration was less in the whole oyster meats (2.87 μmoles/g) than is usually found in fish muscle, or other marine invertebrates. In addition to the adenine nucleotides and inosine monophosphate, uridine triphosphate, guanosine triphosphate, guanosine diphosphate, guanosine monophosphate, and guanosine diphosphate-mannose were found in the fresh oysters. Samples collected in the summer had greater concentrations of nucleotides than similar winter samples. Inosine monophosphate formed rapidly from adenosine triphosphate during storage at 0–2°C, while the turnover rate of inosine monophosphate was slow and reflected low 5′-nucleotidase activity.

ETHYLENE OXIDE RESISTANCE OF MICROORGANISMS IMPORTANT IN SPOILAGE OF ACID AND HIGH-ACID FOODS. D. F. BLAKE & C. R. STUMBO. *J. Food Sci.* **35,** 26–29 (1970)—Resistance values are presented for spores of *Bacillus coagulans*, conidiospores of *Aspergillus niger*, and vegetative cells of *Lactobacillus brevis, Leuconostac mesenteroides, Hansenula anomala* and *Saccharomyces cerevisiae* exposed to a mixture of ethylene oxide (12%) and *dichlorodifluoromethane* (88%). The resistance parameter D was determined to characterize the resistance of each organism displaying logarithmic death. Values of z were determined for spores of *B. coagulans*. Most resistant were the spores of *B. coagulans*, about 1.8 to 6 times more resistant than the vegetative cells of *L. mesenteroides* and *L. brevis*. Vegetative cells of *H. anomala, S. cerevisiae* and conidiospores of *A. niger* displayed non-logarithmic death.

INHIBITORY EFFECTS OF Pseudomonas ON SELECTED Salmonella AND BACTERIA ISOLATED FROM POULTRY. J. L. OBLINGER & A. A. KRAFT. *J. Food Sci.* **35,** 30–32 (1970)—A perpendicular streak technique was used as a preliminary screening procedure to determine relative degrees of inhibition exhibited by known strains of *Pseudomonas* against sensitive *Salmonella* and known organisms isolated from poultry. Spectrophotometric analysis was also used to measure inhibitory activity produced by different concentrations of filtrates from *Pseudomonas* cultures against sensitive organisms. The production of pigment appeared to be closely linked to the relative ability of different *Pseudomonas* cultures to produce inhibition. *Pseudomonas* strains were inhibitory to strains of *Salmonella, Staphylococcus, Escherichia coli*, and *Streptococcus*. None of the inhibition producing strains of *Pseudomonas* isolated from poultry were mutually repressive.

IN THIS ISSUE

METMYOGLOBIN FORMATION IN BEEF STORED IN CARBON DIOXIDE ENRICHED AND OXYGEN DEPLETED ATMOSPHERES. D. A. LEDWARD. *J. Food Sci.* **35,** 33–37 (1970)—At 0 and 7°C and constant humidity, the formation of metmyoglobin in beef was maximal at low partial pressures of oxygen (7.5 ± 3 Hg at 7°C and 6 ± 3 mm Hg at 0°C) for semitendinosus muscles. Carbon dioxide concentrations of 10% and higher had negligible effect on the formation of metmyoglobin, provided the oxygen pressure was above about 5%. At higher partial pressures of carbon dioxide, absorption of carbon dioxide increased and the pH of the surface decreased. In air, the formation of metmyoglobin varied widely from muscle to muscle.

COMPARISON OF CARBONYL COMPOUNDS IN MOLDY AND NON-MOLDY COCOA BEANS. A. P. HANSEN & P. G. KEENEY, *J. Food Sci.* **35,** 37–40 (1970)—Carbonyl compounds in moldy and non-moldy cocoa beans were converted to dinitrophenylhydrazones and separated into monocarbonyl classes. Growth of mold was always accompanied by relatively large increases in carbonyl concentrations. Increases in total monocarbonyl values ranged from 20 to 500% and averaged almost 300% for the eight pairs of samples analyzed. Compared to non-moldy beans, moldy cocoa beans contained greater concentrations of methyl ketones, 2-enals and 2,4-dienals, but saturated aldehyde concentrations were quite often lower. TLC revealed the presence of C_3, C_1, C_6, C_{17} and several unidentified methyl ketones. Most of the ketones detected in moldy beans were also found in non-moldy beans but in lower concentrations.

ANTHOCYANIN PIGMENTS IN TINTO CÃO GRAPES. F. W. H. LIAO & B. S. LUH. *J. Food Sci.* **35,** 41–46 (1970)—The R_f measurement of the pigments and their hydrolysis products, together with the alkaline degradation of the aglycone, confirmed the chemical structures of the anthocyanins as malvidin 3-monoglucoside, peonidin 3-monoglucoside, cyanidin 3-monoglucoside, petunidin 3-monoglucoside, petunidin 3-monoglucoside acylated with caffeic acid, malvidin 3-monoglucoside acylated with caffeic acid, malvidin 3-monoglucoside acylated with p-coumaric acid, peonidin 3-monoglucoside acylated with p-coumaric acid, and cyanidin 3-monoglucoside acylated with caffeic acid. Malvidin and peonidin were not present in the original sample. They were formed during the extraction and purification procedures. Malvidin 3-monoglucoside and malvidin 3-monoglucoside acylated with *p*-coumaric acid were the dominant anthocyanins present in Tinto cão grapes.

RESPIRATION OF POTATO MITOCHONDRIA AND WHOLE TUBERS AND RELATION TO SUGAR ACCUMULATION. L. E. PAEZ & H. O. HULTIN. *J. Food Sci.* **35,** 46–51 (1970)—The increase in sugar content of potato tubers with decrease in storage temperature and the decrease in sugars with increase in temperature is accounted for in only a minor way by the corresponding decrease or increase in respiratory activity of the tubers. The maximal possible contribution of respiratory activity to the changes in sugar content varied from less than 1% to 13% depending on the particular storage conditions. Unlike whole tubers, mitochondria displayed a temperature response typical of an enzymic reaction with an apparent energy of activation of 14,000 cal/mole with succinate as substrate. The potential mitochondrial activity would not limit respiration of whole tubers at any temperature studied.

SODIUM CHLORIDE EFFECT ON AUTOXIDATION OF THE LARD COMPONENT OF A GEL. R. ELLIS, A. M. GADDIS, G. T. CURRIE & F. E. THORNTON. *J. Food Sci.* **35,** 52–56 (1970)—Stable gels composed of lard, sodium carbomethoxy cellulose and water were used for the examination of factors involved in the pro-oxidant activities of sodium chloride, heme compounds and other additives. Sodium chloride had a direct pro-oxidant action on the lard of freezer-stored and dehydrated gels, but inhibited the oxidation of hydrated gels at 20°C. Heme catalysis was accelerated by sodium chloride.

STUDIES IN MEAT TENDERNESS. 8. Ultra-Structural Changes in Meat During Aging. C. L. DAVEY & M. R. DICKSON. *J. Food Sci.* **35,** 56–60 (1970)—During the aging of beef, the external loading required to stretch sternomandibularis muscle to its fullest extent declines 5–10-fold from the maximum of 2 Kg/cm² muscle attained at rigor onset. This loss of tensile strength is due to a weakening of the myofibrillar structures at the junction of the I filaments with the Z discs of the sarcomeres. The Z discs undergo progressive changes and lose ground substance as aging proceeds. There is no obvious structural change in the I filaments during aging although a weakening in the association of the constituent proteins of these structures apparently occurs.

INFECTION ROUTES OF BACTERIA INTO CHICKEN EGGS. D. V. VADEHRA, R. C. BAKER & H. B. NAYLOR. *J. Food Sci.* **35,** 61–62 (1970)—A study was undertaken to determine the route of bacterial passage into chicken eggs and their consequent spoilage. The blunt end was found to be most vulnerable to infection while the narrow end was the least vulnerable and the equitorial region was in between the two.

CHEMICAL DESTRUCTION OF *Aspergillus niger* CONIDIOSPORES. M. K. C. CHENG & R. E. LEVIN. *J. Food Sci.* **35,** 62–66 (1970)—Destruction of *A. niger* conidiospores at 20°C (68°F) by 20 ppm NaClO and 20 ppm iodine as iodophor yielded *D* values of 0.61 and 0.86 min, respectively at pH 3.0 and 1.31 and 2.04 min, respectively at pH 7.0. On the basis of molar concentrations, iodine was slightly more effective than chlorine. A *D* value of 0.026 min was obtained with 4% NaOH at 60°C (140°F) indicating 4% NaOH at 60°C to be far more germicidal than 20 ppm of either halogen compound at 20°C. One per cent NaOH at 30°C resulted in an immediate and rapid release of amino acids presumably from the spore wall during the first two minutes of contact and a slower rate of release of RNA, with DNA released at the slowest rate.

FACTORS AFFECTING THE DISTRIBUTION OF LACTATE DEHYDROGENASE BETWEEN PARTICULATE AND SOLUBLE PHASES OF HOMOGENIZED TROUT SKELETAL MUSCLE. RONALD L. MELNICK & H. O. HULTON. *J. Food Sci.* **35,** 67–72 (1970)—Lactate dehydrogenase (LDH) of trout skeletal muscle was solubilized from the particulate fraction by high ionic strength and high pH. Reduced diphosphopyridine nucleotide (DPNH) also was effective in solubilizing the enzyme. The oxidized form of the coenzyme (DPN^+) was much less effective. Pyruvate and lactate had little effect in themselves but acted synergistically with the pyridine nucleotides. Although reduced triphosphopyridine nucleotide (TPNH) was as effective as DPNH, several other metabolites structurally related to DPNH were relatively ineffective. Solubilization by DPNH was dependent on tissue concentration in the suspending medium; the lower the tissue concentration, the more readily LDH is solubilized by DPNH.

ABSTRACTS:
IN THIS ISSUE

PHYSICOCHEMICAL NATURE OF BANANA PSEUDOSTEM STARCH. H. S. SHANTHA & G. S. SIDDAPPA. *J. Food Sci.* **35,** 72–74 (1970)—Starch was isolated from banana pseudostem and its properties were compared with those of potato, corn and tapioca. The starch granules are irregular in shape and are bigger in size than those of the fruit starch. At 60°C, the granules start swelling, gradually increase in size and attain their maximum size at 75°C and do not rupture even after heating to 100°C. The intrinsic viscosity of the starch (2.05) is similar to that of potato starch (2.00). The amylose content of the starch compares well with those of banana fruit and potato (tuber) starch (21%). In general, banana pseudostem starch resembles potato starch.

ACCUMULATION OF STARCH IN BANANA PSEUDOSTEM AND FRUIT. H. S. SHANTHA & G. S. SIDDAPPA. *J. Food Sci.* **35,** 74–77 (1970)—The rhizome, pseudostem and the unripe fruit of the banana have a high starch content. Its concentration is higher in the middle fleshy leaf sheaths and increases gradually toward the rhizome downwards along the length of the pseudostem. During the growth of the plant, starch accumulates in the pseudostem and reaches its maximum at the time of inflorescence and remains practically constant thereafter until the harvesting of the mature bunch. After the removal of the mature bunch, there is a gradual decrease in the accumulated starch, when the stem is allowed to stand in the field. If, however, it is felled and stored in the shade, the decrease in starch content is rapid and even after a period of two days, the starch content is reduced to such a great extent that its extraction becomes rather uneconomical.

WATER-SOLUBLE FLAVOR AND ODOR PRECURSORS OF MEAT. 3. Changes in Nucleotides, Total Nucleosides and Bases of Beef, Pork and Lamb during Heating. R. L. MACY JR., H. D. NAUMANN & M. E. BAILEY. *J. Food Sci.* **35,** 78–80 (1970)—Inosinic acid was the predominant nucleotide in all three species and it was degraded by heating. Adenylic acid increased during cooking in meat from all three species. Cytidylic, uridylic and guanylic acids were present in relatively low concentrations in meat from all three species and changed little during cooking. A rapid method for estimating total nucleotides resulted in greater variation than a specific method for measuring individual nucleotides.

WATER-SOLUBLE FLAVOR AND ODOR PRECURSORS OF MEAT. 4. Influence of Cooking on Nucleosides and Bases of Beef Steaks and Roasts and Their Relationship to Flavor, Aroma and Juiciness. R. L. MACY JR., H. D. NAUMANN & M. E. BAILEY. *J. Food Sci.* **35,** 81–83 (1970)—Cooking resulted in significant increases in adenylic acid, total purine nucleosides and bases of eighty beef roasts of eight different cuts. It decreased the contents of inosinic acid, guanylic acid and sum of individual nucleotides (adenylic, cytidylic, uridylic, inosinic and guanylic acids) in these samples. Significant differences were also found between the various constituents of raw and cooked samples of the beef cuts.

WATER-SOLUBLE FLAVOR AND ODOR PRECURSORS OF MEAT. 5. Influence of Heating on Acid-Extractable Non-Nucleotide Chemical Constituents of Beef, Lamb and Pork. R. L. MACY JR., H. D. NAUMANN & M. E. BAILEY. *J. Food Sci.* **35,** 83–87 (1970)—Changes in chemical constituents during cooking of the various beef cuts were greatest in Choice clod and Good calf clod roasts, followed by round roasts and smallest changes occurred in rib steaks. Cooking caused significant increases in creatinine and decreases in amino and non-amino nitrogen, creatine and total carbohydrates. Creatine-creatinine was a better index of sensory quality than other chemical constituents studied.

A REEXAMINATION OF THE TWO-STAGE TRIANGLE TEST FOR THE PERCEPTION OF SENSORY DIFFERENCES. N. T. GRIDGEMAN. *J. Food Sci.* **35,** 87–91 (1970)—A review of the theory and practice of the two-stage triangle test for the sensory perception of small differences leads to the conclusion that its disadvantages usually outweigh its advantages. When the test is used its information content is difficult to assess, and a "least objectional" method, based on a scoring scheme and statistical significance procedures, is here proposed.

RESISTANCE OF *Clostridium perfringens* TO VARYING DEGREES OF ACIDITY DURING GROWTH AND SPORULATION. L. H. FISCHER, D. H. STRONG & C. L. DUNCAN. *J. Food Sci.* **35,** 91–95 (1970)—The level of acidity, length of exposure of the cells, the growth medium employed and the phase in the growth curve influenced the survival of *C. perfringens*. Exposure of cells grown in DS-sporulation medium to buffer pH 6.0 had little effect on the survival over the 8 hr test period, with somewhat greater sensitivity of cells being demonstrated at pH 4.5. Exposure of cells, similarly produced, to buffers pH 1.0 or 2.0 was much more effective in reducing the percentage of survival, particularly during early log phase and at the onset of sporulation. Incubation at temperatures of 37° or 0°C, during the time of treatment with the test buffers pH 1.0 or 2.0, produced no consistent change in the percentage of survivors when the cells were grown in FTG.

DETERMINING THE SENSORY ODOR LEVELS OF AROMA COMPOUNDS OF ALCOHOLIC BEVERAGES. P. SALO. *J. Food Sci.* **35,** 95–99 (1970)—Sensory odor thresholds of four alcohols, five esters, seven acids and diacetyl found in alcoholic beverages were determined by triangular test. Among alcohols threshold levels ranged from 5 to 8 ppm except for isobutyl alcohol which had 75 ppm, three esters had 0.2–0.6 ppm, two had 14–17 ppm, four acids had 4–9 ppm, two had 15–20 ppm and isovaleric acid had 0.7 ppm. Diacetyl had 0.0025 ppm. Standardizations were made using percentage-above-chance-scores; usefulness of the panel was tested. Results showed a fair goodness-of-fit to the model. Smell perception varied with the logarithm of stimulus, and distribution of scores followed the normal probability function.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF FREE AND STRETCHED RABBIT MUSCLE. E. M. BUCK, D. W. STANLEY & E. A. COMMISSIONG. *J. Food Sci.* **35,** 100–102 (1970)—Stretched muscles were significantly more tender as evidenced by lower shear values. They also exhibited significantly longer sarcomeres as compared to their paired controls allowed to pass through rigor without restraint. Greater amounts of total protein were extracted from stretched muscles in all trials except one. Significantly greater amounts of actomyosin were extracted from stretched muscles in all trials. The actomyosin results were unexpected since it has been suggested by several workers that actomyosin formation is directly related to toughness in muscle. Some possible explanations for the results obtained in this study are discussed.

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Pectin Methyl Esterase Activity in Southern Peas (Vigna sinensis)

SUMMARY—*Pectin methyl esterase (PME) activity was investigated in vitro in Southern peas (Vigna sinensis). Experiments were conducted to determine the effect of NaCl concentration, pH, temperature, maturity, frozen storage and rinsing the peas on PME activity.*

The optimum salt level for maximum PME activity in the Purple Hull Pink Eye (PHPE) and Princess Ann (PA) varieties was ascertained to be 0.25 M. The pH optima were: PHPE, 8.5; and PA, 7.5 to 8. The remaining studies were made using peas of the PHPE variety which were harvested at 3 stages of maturity. Maximum enzymatic activity occurred at 50°–60°C. Partial inactivation occurred at 65°C. The mean Q_{10} was 1.35 over the range of 30°–50°C.

PME activity was dependent upon the maturity of the peas. The most immature peas had an activity level about 2.5 times that found in the most mature peas. Peas subjected to frozen storage had a higher activity than the fresh peas; the increased activity was more pronounced in the more mature peas.

Rinsing the peas removed significant amounts of the enzyme. A greater proportion of PME was removed from the more immature peas than from the more mature ones. With the immature, frozen peas PME activity was reduced 22.7% by rinsing; whereas, in the fresh counterpart the reduction was 11.1%. The rinse water from frozen peas contained more PME activity than did rinse water from fresh peas. Also, the activity in rinse water from the most immature peas was about 2.7 times that from the most mature ones. Upon standing, PME activity in the slurries prepared from frozen peas continued to increase up to 3–4 hr.

INTRODUCTION

PECTIN methyl esterase (PME, 3.1.1.11) is widely distributed in higher plants (Lineweaver et al., 1951) and is associated with the de-esterification of the pectin substances in the tissues (Hsu et al., 1965; Kertesz, 1951; McColloch et al., 1949; McCready et al., 1954). As a result of the activity of PME and its influence on texture, this enzyme has received considerable attention.

Presently it is difficult, if not impossible, to study the action of PME in situ. Consequently, research has dealt primarily with ascertaining in vitro the response of PME to various chemical and physical conditions. These studies have included determinations of the effect of salt concentrations, pH, temperature, frozen storage, and maturity of the fruit on PME activity (Aung et al., 1965; Hills et al., 1947; Hsu et al., 1965; MacDonnell et al., 1945; Van Buren et al., 1962).

NaCl, the most widely used salt, promotes maximum activity of PME from many sources at concentrations of 0.15–0.2 *M* when the pH is in the range of 7–8 (Deuel et al., 1958; Kertesz, 1955; Lineweaver et al., 1951; MacDonnell et al., 1945; Rouse et al., 1955). PME of many plants is most active at temperatures between 45° and 55°C (Hills et al., 1947; Manabe et al., 1965; Van Buren et al., 1962). The temperature coefficient for PME is fairly low (Lineweaver et al., 1951); the Q_{10} under specified conditions for snap bean PME is 1.4 (Van Buren et al., 1962) and for tomato PME, 1.43 (Hills et al., 1947).

Generally, freezing and frozen storage do not destroy PME activity appreciably in plant tissues (Manabe et al., 1965; Van Buren et al., 1962).

Maturity of the plant tissue may influence the relative PME activity (Lineweaver et al., 1951). An increase in enzymatic activity upon maturity has been demonstrated in tomatoes (Hills et al., 1947), cherries (Al-Delaimy et al., 1966), oranges (Rouse et al., 1962), and persimmons (Nakayama et al., 1966).

Although the functions of PME in plant tissues has not been elucidated, there are reports which indicate that PME demethylates component pectic substances of plant tissues under certain conditions. Deshpande et al. (1965) studied the effect of heat treatments and polyvalent cations on texture of canned tomatoes. They showed that tomatoes subjected to mild heat reacted more favorably with cations than did raw tomatoes. The authors concluded that this condition was probably due to enzymatic action, resulting in some de-esterification during the transient period of heating.

Often changes take place in Southern peas during the post-harvest and processing periods which produce an undesirable toughness that is not overcome by prolonged cooking. The reason for the development and persistence of this hardness is not known, but it may result from the bonding of various polyvalent cations with the demethylated pectic substances within the cotyledons or seed coat (Kertesz, 1939). The amount of polyvalent cations within the peas (Howard et al., 1962; Wade et al., 1951) and in the water (Bigelow et al., 1923) used for processing could be sufficient to produce insoluble pectic substances and toughening.

Literature was not available concerning PME in Southern peas. Therefore, before attempting to relate PME activity to texture, the nature of the enzyme should be determined. The objectives of this study were to determine in vitro the response of PME to variations of NaCl concentration, pH and temperature; to study the influence of rinsing, freezing and cold storage of the peas on enzymatic activity; and to ascertain the influence of maturation on PME activity.

MATERIALS & METHODS

Source and maturity of peas

Southern peas of the Purple Hull Pink Eye (PHPE) and Princess Ann (PA) varieties were raised on the University Plant Science Farm at Knoxville by the Department of Horticulture. The former variety was harvested by hand at 3 stages of maturity. The stages, as determined from the color of the seed pods, were: green pods, most immature; green-purple pods, intermediate maturity and glossy reddish-purple pods, most mature. The maturity range represented that of the peas (mature, dry peas excluded) normally harvested for freezing. The maturation rate was such that the green pods became purple within 3–4 days. Peas of the PA variety were harvested by hand and combined to provide a composite of different stages of maturity. No mature, dry peas were included.

Handling and freezing of the peas

The peas were shelled by hand and divided into two lots. One lot was designated for assay as soon as possible in the fresh state and the other lot was frozen for assay at a later date. The peas to be frozen were packaged in polyethylene bags (each *ca.* 1 lb capacity) and placed in an air-blast freezer (−25°C) overnight. After freezing, the peas were moved to and held in a still-air freezer (−18°C) for at least 84 days.

Factors considered in assay of PME

PME of fresh peas of the PHPE and PA varieties was studied to ascertain the effect

of different levels of NaCl and pH on enzymatic activity. The additional effects of temperature, maturity and rinsing of peas were evaluated for the PHPE variety. Frozen PHPE peas were used for determining the effect of frozen storage, maturity and rinsing on PME activity.

Sample preparation for PME assay

The source of PME was a slurry of the peas. The slurry was prepared by blending the peas with a 2% solution of NaCl (1:2, w/v) in a Waring blendor for 4 min (the peas were blended in distilled water when the level of salt was the variable under study). A small amount of Antifoam A spray (Dow Corning Corp., Midland, Mich.) was added to the mixture before blending to reduce foaming. Before the frozen peas were mascerated, they were allowed to reach the melting point, thus permitting satisfactory blending.

For the test mixture in which PME activity was measured, 30 g of the enzyme-containing slurry, 50 ml 2% pectin N.F. (w/v) (Sunkist Growers, Inc., Corona, Calif.), 70 ml distilled water and a sufficient amount of NaCl to bring the concentration to the desired level were mixed. The final concentration of pectin was 0.67%.

Procedure for PME assay

The temperature of the mixture was raised to 30°C (used for convenience) by placing the beaker containing the mixture in a water bath of 75°C and stirring the contents vigorously. The pH of the mixture then was adjusted to the desired level with 1 *N* NaOH. With the aid of an automatic titrator, the pH was maintained during the reaction period of 5 min. The temperature was maintained by holding the beaker in a constant temperature water bath. The mixture was continually stirred by a magnetic stirrer. Enzymatic activity is reported as PME units, each unit indicating that amount of activity which produced 1 meq of acid per min per g of peas (dry weight) (Rouse et al., 1955).

Initial study to determine optimum conditions for PME assay

At the beginning of the study, experiments were conducted to determine the concentration of NaCl and the pH which would promote maximum enzymatic activity for the PHPE (intermediate maturity) and PA varieties. To ascertain the level of salt, the concentration of NaCl in the test mixture was varied from 0 to 0.75 *M*. The pH and temperature were held at 7.5 and 30°C. Next, the pH which would promote maximum activity at the optimum salt level previously determined was ascertained by adjusting the mixture to and titrating at pH values from 5 to 9.5. Again, the temperature was held at 30°C.

Further studies of PME activity

Further experimentation using the PHPE variety was conducted under the "optimum" conditions (0.2 *M* NaCl and pH 7.5) determined by the initial study. The additional experiments included determinations of the effect of temperature, maturity, frozen storage and rinsing of peas on the activity of PME.

Temperature. The effect of different temperatures ranging from 25° to 65°C on the activity of PME was studied, utilizing the intermediate stage of maturity. Adjustments in temperature were made by the procedure previously given.

Maturity. The investigation of the effect of maturity was conducted by utilizing fresh and frozen peas of the 3 stages of maturity.

Frozen storage of peas. Peas of the 3 stages of maturity were removed from the freezer after 84 days of storage and assayed for PME activity. An additional study was made to determine if the PME activity changed in the slurries of previously frozen peas (intermediate maturity) upon standing. 30-g aliquots of the slurry were added to beakers and allowed to stand up to 4 hr at 6° and 24°C. At 1 hr intervals the test mixtures were prepared and titrations were conducted.

Rinsing of the peas. This experiment was conducted in 2 parts. 100 g of peas (fresh and frozen stored peas of the 3 stages of maturity) were covered with 100 ml distilled water in a 500-ml Erlenmeyer flask and placed on a wrist action shaker for 30 min. After the period of shaking, the solution was decanted and assayed for enzymatic activity. Each test mixture contained 30 ml of the solution as the source of the enzyme. The reaction period was extended to 10 min. For this study one PME unit is the activity which produced 1 meq of acid per ml of rinse water.

After decantation, the peas were rinsed again with running water (*ca.* 500 ml) to remove the remaining external soluble protein. These peas then were utilized to determine the residual PME activity.

Determination of moisture and protein contents

The moisture content of the peas was determined by freeze-drying 25-g samples in duplicate to constant weight. The protein content (dry basis) of 2 g of wet peas was measured by the Kjeldahl method ($N \times 6.25$) (AOAC, 1965).

Statistical analyses

Samples were assayed in duplicate for PME activity when the effect of salt concentration, pH, temperature, rinsing of the peas on PME activity in rinse water and standing of frozen peas slurries on the enzymatic activity were considered. All other determinations (maturity, freezing and residual PME activity after rinsing) were made in triplicate. The data were analyzed by the analysis of variance arrangement, using the ANOVAR program (Bone, 1963) adapted for computation by the IBM 7040 computer at the University Computing Center. The Duncan's Multiple Range Test and the Least Significance Difference Test were used to evaluate differences among mean values (Li, 1959).

RESULTS & DISCUSSION

THE PROTEIN content of the most immature peas (PHPE variety) was 23.4% and, of the most mature peas, 26.7%. Since the peas represented a maturation period of 3 to 4 days, the daily mean increase of protein was *ca.* 0.94%. The moisture level decreased from 75.8% to 62.5% during this period.

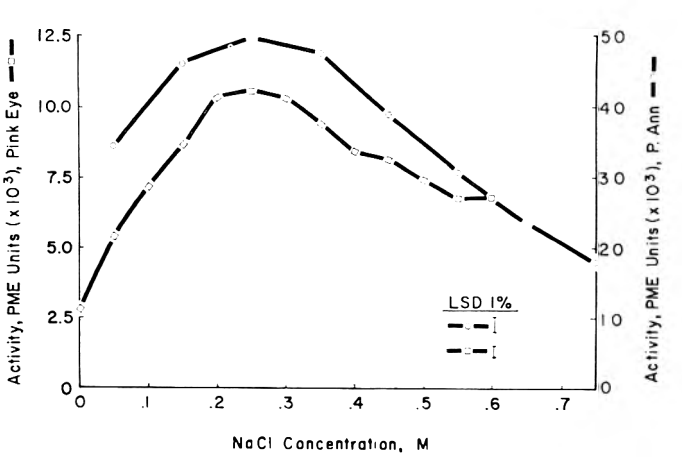

Fig. 1—Activity of pectin methyl esterase of Southern peas at different concentrations of NaCl. Conditions of assay: pH 7.5 and 30°C.

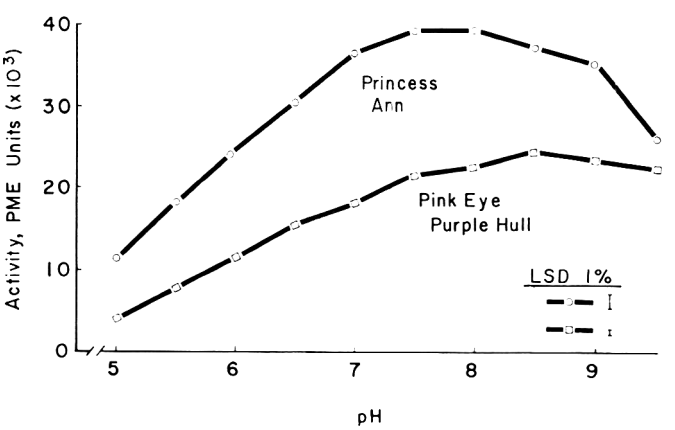

Fig. 2—Activity of pectin methyl esterase of Southern peas at different pH levels. Conditions of assay: 0.2 M NaCl and 30°C.

Figure 1 presents the PME activity values of the test mixtures which contained different concentrations of NaCl. Maximum activity for PME in the PA variety was obtained at 0.25 *M* NaCl. Raising or lowering the level of salt reduced activity. A similar response was observed for the PHPE variety, with maximum activity occurring at salt concentrations between 0.2 and 0.3 *M*. A 0.2 *M* concentration was selected for assaying subsequent samples for PME. However, a 0.25 *M* concentration would probably have been more practical, since at this level a small inadvertent variation in the concentration of salt in the assay mixture would not have produced a significant difference in activity when other variables were being tested.

The effect of pH on PME activity is presented in Figure 2. PME in the PA variety was most active at pH 7.5–8. When the pH was altered from this level, the activity decreased. PME activity in the PHPE variety was maximum at pH 8.5. The pH value of 7.5 selected for assay of PME in subsequent treatments was erroneously taken from the results obtained by analyzing peas of the PA variety. A pH of 8.5 would have been a more suitable level.

The PME activity of peas of the PA variety was higher than peas of the PHPE variety. This could be due to varietal differences or to a greater proportion of immature peas in the PA sample.

The response of PME to changes in temperature was similar to that reported for PME from other sources. Enzymatic activity increased about 2-fold when the temperature was raised from 25° to 50°C (Fig. 3). No difference in activity was found between 50° and 60°C, but at 65°C the activity was reduced sharply. The mean Q_{10} was 1.35 over the range of 30° to 50°C.

Table 1—Effect of maturity, frozen storage, and rinsing on activity[1] of pectin methyl esterase in Southern peas.

Maturity		Frozen storage		Rinsing	
Stage	Mean	Days	Mean	Treatment	Mean
Green	48.3 a	0	31.5 p	Unrinsed	33.9 x
Green-purple	27.7 b	84	31.9 n	Rinsed	29.5 y
Purple	19.2 c				

[1] PME Units ($\times 10^3$).
Means within a variable not followed by the same letter are significantly different (Maturity and Rinse, $P < 0.01$; Storage $P < 0.05$).

Table 2—Effect of maturity x frozen storage x rinsing interaction on activity[1] of pectin methyl esterase in Southern peas.

Rinse treatment	Maturity (pod color) Green	Green-purple	Purple
	Fresh, 0 days storage		
Unrinsed	52.3 a	28.1 e	18.3 h
Rinsed	46.5 b	27.3 e	16.7 i
	Stored, 84 days, −18°C		
Unrinsed	53.2 a	30.4 d	21.2 g
Rinsed	41.1 c	25.0 f	20.7 g

[1] PME units ($\times 10^3$).
Means not followed by the same letter are significantly different ($P < 0.01$).

Table 1 shows the effects of maturity, frozen storage and rinsing of the peas on enzymatic activity in the PHPE variety.

The maturity level was the major factor influencing activity. The more immature the peas, the higher the activity. Peas with green pods had about 2.5 times as much PME activity as peas with purple pods. There was an inverse relationship (by observation) between PME activity and protein content in the peas.

Peas held at −18°C for 84 days had a slightly higher activity ($P < 0.05$) than the fresh peas. The increase was probably due to the liberation of PME by mechanical disruption of the cellular materials since PME is adsorbed strongly to the water-insoluble cellular constituents (Kertesz, 1955; McColloch et al., 1949). According to Newcomb (1955), PME activity appears to be localized at the surface of the cell in intimate association with the cell wall.

The interaction between maturity, frozen storage and rinsing of the peas is presented in Table 2. In all treatments there was a progressive decrease of PME activity with maturity. Freezing caused the more mature, unrinsed peas (green-purple and purple) to exhibit a higher activity than the fresh counterparts. This indicates that the enzyme might have become more strongly affixed to the cellular constituents as the peas became more mature and was subsequently released by freezing.

Rinsing did not lower the activity in the most mature, frozen peas (purple pods). Apparently, some of the PME that was "activated" was located well within the cotyledons and was not subject to dissolution. Rinsing had the greatest effect on green, frozen peas where the enzymatic activity was reduced by 22.7%. However, in fresh peas of the same maturity level, rinsing decreased the activity by 11.1%.

The PME activity values of the rinse water from fresh and frozen peas of the 3 maturity levels are presented in Table 3. The presence of active enzyme in the aqueous solution shows that some of the PME was dissolved from the pea tissues. The rinse water from the frozen peas had a mean activity that was 2.7 times that

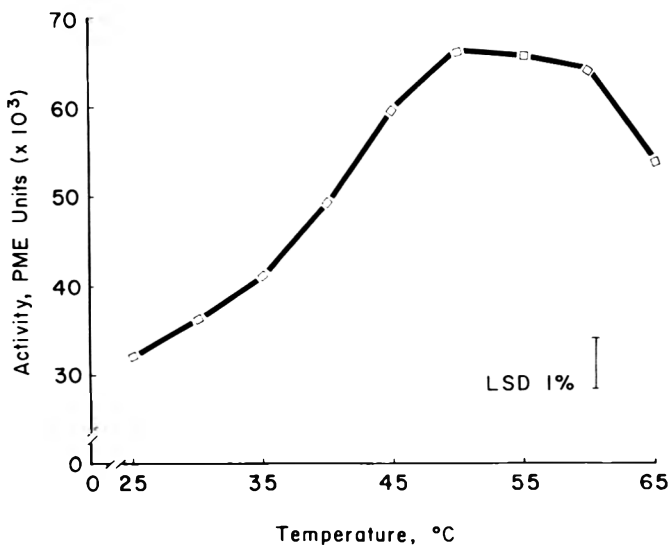

Fig. 3—Activity of pectin methyl esterase of Southern peas (Purple Hull Pink Eye variety) at different temperatures. Conditions of assay: 0.2 M NaCl and pH 7.5.

Table 3—Pectin methyl esterase activity[1] of rinse water from Southern peas of three maturity levels.

Storage, −18°C, days	Maturity (pod color) Green	Green-purple	Purple	Storage, mean
0 (fresh)	0.24 c	0.14 d	0.08 d	0.15 y
84	0.59 a	0.36 b	0.27 c	0.40 x
Maturation, mean	0.41 m	0.25 n	0.17 p	

[1] PME units ($\times 10^3$).
Means within a variable not followed by the same letter are significantly different ($P < 0.01$).

Table 4—Periodic changes in pectin methyl esterase activity[1] of slurries prepared from frozen Southern peas.

Duration, hr	Temperature, °C		Duration, mean
	6	24	
0	24.0	23.4	23.7 c
1	26.2	27.2	26.7 b
2	27.2	28.5	27.9 b
3	28.5	30.1	29.3 a
4	29.2	30.0	29.6 a
Temperature mean	27.0 n	27.8 m	

[1] PME units ($\times 10^3$).

Means within a variable not followed by the same letter are significantly different (Duration, $P < 0.01$; Temperature, $P < 0.05$).

found in the rinse water of fresh peas. The activity in the rinse water from both fresh and frozen peas decreased as more mature peas were utilized.

Table 4 shows the periodic increases of PME activity when the slurries of previously frozen peas were allowed to stand up to 4 hr. There was a progressive increase for 3 hr, but no significant changes occurred between the 3- and 4-hr period. The increase in PME activity was 1.25-fold. The gradual increase in available PME might have resulted from a continuous release of PME from the cell wall constituents or even from within the cells. Further experimentation is needed to elucidate the site within the tissue where PME is located.

The question arises concerning the role of PME within the intact peas. Although it is generally assumed that the enzyme in the uninjured plant tissue is essentially inactive (Van Buren et al., 1962), there are several ways by which PME in Southern peas may become activated. First, the shelling operation may initiate enzymatic reactions by bruising the tissues. It is possible that the enzyme and the substrate could be brought into intimate proximity within the damaged areas, permitting the enzyme to function (Lineweaver et al., 1951).

In addition, the peas are sometimes held, before and/or after shelling, under conditions which often cause the peas to reach relatively high temperatures. These elevated temperatures, due primarily to the heat of respiration, often reach the point where PME would function in vitro at the maximum rates. At the peak of the harvest season, facilities are usually inadequate for processing the large volume of peas without undue delay. As a means of alleviating this situation, the peas are placed in large tanks filled with water and held until they can be processed. With a portion of the PME being water-soluble (the relative amount would depend on the stage of maturity), conditions would favor demethylation of pectic substances of the peas.

Southern peas are subjected to many conditions during the harvest, post-harvest, and processing operations which favor partial demethylation of the inherent pectic substances. Reaction of these products of demethylation with cations could permit subsequent adverse textural changes to take place within the peas. In view of these possibilities, further studies are planned to ascertain the action of PME on the methylated pectic substances and to relate this action to textural changes of Southern peas.

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Role of Cuticle in Spoilage of Chicken Eggs

SUMMARY—The role of cuticle (mucoprotein layer on the egg shell) in preventing spoilage of eggs by microorganisms was studied. Eggs collected from the uterus or eggs treated with 5% EDTA solution to remove cuticle spoiled at a much faster rate than the normally laid eggs. The weight of the egg shell and its membranes did not affect the spoilage of eggs collected from the uterus. The protection provided by the cuticle was found to last at least up to 96 hr after the eggs are laid.

INTRODUCTION

THE ABILITY of eggs to resist spoilage by microorganisms is largely due to the exterior structures of the egg, including the shell and the inner and outer shell membranes. The egg albumen proteins, including lysozyme, conalbumin, avidin and others, also have bacteriostatic properties. The role of the shell and its membranes has been studied by Lifshitz et al. (1964), Garibaldi et al. (1958), Kraft et al. (1958) and Brown et al. (1965). No exact mechanism of bacterial penetration has been postulated, but it has been generally agreed by these workers that the inner shell membrane offers the greatest resistance to bacteria.

The role of the cuticle (bloom) in preventing spoilage has not been fully studied. There is a common belief that the protective action of the cuticle, if any, prevails for just a few hours after the eggs are laid. This study was undertaken to determine the role played by the cuticle in the spoilage of eggs and the length of time such a protective action is available.

MATERIALS & METHODS

Eggs

To study the effect of the cuticle on spoilage, eggs were collected in a commercial dressing plant from the uterus of hens while the birds were being eviscerated. The collection was done in the early part of the morning so that the eggs were of the same age. A portion of the eggs was tested to make sure the eggs were devoid of cuticle. A set of control eggs was obtained from the Cornell University farm. All of the eggs used in the study were from one strain of White Leghorns. The eggs from the uterus were dried at room temperature for 6–8 hr before washing as were the control eggs.

Shell-less eggs

At the slaughter house, a number of immature eggs which had no shell were also obtained, and these eggs will be referred to as shell-less eggs.

Removal of cuticle

For this part of the experiment, the cuticle was artificially removed by dipping eggs in 5% Ethylenediamine tetra acetic acid (sodium salt) solution for 10 min. The mucilaginous layer was removed by rubbing the surface of the shell.

Testing the cuticle

The eggs were dipped in 5% EDTA solution. The solution was then tested for the presence of protein by the procedure of Lowry et al. (1951).

Washing of eggs

The eggs were washed in an immersion type of egg washer using a sanitizer-detergent according to the directions of the manufacturer. The washing time was 3 min and the temperature was 45°C. The eggs were then rinsed with water at 40°C to remove any residual sanitizer-detergent.

Exposure to infection

Eggs were exposed to infection with *Pseudomonas aeruginosa*. The dip water was 15°C and had a count of approximately 100,000 *P. aeruginosa* per ml. The eggs were left in the dip water for 5 min.

Spoilage

The infected eggs were incubated at room temperature (21–23°C). The spoilage was determined by candling eggs using ultraviolet light. Eggs showing a greenish fluorescence were considered spoiled.

Length of protection by cuticle

The length of protection afforded by the cuticle was studied by collecting eggs from three different strains of White Leghorn hens. 20 eggs were collected at 0, 1, 3, 6, 24, 48, 72 and 96 hr post lay. Except for 0 hr eggs, all the eggs were heated in an incubator at 42°C for 1 hr to bring them to the same temperature as eggs when laid. The eggs were nest clean and were not washed before exposing to infection.

RESULTS & DISCUSSION

DATA PRESENTED in Table 1 show the importance of cuticle on the spoilage of eggs artificially exposed to infection with *P. aeruginosa*. Normally laid eggs which had the cuticle showed a spoilage of 28 and 37% in two trials after 15 days of incubation following infection. When normal eggs were treated with 5% EDTA solution to remove the cuticle, the spoilage rate increased to 92 and 100% after 11 days of storage at room temperature. Eggs collected from the uterus behaved very similarly to EDTA treated eggs and the spoilage was 98, 100 and 100% in three trials after 15 days of storage.

The eggs collected from the uterus or the ones treated with EDTA solution had a more rapid rate of spoilage. After 3 days of infection, these eggs showed a spoilage of over 20% while none of the normally laid eggs showed any spoilage.

The difference between normal eggs and eggs collected from the uterus were essentially due to cuticle. Romanoff et al. (1949) reported that the cuticle is deposited on the egg in the vagina just be-

Table 1—Effect of cuticle on spoilage of eggs after infection with Pseudomonas aeruginosa

Treatment	Trial	Spoilage time, days								
		3	5	7	9	11	15	20	25	30
		Percentage of spoilage								
Normally laid	1	—	—	—	—	7	28	57	80	93
	2	—	—	3	8	22	37	71	91	100
Eggs from uterus	1	8	35	41	47	64	98			
	2	43	61	74	89	100				
	3	38	50	68	86	91	100			
Shell-less eggs	1	100								
	2	100								
EDTA Treated eggs	1	—	12	28	45	62	82	100		
	2	—	18	32	51	70	86	100		

Table 2—The influence of weight and thickness of egg shell and membranes on spoilage by Pseudomonas aeruginosa.

Incubation time (days) at 21–23 °C	Weight of shell and its membrane, g			Thickness of shell and its membrane, mm		
	4.0–4.5	4.6–5.0	5.1–5.5	0.25–0.30	0.31–0.35	0.36–0.40
	Numbers showing spoilage[1]					
5	9	7	14	10	17	3
7	1	2	1	2	2	
8	—	2	1	1	2	
9	1	5	8	3	9	2
11	—	1	1	1	1	

[1] A total of 54 eggs were studied.

fore it is laid, which means the eggs from the uterus should not have any cuticle. This was confirmed by dipping the eggs in 5% EDTA. The test for cuticle as determined by the presence of protein was negative.

The importance of the cuticle was further confirmed when the normal eggs were treated with EDTA solution, and it was observed that these eggs spoiled much faster than the untreated eggs. The EDTA solution was tested for calcium and the result showed that such a treatment did not remove any calcium and thereby weaken the shell structure.

The importance of shell and cuticle as a protective agent is clear from the data obtained with shell-less eggs. In two trials the spoilage was 100% within 3 days of incubation while normally laid eggs had to be incubated for over 30 days for a similar degree of spoilage.

Since the eggs were collected from the uterus, it was possible they may not have fully developed. The amount of shell deposited could be different and thus may affect on spoilage. This was studied by determining the effect of weight and thickness of egg shell and its membrane on the spoilage of eggs collected from the uterus.

Table 3—Effect of strain in post-lay time on spoilage of eggs when infected with Pseudomonas aeruginosa.

Strain	Time elapsed after eggs were laid, hr								Total for each strain
	0	1	3	6	24	48	74	96	
	Number showing spoilage after 15 days[1]								
A	3	0	5	3	2	5	9	1	28
C	4	2	10	13	7	11	15	11	83
K	4	1	8	9	2	11	8	6	54
Total for all strains	11	3	23	25	11	27	32	18	

[1] 20 eggs for each group.

Table 2 shows that the weight of the shell and its membranes varied from 4.0 to 5.5 g, while the thickness varied between 0.25 and 0.400 mm. These values are comparable to normally laid eggs.

No definite correlation was observed between the weight of shell and its membranes and spoilage, indicating that the eggs obtained from the uterus were mature. The egg shell weight was not a factor in spoilage and the differences in spoilage were due to cuticle alone. The egg shell and membrane thickness showed similar results.

The length of time the cuticle affords protection is shown in Table 3. Using three strains of birds, no definite pattern of spoilage was observed. It appears that the protection afforded by the cuticle is not altered at least up to 96 hr after lay. However, the eggs laid by three strains of birds showed considerable differences in spoilage. Strain A was most resistant to spoilage while C was the most sensitive to spoilage.

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Chicken Myofibril Fragmentation in Relation to Factors Influencing Tenderness

SUMMARY—*Susceptibility of chicken pectoralis major myofibrils to mechanical fragmentation was investigated, after various periods of aging, as an index of tenderness. Treatments were used which accelerated, retarded or prevented post-mortem glycolysis. Fragmentation, with breaks always beside the Z line, was measured by microscopic examination of homogenized muscle. Fragmentation of pre-rigor muscle produced small, contracted and poorly defined particles. As rigor mortis developed, fragments became longer, more rigid and clearly defined. With additional aging after full rigor, homogenization produced progressively smaller myofibrillar fragments consisting of 1 to 6 sarcomeres. Muscle was least tender (as measured by shear force) when it was in full rigor and tenderized with subsequent aging. Retardation of the onset of rigor mortis extended the time required for tenderization. Although the fragmentation pattern generally corresponded to changes in tenderness in glycolysing muscle, fragmentation was not found to be an accurate index of tenderness. Sarcomeres did not lengthen during the aging period after rigor mortis developed.*

INTRODUCTION

TENDERNESS changes in muscle during rigor mortis development and post-mortem aging have been the subject of extensive research. However, an understanding of the mechanisms involved in these changes, particularly after rigor development, has not been reached. Radouco-Thomas et al. (1959) found that antemortem epinephrine injection retarded muscle fiber disintegration during aseptic storage. Sharp (1963) reported that muscle broke up into short segments of fibers and fibrils after long term aseptic storage (up to 65 days). He indicated that the degree of disintegration during homogenization was related to the degree of intracellular proteolysis.

Phase contrast micrographs by Davey et al. (1967) of beef muscle aged 4 days at 15°C showed that the Z line disappeared and that the myofibrils distintegrated into individual A bands upon brief homogenization. A recent paper by Takahashi et al. (1967) reported that myofibrils of chicken muscle were fragmented into progressively smaller segments when homogenized after increasing periods of aging. The rate of increase in fragmentation was most rapid during the first 8 hr post mortem which corresponds with the period of most rapid tenderization (Pool et al., 1959).

The degree of toughening and of subsequent tenderization in poultry muscle is dependent on the rate and extent of post-mortem glycolysis (de Fremery et al., 1960). The resistance to shear of fully aged (24 hr) muscle was increased by subjecting pre-rigor birds to increased time or temperature of scalding and to beating with mechanical pickers (Pool et al., 1959). Conversely, prevention of post-mortem glycolysis with either epinephrine or iodoacetate eliminated toughening during the development of rigor mortis (de Fremery et al., 1963).

The purpose of this study was to investigate the effect of acceleration, retardation and prevention of glycolysis on the mechanical fragmentation of myofibrils. The possibility of using myofibrillar fragmentation as an index of tenderness was also investigated.

EXPERIMENTAL

Slaughter and chilling

Commercial, meat type fryers, approximately 2 kg live weight, were used in these experiments. All birds were killed by a throat slash, and the thoracic region without the skin was separated from the remainder of the carcass in such a way that the attachments of the pectoralis muscles to the bone structure were not broken. The thoracic region was placed in a plastic bag in a 15°C bath at 15 min post mortem. After aging 2 hr, it was transferred to a 3°C cold room. The initial chilling temperature of 15°C was used to prevent possible low temperature stimulation and cold shortening of the muscle (Locker et al., 1963).

Alteration of glycolysis

In addition to the control procedure just described, glycolysis was accelerated, retarded or prevented by six methods. Heating or beating the muscle was used to accelerate glycolysis. The muscle was heated by placing the excised thoracic region in a 50°C bath at 5 min post mortem until the internal temperature rose to 45°C (6 min). The muscle was then removed from the bath and held at room temperature until the start of the standard chilling procedure at 15 min post mortem.

Muscle was beaten by placing the breast area of an unscalded, intact carcass on a rotary drum picker (30 sec per side). Glycolysis was retarded by either sodium pentobarbital injection (35 mg/kg intravenous) 5 min before slaughter or electrical stunning for 5 sec before throat slash. In either case there was no death struggle. Neutralized sodium iodoacetate (1 mmole/kg intravenous) injected 3 to 4 min before slaughter was used to block glycolysis. Epinephrine (15 μmole/kg subcutaneous) injected 16 hr pre-slaughter, depleted the glycogen stores in the muscle and prevented post-mortem glycolysis.

Sampling procedure

Portions of the pectoralis major were removed from the thoracic region after specified aging periods without disturbing the attachment of the surrounding muscle. Part of the muscle was clamped between two aluminum plates held apart with 1 cm spacers and cooked 10 min in boiling water (de Fremery et al., 1960). After the cooked muscle was chilled, it was cut into 1 cm square strips and shear force measurements were made with a Warner-Bratzler type shear apparatus. The remainder of the muscle sample was minced with scissors and the pH was measured on a slurry composed of 2 g minced muscle and 5 vol of 5 m*M* Na iodoacetate (Marsh, 1952).

Fragmentation

Mechanical fragmentation of the myofibrils was accomplished by homogenizing 2 g minced muscle with 20 vol of 0.25*M* sucrose, 0.04*M* imidazole and 2 m*M* ethylenedinitrilo tetraacetic acid (EDTA), pH 7.2 for a total of 45 sec with two 30 sec cooling periods (Stromer et al., 1967). Photomicrographs were made of the suspension using a phase contrast microscope. Sarcomere lengths were measured from these pictures, and a subjective score was given for fragmentation.

Subjective scores

Five subjective scores were given for mechanical fragmentation of myofibrils (Fig. 1). The A score was given for myofibrils from muscle considered to be in a pre-rigor condition at the time of homogenization. These suspensions contained a large proportion of amorphous material and the myofibrillar segments were short, heavily contracted and poorly defined. Myofibrils scored as B were long, curved, somewhat poorly defined and in various stages of contraction.

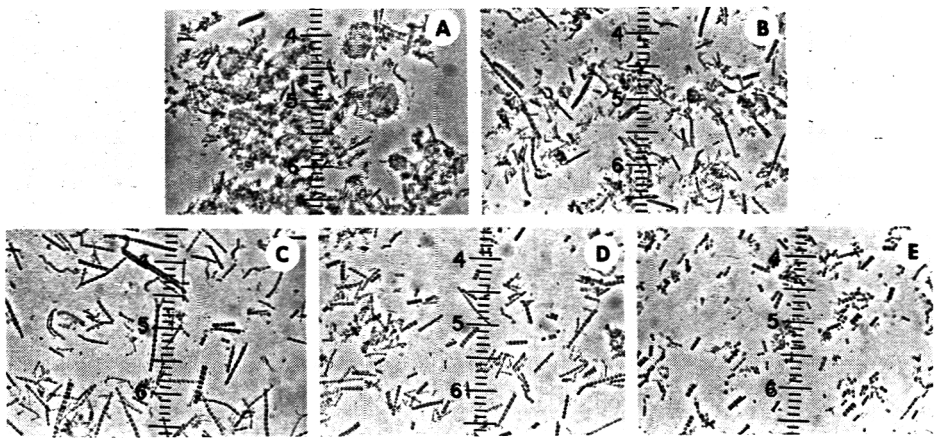

Fig. 1.—Representative micrographs of the five fragmentation scores (each division equals 3.1 μ).

The B score appeared to cover muscle just going into rigor at the time of homogenization.

Myofibrils that were long, rigid appearing and well defined with relatively uniform sarcomere lengths were scored C. No amorphous material was present in these suspensions, and very few short segments were present. Suspensions given a D score were similar in appearance to those scoring C except that they contained a mixture of about equal proportions of short and long myofibrils. Preparations containing predominantly short fragments of 1 to 6 sarcomeres in length were scored E.

RESULTS

Control

Control birds (Fig. 2) killed by a throat slash and allowed to struggle freely, yielded muscle averaging pH 6.3 at 30 min post mortem and pH 6.0 after 2 hr of aging. The standard deviations of the means were large, reflecting the different degrees of excitement and struggling of the individual birds. Sarcomere lengths increased from 1.5 μ in muscle homogenized at 30 min and 1 hr post mortem to 1.9 μ when homogenized at 6 hr. Shear force values increased to a maximum of 5.3 kg at 1 hr post mortem then declined rapidly to 2.7 kg at 6 hr reaching 1.2 kg after 24 hr of aging.

The time course of changes in the above three factors indicates that full rigor was established between 1 and 2 hr post mortem. The appearance of the fragmented myofibrils also indicates that rigor mortis developed during the above mentioned time interval. Samples were scored as A (short pre-rigor fragments) or B (long, flexible myofibrils) through 1 hr post mortem. By 2 hr, in addition to the B score, one third of the samples were given a C score (long, rigid myofibrils). Subsequently, the degree of fragmentation increased until all samples were scored as E (fully fragmented) at 24 hr post mortem.

Fast glycolysis

Acceleration of glycolysis by either heating or beating the muscle (Fig. 3) resulted in pH values near 6.1 within 30 min after slaughter. Average sarcomere lengths of the homogenized myofibrils appeared to be slightly shorter during the first hour post mortem but did not change appreciably throughout the 24 hr aging period. Shear values were maximal 30 min after death at 5 to 6 kg but were approaching 2 kg after 6 hr of aging. Fragmentation scores were primarily B at 30 min and 1 hr reflecting the more rapid onset of rigor mortis. However, fragmentation scores at subsequent sampling times were similar to those of the control muscle. An exception was the high proportion of D scores in heated muscle aged 24 hr. Myofibrillar preparations from this muscle contained a relatively few long thick particles composed of many myofibrils in side by side aggregation.

Slow glycolysis

Both anesthetization with sodium pentobarbital and electrical stunning resulted in high pH values 30 min after slaughter (Fig. 4). The muscle of pentobarbital treated birds was pH 7.1 at 30 min and fell gradually to pH 6.1 after aging 9 hr. Some tetanic contractions were produced by the electrical stunning procedure and consequently at 30 min the muscle averaged pH 6.6. Subsequently, the rate of pH drop was similar to that of anesthetized birds, and by 6 hr post mortem, the muscle had reached pH 6.0.

The short sarcomere lengths of the homogenized myofibrils indicated a pre-rigor condition in the muscle of anesthetized birds until after 6 hr of aging. Conversely, the onset of rigor in muscle of electrically stunned birds was well underway by 2 hr post mortem. The high initial pH and consequent later development of rigor delayed attainment of maximum

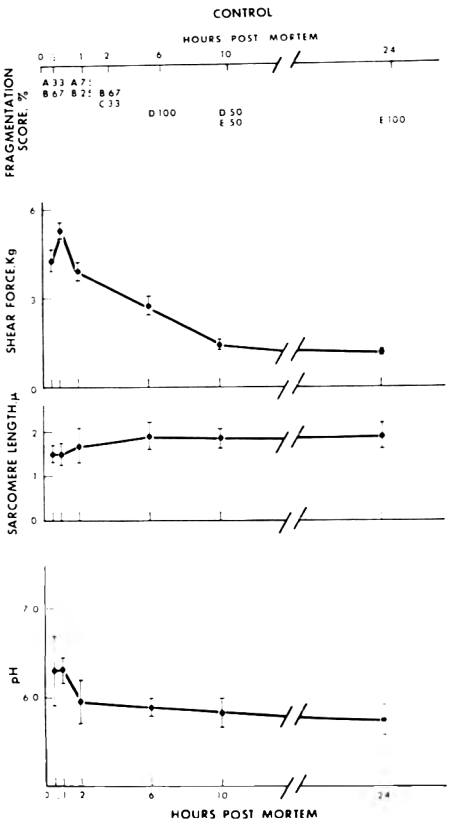

Fig. 2—Post-mortem time course of changes in control muscle samples.

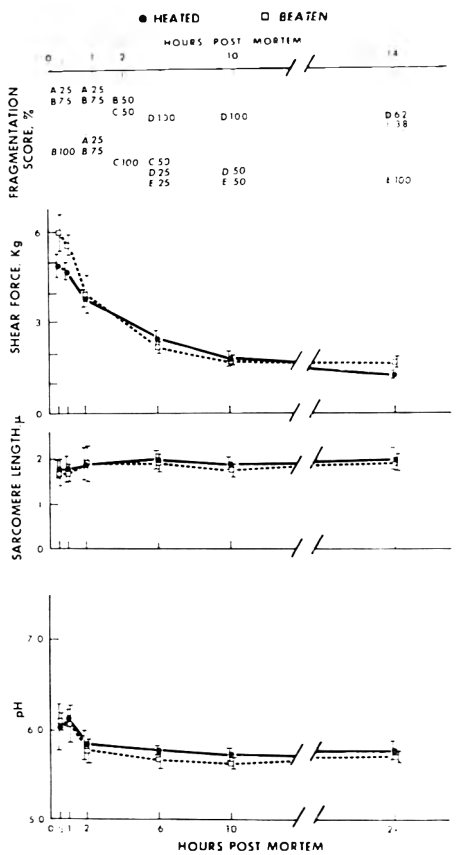

Fig. 3—Post-mortem time course of changes in muscle treated to accelerate glycolysis.

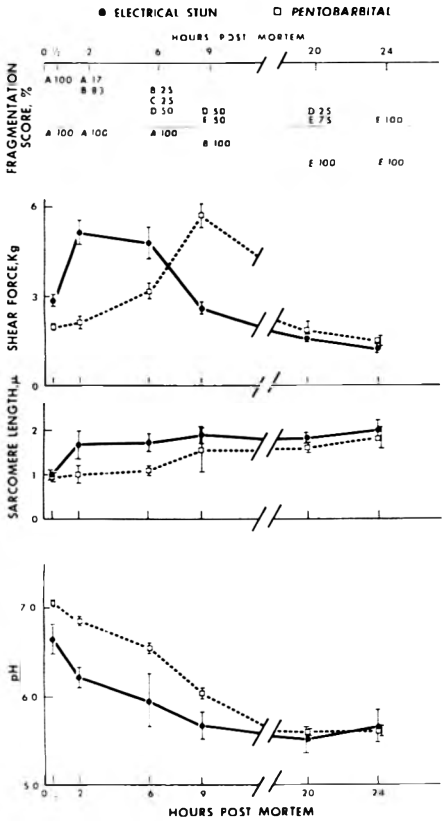

Fig. 4—Post-mortem time course of changes in muscle treated to retard glycolysis.

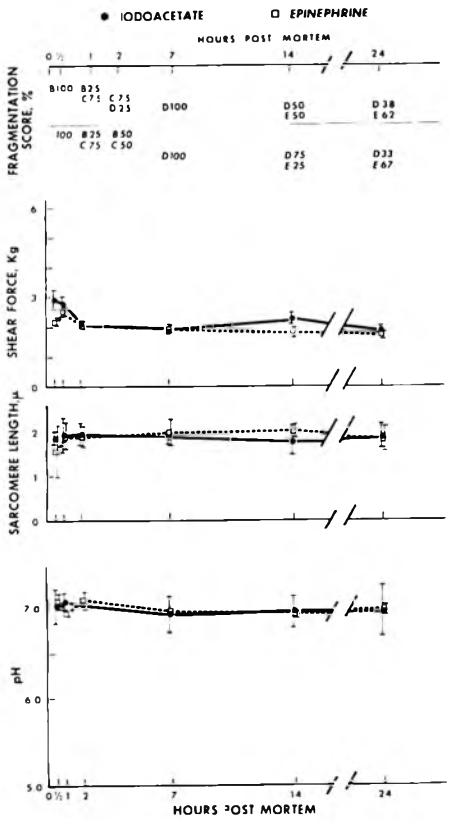

Fig. 5—Post-mortem time course of changes in muscle treated to prevent glycolysis.

shear forces and subsequent tenderization. The shear force for muscle from anesthetized birds increased slowly from 2 kg at 30 min post mortem to 5.7 kg at 9 hr then decreased to 1.6 kg after aging 24 hr.

Electrically stunned birds yielded muscle which followed a similar pattern of shear force changes except that the maximum was reached between 2 and 6 hr post mortem. Changes in fragmentation score were retarded by the delay in rigor development. Homogenates of pre-rigor muscle always yielded small, contracted, poorly defined myofibrils and were given an A score. However, once the muscle began to lose extensibility, the usual progression of fragmentation scores was observed. After aging 20 to 24 hr, the myofibril preparations from both groups were completely fragmented and scored E.

No glycolysis

Pre-slaughter glycogen depletion with epinephrine and blocking of glycolysis with iodoacetate were both effective in preventing a pH drop post mortem and in establishing rigor mortis soon after death (Fig. 5). Values for pH, sarcomere length and shear force did not change appreciably during the 24 hr aging period. However, fragmentation scores did change. There were no A scores since the muscle was in rigor immediately after death, but the progression of fragmentation through the other scores was similar to that noted in any of the other treatments after the establishment of rigor mortis. An exception was that some samples of both iodoacetate and epinephrine treated muscle retained a mixture of long and short myofibrils (D score) after aging 24 hr.

DISCUSSION

THE DELAY in development of maximum toughness due to slow onset of rigor mortis was noted previously by Stadelman et al. (1961). Sodium pentobarbital was administered orally in their experiment and produced a less pronounced but longer period of toughness than noted in the present experiment. Electrical stunning is used commercially particularly in turkey processing, and the results of this investigation implicate it as a factor which might lengthen the aging period required for tenderization. The onset of rigor mortis appears to initiate the tenderization process. Thus early development of rigor was found to speed tenderization. It should be noted that the muscles of electrically stunned birds were not subjected to either heating or beating as would be the case in normal processing. A combination of the treatments might tend to cancel the effects.

The heating and beating treatments used in this study accelerated the onset of rigor mortis but did not produce the residual toughness previously found to result from scalding and beating in mechanical pickers (Pool et al., 1959; de Fremery et al., 1960; Wise et al., 1961). The probable explanation is that relatively mild treatments were used in these experiments. Muscles were not subjected to both heating and beating as in a scalding and picking operation. Also, the temperature of the heated muscle was not elevated for longer than 10 min before being lowered rapidly to 15°C.

Observations in this study support the findings of Fukazawa et al. (1963) that breaks in the myofibrils always take place in the I band region of the sarcomere and that the A band is never broken. Likewise the present observations do not indicate that breaks are the result of complete disintegration of the Z line. Although Davey et al. (1967) found that the Z line disappeared in 4-day-aged beef muscle, it was present in short term aged (<24 hr) chicken muscle. Observations with the light microscope indicated that the bulk of the Z line was either attached to the broken end of a fragment with no I filaments protruding on the break side or absent from protruding I filaments at a break. This would support the suggestion of Takahashi et al. (1967) that fragmentation is due to a weakening of bonds between the thin actin containing filaments and the Z line.

Although fragmentation takes place at the same time as tenderization in muscle, they may not have a direct cause and effect relationship. As previously reported by de Fremery et al. (1963), prevention of post mortem glycolysis renders muscle tender without aging. Muscle, in which glycolysis had been blocked and in which rigor developed immediately, did not exhibit a change in shear force values during the aging period. But, this muscle did offer appreciable resistance to mechanical fragmentation during the first 2 hr after slaughter, as indicated by the subjective scores.

The apparent delay in fragmentation when rigor mortis developed slowly as in the muscle of anesthetized birds may have been due to masking of fragmentation changes by the pre-rigor condition of the muscle. Almost all of the treatment groups show examples where changes in shear force are not reflected by changes in fragmentation score. The results of this study do not indicate that mechanical fragmentation of myofibrils offers a reliable index of tenderness. Mechanical breaking of the myofibrils during homogenization is very likely a manifestation of the structural weakening that takes place during tenderization. However, either localized areas of weakening or widely dis-

tributed breaks in myofibrils may cause appreciable tenderization and not be detected by microscopic examination.

In contrast to the findings of Takahashi et al. (1967), no indication of reversal of contraction was found during the aging period. The short sarcomere lengths noted in the early stages of aging were considered to have been established during homogenization of pre-rigor muscle. Considerable variability was noted in sarcomere length, particularly at the time of rigor onset. However, once the muscle was in rigor, the average sarcomere length remained constant regardless of the degree of fragmentation. Attachment of all muscle samples to the bone structure until the time of sampling, in the present study, probably accounts for the different findings of the two investigations.

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Flavor Components in Cognac

SUMMARY—Flavor components of a genuine cognac were identified by means of CGLC/MS. The complex extract was fractionated on a packed column preceding further separation on a capillary column. The identification of fatty acids, phenolic acids and some of the carbonyl compounds was achieved by other chromatographic methods. 81 components, of which 24 have not previously been reported in cognac and other grape brandies, were identified.

INTRODUCTION

THE AROMA of cognac has previously been studied by a number of investigators. Baraud (1961) identified and quantified some of the alcohols and esters by means of gas chromatography in a pentane/-ether extract of the liquor. The same author (Baraud, 1964) published a survey of these compounds in cognac, calvados and rum.

Bober et al. (1963), Deluzarche et al. (1967) and Fouassin (1959) analyzed cognacs for their most abundant constituents, such as alcohols, esters and carbonyl compounds, with the aid of GLC. Otsuka et al. (1965) isolated the phenolic constituents from cognac from a precipitate by lead acetate and identified vanillic acid and ellagic acid.

The identification of some of the fatty aldehydes, aromatic hydroxyaldehydes and furfural as their 2,4-dinitrophenylhydrazones by paper chromatography and thin-layer chromatography was carried out by Lichev et al. (1958) and Ronkainen et al. (1962). Recently, various brands of whisky, cognac and rum were analyzed for their fatty acids by Nykänen et al. (1968). Identification was based on the retention times of the free fatty acids C_2-C_{10} and of the methyl esters of the free fatty acids C_8-C_{18}.

Apart from the analytical studies on cognac, data are available in the literature on the composition of the aroma of other grape brandies and grape brandy fusel oils (Dzhanpoladyan, 1962; Frey et al., 1956; Otsuka et al., 1965; Rodopulo et al., 1963; Singer et al., 1965; Webb et al., 1952). Identifications of components were mainly based on chromatographic retention parameters, though at times results were supported by chemical group separations.

This paper presents the results of the analysis of a genuine cognac, carried out with the aid of a coupled capillary gas chromatography and mass spectrometry arrangement (CGLC/MS). This technique was previously used by other investigators (Buttery et al., 1963; Day et al., 1964; Mc Fadden et al., 1963, 1965; Maarse et al., 1966). An extract of the cognac was separated into fractions on a packed GLC-column preceding the analysis of these fractions by means of CGLC/MS. The identification of the different components was therefore mainly based on their mass spectra.

Where sufficient amounts of pure compounds could be collected from the packed column, the identification was supported by infrared spectrometry. Carbonyl compounds were isolated from the cognac in the form of their 2,4-dinitrophenylhydrazones and analyzed by thin-layer chromatography. The phenolic acids and fatty acids were identified separately by comparing their retention values in paper chromatography and gas chromatography respectively with those of au-

thentic samples.

EXPERIMENTAL

Cognac

A genuine cognac was investigated. Samples were obtained in bottles of 0.75 l and were found to contain 38.3 vol % ethanol.

Cognac extract

The cognac was diluted with water to 15 vol % ethanol to reduce the amount of ethanol in the extract. 1.5 l of diluted cognac was extracted with pentane/ether (2:1) for 16 hr in a modified Kutscher-Steudel extraction apparatus. After drying over Na_2SO_4 the solvent was removed by careful distillation through a Vigreux column.

Identification

Mass spectrometry and infrared spectrometry. 50 μ. samples of the extract were separated into fractions on a Becker gas chromatograph (Delft, Holland), equipped with a katharometer and fitted with a 4 m Al column (i.d. 4 mm), packed with 20% LAC-1-R-296 on Chromosorb W 60-80 mesh. The oven was operated isothermally at appropriate temperatures and H_2 was used as the carrier gas with a flow rate of 50 ml/min. The emerging fractions were collected in traps according to Badings et al. (1965), cooled in solid CO_2.

The collected samples were injected on a capillary column, coated with polypropylene glycol. The effluent of the latter was admitted directly to the inlet of a conventional single focusing 60° magnetic sector field mass spectrometer (Atlas, CH_4 Bremen, Germany). Whenever sufficient quantities of pure compounds were available, mass spectral analysis was supplemented by infrared spectroscopic measurements on a Perkin-Elmer spectrometer, model 13.

Fatty acids. Free fatty acids C_2-C_8 were separated from cognac using a modified method of Nordström (1963). To 100 ml of cognac, previously diluted to 20 vol % ethanol, a solution of 1 g $NaHCO_3$ in 10 ml of water was added. The mixture was extracted with 40 ml pentane/ether (2:1). After the addition to the aqueous phase of 2 ml of concentrated H_3PO_4 and 10 g NaCl, the solution was distilled under atmospheric pressure until a residue of 15 ml remained. The distillate, which contained the acids, was made alkaline with 0.1 *N* NaOH.

After vacuum distillation until dry, the acids were regenerated from the salts by the addition of 10 drops of 10 *N* H_2SO_4, and extracted with 2 ml of ether. The acids in the extract were identified gas chromatographically by comparing their retention times with those of a mixture of known acids.

Fatty acids C_8-C_{18} were separated from cognac, following the procedure of Nickerson (1966). This method leads to the isolation of the acids present in the free state and in the form of their esters. 100 ml of diluted cognac (20 vol % ethanol) were extracted three times with portions of 50 ml pentane. To the joined extracts was added a solution of 1 g NaOH in 25 ml ethanol. The pentane was removed by distillation and the remaining ethanolic solution was refluxed for $^1/_2$ hr to saponify the esters. The acids were liberated by the addition of 3 ml H_3PO_4 and 75 ml of water and subsequently extracted with pentane. After the conversion of the acids into their methyl esters with methanol/H_2SO_4, the esters were analyzed gas chromatographically on a LAC-1-R-296 column.

Carbonyl compounds. From the cognac, which had previously been acidified to 2 *N* by the addition of HCl, carbonyl compounds were isolated in the form of their 2,4-dinitrophenylhydrazones and analyzed by thin-layer chromatography according to Dhont et al. (1966).

Phenolic acids. 500 ml of cognac were vacuum distilled at room temperature until dry. Phenolic acids were isolated from the residue by extraction with a solution of 10% $NaHCO_3$ in water. Identification was achieved by two-dimensional paper chromatography according to Yang et al. (1962).

RESULTS & DISCUSSION

FIGURE 1 shows a chromatogram obtained by injecting 50 μl of a cognac extract on a packed LAC-1-R-296 column. The shaded portion of the chromatogram indicates one of the collected fractions. Figure 2, showing the chromatogram obtained on reinjecting this fraction on a polypropylene glycol coated capillary column, illustrates the complexity of the fractions trapped.

The components, identified by means of CGLC/MS in the various fractions are listed in Table 1. Also shown are the components identified applying the other methods as described under "Experimental."

81 components were identified with certainty; of these, 24 have not been previously referred to in the literature as occurring in cognac and other grape brandies.

The isolation of fatty acids C_8-C_{18} involved the saponification of the esters of these acids. In cognac, they will be present in the free state as well as in the form of their esters. The ethyl esters will be prevalent as ethanol is by far the most abundant alcohol.

Concerning the acetals identified in the extract, it should be realized that conditions during the extraction procedure are very favorable for their formation since the amount of water in the organic medium is very low (Galetto et al., 1966; Weurman, 1966). Their amount in the original cognac will be considerably lower than in the extract. However, the amounts of aldehydes and acetals in the original cognac are governed by the equilibrium constants. Consequently, both acetals and aldehydes are present. Therefore the mass spectral identification of the acetals in the extract was accepted as proof of the presence of the aldehydes in cognac.

Cognac is stored in oak barrels for a number of years before it is released to the market. It is therefore likely that the phenolic acids identified in the cognac originate from the barrels. They are probably formed by alcoholysis of the

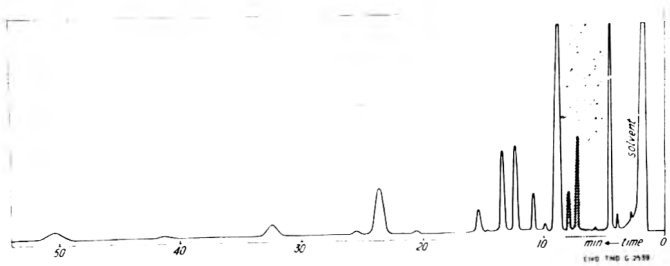

Fig. 1—Chromatogram of a cognac extract. Packed column, 4 m × 4 mm—ID filled with LAC-1-R-296 (20% by weight) on Chromosorb W, 60-80 mesh. Temperature 150°C. The shaded area indicates one of the fractions (Fig. 2) condensed in a cold trap.

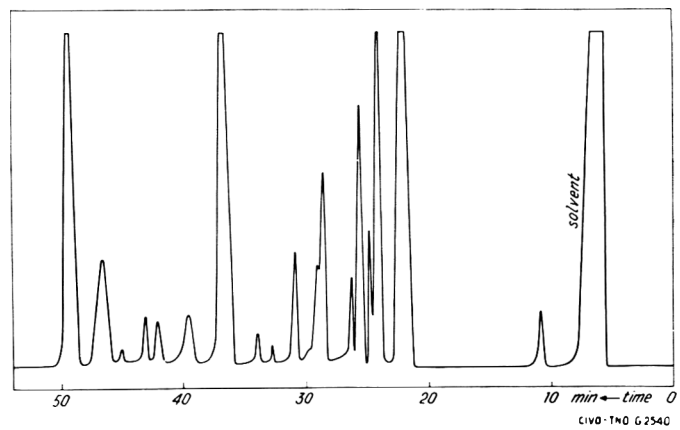

Fig. 2—Chromatogram of a fraction of cognac extract, (Fig. 1). Capillary column, 50 m × 0.25 mm—ID coated with polypropylene glycol; starting temperature 70°C, programmed to 140°C at 1.25°/min.

lignin in the wood (Otsuka, 1965.)

The methyl furfural and furfural could be formed from sugars during the distillation of the wine or during the storage in the oak barrels (Dzhanpoladyan, 1962).

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Table 1—Flavor components identified in cognac.

Component	MS[1]	GLC[1]	other[2]	Ref.[1]
Alcohols				
Ethanol	−	+		+
Propanol-1	+	+		+
Propanol-2	+	−		+
n-butanol	+	−		+
2-methyl-propanol-1	+	+		+
n-butanol-2	−	+		+
3-methyl-butanol-1	+	+		+
n-hexanol-1	+	+		+
n-hexenol	(+)	−		−
n-octanol-1	+	+		+
2-phenyl-ethyl-alcohol	+	+	IR	−
α-terpineol	+	+		−
Carbonyl compounds				
Acetaldehyde	+[3]	+	TLC	+
Propionaldehyde	+[3]	−		+
n-butanal	+[3]	−		+
n-pentanal	−		TLC	−
2-methyl-propanal	+[3]	+		+
2-methyl-butanal	+[3]	+		+
3-methyl-butanal	+[3]	+		+
2-pentenal	−	−	TLC	−
n-hexanal	−	+	TLC	−
n-dodecanal	−	−	TLC	−
Acrolein	−	−	TLC	+
Butanone	+	−		−
Hexanone-2	(+)	−		−
Nonanone-2	+	−		−
Furfural	+	+	TLC	+
5-methyl-furfural	+	+	TLC	+
Benzaldehyde	+	+		−
p-hydroxy-benzaldehyde	−	−	TLC	+
Carvomenthone	(+)	−		−
An ionone	(+)	−		−
Acids				
Acetic acid	−	+		+
Propionic acid	−	+		+
i-butyric acid	−	+	TLC	+
n-butyric acid	−	+		+
i-valeric acid	−	+		+
Caproic acid	−	+	TLC	+
Caprylic acid	−	+	TLC	+
Capric acid	−	+	TLC	+
Lauric acid	−	+		+
Myristic acid	−	+		+
Palmitic acid	−	+		+
Palmitoleic acid	−	+		+
Margaric acid	−	+		+
Stearic acid	−	+		+
Linoleic acid	−	+		+
Oleic acid	−	+		+
p-hydroxy-benzoic acid	−	−	PC	−
Vanillic acid	−	−	PC	+
Syringic acid	−	−	PC	+
Ferulic acid	−	−	PC	−
Gallic acid	−	−	PC	+
Protocatechuic acid	−	−	PC	+

Component	MS[1]	GLC[1]	other[2]	Ref.[1]
Esters				
Methyl formate	+	−		−
ethyl formate	+	+		+
i-amyl formate	(+)	−		−
Hexenyl formate	(+)	−		−
Methyl acetate	+	−		−
Ethyl acetate	+	+		+
n-propyl acetate	+	+		−
i-butyl acetate	+	+		+
i-amyl acetate	+	+		+
β-phenyl-ethyl acetate	+	−		−
Ethyl propionate	+	+		+
Ethyl isobutyrate	+	+		−
Ethyl *n*-butyrate	+	+		−
Ethyl *n*-valerate	(+)	−		+
Ethyl isovalerate	+	−		−
Ethyl caproate	+	+		+
i-amyl caproate	+	+		+
2-methyl-butyl caproate	+	−		+
Ethyl heptanoate	+	−		+
Ethyl caprylate	+	+		+
i-butyl caprylate	+	−		+
i-amyl caprylate	+	+		+
Ethyl nonanoate	+	−		+
Ethyl caprate	+	+		+
i-amyl caprate	+	+		+
Ethyl laurate	+	+		+
Ethyl tetradecanoate	+	+		+
Ethyl lactate	+	+		−
2-ethyl furoate	(+)	−		−
Ethyl levulinate	(+)	−		−
Ethyl malate	(+)	−		−
Miscellaneous				
1,1-diethoxy-propane	+	−		−
1,1-diethoxy-butane	+	−		−
1,1-diethoxy-ethane	+	+		+
1,1-diethoxy-isobutane	+	+		−
1,1-diethoxy-3-methylbutane	+	+		−
1,1-diethoxy-2-methylbutane	+	+		−
Hydrocarbon $C_{12}H_{26}$	(+)	−		−
Hydrocarbon $C_{18}H_{38}$	(+)	−		−
Dibutyl ether	(+)	−		−
a-xylenol	(+)	−		−
Phenol	(+)	−		−
Dihydro furan	(+)	−		−

[1] +, positive identification (Ref. already found in literature); −, no identification.
(+) No definitive identification by lack of material.
[2] Only indicated if identification by some other method was achieved.
[3] Identified as their acetals.

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The Effects of Freezing on the Survival of Salmonella and E. coli in Pacific Oysters

SUMMARY—A mixed inoculum of Salmonella derby or S. typhimurium and Escherichia coli I was injected into the intestinal region of Pacific oysters (Crassostrea gigas) which were then frozen by four methods. Frozen oysters were stored at 0°F, and survival of the inoculated bacteria was determined over a period of two weeks. In separate experiments, inoculated oysters were homogenized and then stored, unfrozen, at 32°F and −30°F (frozen). Routinely, bacterial counts and pH readings were taken of all samples during the course of experiments.

Both species of Salmonella proved to be highly sensitive to freezing, regardless of the freezing method, and showed a survival of 1% or less after 48 hr. E. coli proved less sensitive, showing a wide and capricious variability of survival during the first week of storage, with survival ranging from 10 to 30%. Generally, however, most samples showed a decline comparable to that of salmonellae after two weeks' storage. Because of the fluctuation in E. coli counts after freezing, it is difficult to correlate the numbers of E. coli in frozen shellfish with the count in unfrozen shellfish. Therefore, it would be inappropriate to apply coliform standards for fresh oysters to the frozen product.

In separate studies using inoculated oyster homogenates held at 32° and −30°F for 168 hr, a higher survival rate of E. coli and salmonellae was noted in samples held at −30°F. However, since results obtained were based solely on bacterial counts, it is not possible to say with certainty that these results indicate a protective effect by oyster homogenates against the adverse effects of freezing. Significantly, the results of these experiments did not agree with results obtained with whole oysters, thus indicating the inadvisability of attempting to apply results of homogenate studies to the whole oyster.

INTRODUCTION

OYSTERS exposed to water polluted with domestic sewage can accumulate populations of human enteric bacteria which may include enteropathogenic types. A number of studies have shown that such organisms will persist and may even multiply within the oysters during storage and transport at temperatures between 32° and 48°F. Thus, Tonny et al. (1926) showed that typhoid bacilli survive during the transport of shellfish. Later, Kelly et al. (1954) demonstrated that *Shigella* species could survive for up to five days in artificially polluted oysters and soft clams held at 32°F. Presnell et al. (1961) noted that *Salmonella* species in Gulf Coast oysters held at 39°–44°F were able to survive for at least six days with only a gradual decrease in numbers (one log or less)

Fecal coliforms display essentially the same picture. Hoff et al. (1967) studied the sanitary quality of Pacific oysters held at various temperatures. They reported that numbers of fecal coliforms in oysters held at 0° and 37.4°F for a 20-day period did not increase and may have decreased slightly. Both groups of workers concluded that the fecal coliform count was a good index of bacterial quality in shellfish when the animals were stored and transported at the proper temperatures.

However, with the advent of modern rapid freezing techniques and the use of low storage temperatures which make it possible to ship frozen shellfish, new problems arise. How well do enteropathogens, such as *Salmonella derby* or *S. typhimurium* survive freezing and subsequent storage of shellfish at low temperatures? Is the fecal coliform MPN a true index of the sanitary quality of frozen shellfish? Finally, can the fecal coliform count of the frozen shellfish be used as an index of the sanitary quality of the shellfish before freezing? This paper presents findings on the various aspects of these problems so far as oysters are concerned.

MATERIALS & METHODS

Sample preparation

Shucked, medium sized, Pacific oysters (*Crassostrea gigas*) were purchased from local markets in lots of 50 to 80 oysters for each experiment. The oysters were packaged in polymylar pouches, 3 to 5 animals per pouch, and inoculated. The pouches were heat-sealed and frozen by four different methods. Uninoculated control samples were heated in the identical manner.

Test organisms

Salmonella derby A.T.C.C. 6966, *S. typhimurium* A.T.C.C. 6994 and a strain of *E. coli* type I freshly isolated from human feces were used. The salmonellae were chosen because they had been shown to grow at low temperatures on seafood material (Matches et al., 1968). The inocula were prepared by making serial decimal dilutions of 24 hr trypticase soy broth cultures in sterile phosphate buffer (0.003*M*, pH 7.2).

Inoculation

The oysters were artificially polluted by injecting 1 ml of a mixed culture inoculum into the gut region. The inoculum contained approximately 2.75×10^7 per ml each of *E. coli* I and either *S. derby* or *S. typhimurium*. Zero hr samples were tested for *E. coli* and Salmonella levels. pH readings were also taken. The pH of the gut varied from 5.5 to 6.0, with an average of 5.8. The samples were divided into four lots. One lot was frozen in a plate freezer at −30°F. Another lot was frozen at −10°F in a chest freezer. Of the remaining two lots, one was frozen on the shelves of a sharp freezer at −30°F and the last was frozen in a walk-in storage room held at 0°F.

Storage

Samples were removed from the freezers after 24 hr and stored at 0°F. *E. coli* and *Salmonella* counts were carried out 24, 48, 168 and 336 hr after freezing. *E. coli* counts were determined according to standard methods (1962) and *Salmonella* levels by the Raj-Liston technique (1965).

Homogenate studies

Inoculated oysters were homogenized and aliquots representing 10 g samples of oyster meats were pipetted into sterile screw-capped tubes for freezing and storage.

The oyster homogenates were stored in "walk-in" refrigeration units set at +32° and −30°F. Assays of homogenates were conducted at 24, 48 and 168 hr. The pH readings of homogenates were taken at the same time.

RESULTS

Freezing-temperature experiments

Because of the small size of each unit sample, the temperature decline was equally rapid in each freezing method. These results are shown in Table 1. It is doubtful, therefore, whether any valid conclusions can be drawn concerning the effect of freezing rate on survival as a result of these experiments.

Figure 1 summarizes the results of studies conducted with *S. derby*. These

Table 1—Time in hr required for oysters to freeze and temperatures attained.

Time, hr	Freezing method			
	Plate	Chest	Sharp	0°F Storage room
	Temperature, °F			
0	50	50	55	55
1	30	34	28	40
2	0	5	15	30
3	−30	0	−10	18
4	−30	−10	−30	3
5	−30	−10	−35	0
6	−30	−10	−30	0
7	−30	−10	−30	0

Table 2—Survival rates of Escherichia coli I in individual freezing-storage experiments.

Time, hr	Freezing Method, °F			
	Plate, −30	Chest, −10	0	−30
	Log no. bacteria/unit time			
0	3.4	3.4	3.4	3.4
24	3.4–2.7	3.4–2.7	3.4–3.0	3.0–2.3
48	3.0–2.0	3.0–1.1	3.4–2.5	3.0–1.9
168	3.0–1.9	3.0–1.1	3.0–1.4	3.0–1.9
336	2.3–1.4	2.5–1.4	2.4–1.0	2.2–1.3

Table 3—Survival rates of Salmonella in individual freezing-storage experiments.

Time, hr	Freezing method, °F			
	Plate	Chest	0	−30
	Log no. bacteria/unit time			
0	3.4	3.4	3.4	3.4
24	2.2–1.5	2.2–1.6	2.2–1.7	2.2–1.3
48	1.4–1.0	1.5–1.1	1.6–1.2	1.6–1.3
168	0.8–0.3	1.0–0.7	1.2–1.0	1.1–0.8

results represent the average of 8 replicate runs at each temperature. There appeared to be an initial rapid die off, regardless of freezing method employed, with a 3–4% survival of *S. derby* after 24 hr. Between 24 and 48 hr there was a continued, but more gradual decline, with 1% survival at 48 hr and less than 1% at 168 hr and none detectable after 336 hr by the assay method used.

To investigate the possibility of a species difference in resistance, a series of identical experiments was conducted using *S. typhimurium*. The results of these studies are summarized in Figure 2, which presents the average of 8 replicate runs at each temperature.

The results were similar to those obtained with *S. derby*. There was an initial rapid decline, with a 4% survival at 24 hr, and 1% or less at 168 hr. Statistical analysis of data indicated no significant difference between the responses of the two species (r = wt, etc.).

Data from *E. coli* experiments are summarized in Figure 3. They represent an average of 6 replicate runs at each temperature. There was approximately 5% survival, in all cases, at the end of 336 hr. Average values for all experiments show a gradual decline in numbers during storage, with about a 40% survival of *E. coli* in the first 48 hr. However, the actual values within experiments and between experiments varied widely at each test point. This contrasts with the very close accord in *salmonellae* counts. This can be seen very clearly from Table 2, which gives the results of individual *E. coli* experiments, and Table 3, which presents the results of individual *salmonellae* experiments.

From these experiments it would appear that freezing has a more profound and more consistent effect on *Salmonella* than on *E. coli* I in the naturally contaminated oyster.

Homogenate studies

The results of these studies are summarized in Figure 4 which represents the average of 6 replicate experiments.

Samples stored at both temperatures displayed a consistent two-phase decline in the number of surviving test bacteria. However, the decline in numbers of fecal *E. coli* held at −30°F was noticeably less than in samples held at the higher temperature. At the end of 48 hr, survival of *Salmonella* was greatest in samples stored at −30°F. Overall survival of the test bacteria at the end of 168 hr was 4% or better for samples held at −30°F and 1% or less in samples maintained at 32°F.

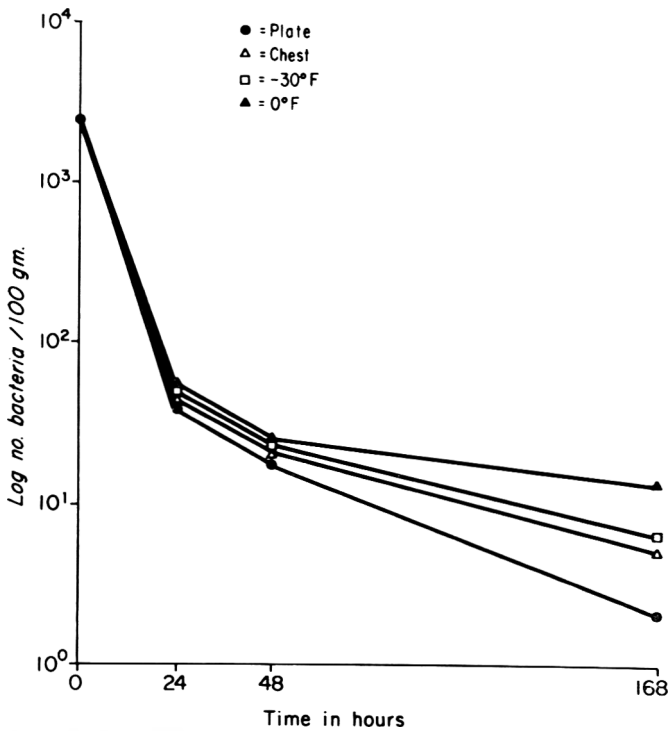

Fig. 1—Survival of Salmonella derby *in Pacific oysters (C. gigas) frozen by four methods and stored at 0°F.*

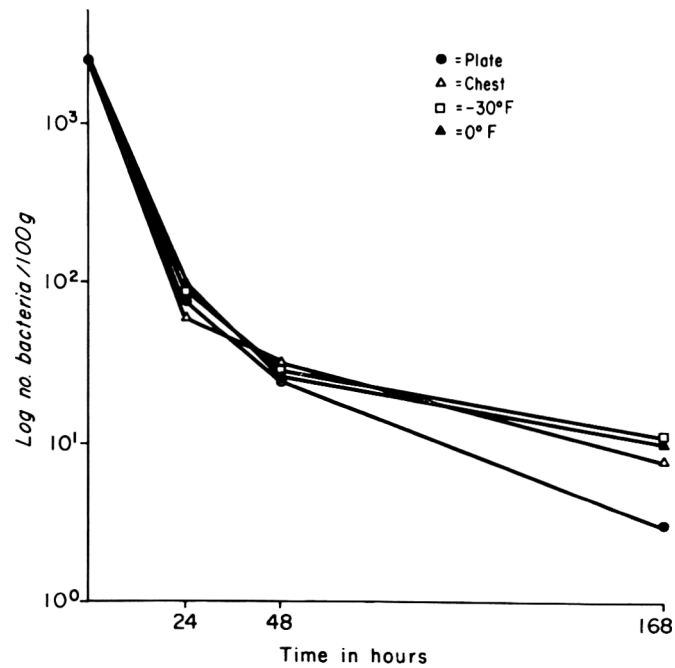

Fig. 2—Survival of Salmonella typhimurium *in Pacific oysters frozen by four methods and stored at 0°F.*

Table 4—Average pH readings of homogenates held at 32° and −30°F.

Time, hr	pH	
	+32°F	−30°F
24	6.75	7.1
48	6.30	7.0
168	5.85	6.95

Average pH readings of homogenates held at both temperatures are given in Table 4.

DISCUSSION

Freezing storage study

Previous reports in the literature have indicated that *salmonellae,* though highly sensitive to freezing, can survive in various foods in reduced number and can survive subsequent storage in the frozen state for considerable lengths of time. Thus, Liston et al. (1962) noted that *salmonellae,* although more sensitive to freezing than *E. coli,* were able to survive for two months or more in frozen fish fillets held at 0°F. Georgala et al. (1963) observed that *salmonellae* were less resistant to freezing than *Staphylococcus aureus,* but would survive storage in the frozen state for a considerable length of time, depending upon the nature of the food in which it was held.

Results of the present study, however, indicate that *salmonellae* in oysters are highly sensitive to freezing and subsequent storage in the frozen state. Freezing the oysters at extremely low temperatures and storing the frozen product at 0°F for 24 hr causes a 95% or greater reduction in the number of *salmonellae.* At the end of one week's storage, the number of *salmonellae* in the oysters may be reduced as much as 99% from the original level present in the unfrozen oysters.

This finding can have public health significance, since it is clearly impossible to determine from the level of *salmonellae* in the frozen oyster the original level of contamination in the unfrozen oyster, nor can this low level of *salmonellae* be taken to indicate the absence of other enteric pathogens. Therefore, as a precautionary measure, the presence of even extremely low numbers of *salmonellae* in frozen oysters should be assumed to indicate the possible presence of a high level of contamination by *salmonellae* in the unfrozen product.

There was no significant change in the pH of samples stored at −30°F. However, there was a steady decline in pH in the 32°F samples. Concomitant with this pH change was an increase in total bacterial counts, as shown by Table 5. Quite possibly, interpopulation competition may have accounted for some of the die off of test bacteria at 32°F.

Escherichia coli seem to be somewhat less sensitive to freezing in oysters than salmonellae. However, their resistance is highly variable. Variability of *E. coli* to the effects of freezing has been noted by other workers. Thus, Weiser et al. (1945)

Table 5—Total plate count and pH change in oyster homogenates stored at 32°F.

Time, hr	Total count	pH
0	175,000	6.9
24	280,000	6.75
48	480,000	6.3
168	530,000	5.85

noted the accelerated death of *E. coli* when grown in peptone blanks and subjected to freeze-thawing. Panes et al. (1959), working with milk samples inoculated with the coli-aerogene group and held at 37.4°–41°F, reported a 100-fold increase in the number of coli-aerogenes present in 35.2% of all samples at the end of 72 hr, but few, if any, *E. coli* survived. Raj et al. (1961b) noted that the test for *E. coli* was by no means specific when applied to frozen seafood. They reported that to obtain satisfactory results, a second passage of *E. coli* in E.C. media was required.

Since the sensitivity of *E. coli* to freezing is highly variable, the number remaining in the oyster during the first week or so of frozen storage is largely unpredictable. This raises a problem of bacteriological control, since it is clearly impossible to use the coli count in the frozen oyster as an index of the original coli count in the unfrozen oyster. As a safety measure, it might be possible to apply a 10× factor to the frozen counts, but this would result in a large overestimation of counts

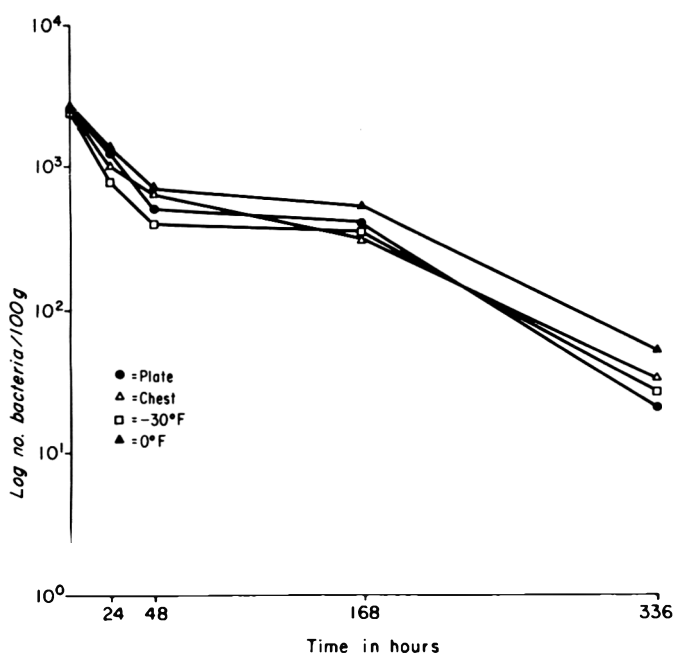

Fig. 3—Average survival rate of E. coli I in Pacific oysters frozen by four methods.

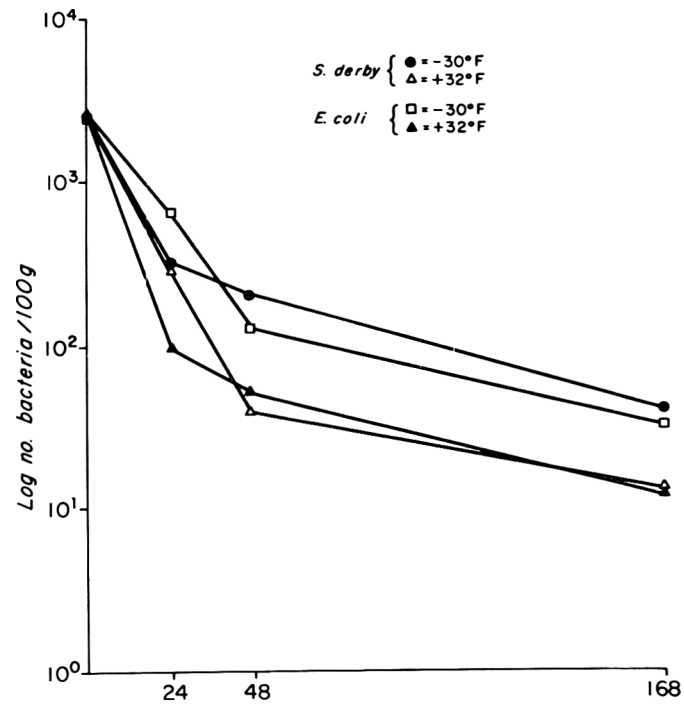

Fig. 4—Survival of S. derby and E. coli I in oyster homogenates held at 32°F and −30°F.

in many cases.

It may be possible to use the coli count of the frozen oyster as an index of the bacterial quality in the frozen state, since *salmonellae* appear to be more sensitive to freezing in oysters than *E. coli*. However, it would be unwise to apply these counts as an index of the presence of other enteropathogenic organisms, particularly the viruses which readily survive the frozen state for prolonged lengths of time (Andrewes et al., 1967). Metcalf et al. (1965), in fact, found that viruses in contaminated shellfish stored at 41°F retained their viability for at least 28 days.

Since the coli counts of frozen oysters are unpredictable, it would seem advisable to find a more reliable indicator organism. Larken et al. (1956); Raj et al. (1961); and Liston et al. (1962) have all demonstrated that enterococci do survive freezing and storage, particularly of seafoods, better than does *E. coli*. Perhaps the enterococci would make suitable indicator organisms for the sanitary quality of frozen shellfish.

Results of blending experiments indicated that the loss of viability by *salmonellae* and *E. coli* was slower in those homogenates held at 32°F than in samples held at −30°F. However, the general trend in these experiments was towards a loss of viability and a reduction in the number of the test bacteria during the entire period of storage, regardless of temperature. Since bacterial counts were the only index employed, it is not possible to say with certainty if the slower loss of viability by the test bacteria in samples held at 32°F truly represents a protective effect by the homogenate.

In these studies the *E. coli* counts displayed a distinct regularity in decline and tended to closely parallel the drop in *Salmonella counts*. Shiflett et al. (1967) found that the loss of viability of *salmonellae* in blended oysters held at 44.6°F was in three stages: A rapid initial stage, a gradual reduction and a final rapid reduction. In the present study using lower temperatures, the loss of viability by *E. coli* and *salmonellae* was in two stages, a rapid initial stage, followed by a continued gradual decline.

Presnell et al. (1961) observed that with a decrease in pH from 6.6 to 5.5 in whole oysters held at 35.6°–47.6°F for 11 days there was a decrease in fecal *E. coli* and an increase in total plate counts. Similar results were noted in the present study with oyster homogenates stored at 32°F. However, the decrease in *E. coli* was more rapid, occurring over a 7-day (168 hr) period, but the total decrease in number (about 90°) was comparable to the decrease observed by Presnell et al. (1961).

The "homogenate" method has been widely used to determine the survival of enteric bacteria in oysters and other shellfish. It has been thought that results obtained with this method are a valid representation of what ensues in the whole oyster. However, a comparison of data from whole oyster and homogenate samples frozen at the same temperature (−30°F) reveals a marked difference in results. *E. coli* in frozen homogenates show a predictable loss of viability which tends to parallel the loss of viability by *salmonellae*. In frozen whole oysters the loss of *E. coli* viability is capricious and unpredictable. The loss of viability by *salmonellae* in frozen homogenates tends to be slower than their loss of viability in frozen whole oysters.

Thus, it is inadvisable, and perhaps even dangerous, to generalize from homogenate studies to the whole oyster. Since the results of homogenate and whole oyster studies can and do differ, some doubt must be cast on the reliability of the homogenate method.

CONCLUSIONS

SALMONELLAE in oysters are highly sensitive to freezing. Fecal coliforms, on the average, are less so. However, the resistance to freezing of *E. coli* I is highly variable. Due to this variability, fecal coliforms are not satisfactory indicators of sanitary quality in frozen shellfish. Therefore, it would seem advisable to use as an indicator another fecal bacteria which possesses greater resistance to freezing.

Raj et al. (1961a) found the enterococci to be more reliable indicators of fecal contamination in frozen seafoods than *E. coli*. Liston et al. (1962) noted that, as compared to *E. coli*, the enterococci showed little or no decrease in numbers in frozen processed foods. Therefore, since the enterococci have been shown to be consistently more resistant to freezing than *E. coli*, they may serve as better indicator organisms of contamination in frozen shellfish.

The use of an oyster homogenate may give *salmonellae* and *E. coli* some protection against the adverse effects of freezing. Nevertheless, both types of bacteria die out in frozen homogenates and in homogenates held unfrozen at 32°F. Significantly, the results of frozen homogenate studies did not agree with results of frozen whole oyster studies, thus suggesting the inadvisability of generalizing from homogenate studies to the whole oyster.

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Free Amino Acids and Other Nitrogenous Fractions in Wine Grapes

SUMMARY—The concentration of eight free amino acids, total nitrogen, amino-acid fraction nitrogen, and nonamino-acid-fraction nitrogen in the juices of 26 red- and 23 white-wine varieties of grapes was determined at early and late stages of fruit maturity. Proline was the most prominent amino acid in 31 of the varieties at early harvest and in 45 of the varieties at late harvest while arginine was the main amino acid in 16 and 3 varieties at early and late harvest, respectively. "Salvador" and "Scarlet" were the only varieties in which α-alanine was the predominant amino acid. The concentration of total nitrogen in the juices of the various varieties ranged from 44 to 256 mg/100 ml and the amino-acid fraction nitrogen ranged from 26 to 171 mg/100 ml juice. The amino acid fraction and nonamino acid fraction nitrogen in the juices ranged from 53 to 76% and 23 to 56% of total Kjeldahl nitrogen respectively. Alanine, γ-aminobutyric acid, arginine, aspartic acid, glutamic acid, proline, serine and theronine accounted for 29 to 72% of the total nitrogen and 47 to 96% of the amino acid fraction nitrogen. Arginine contributed the most nitrogen of the amino acids, accounting for 6 to 44% of the total nitrogen in the juices of the various fruits.

INTRODUCTION

PREVIOUS investigations (Nassar et al., 1966; Kliewer et al., 1966; and Kliewer, 1967b) have dealt with identifying and quantitatively determining the concentration of free amino acids in various parts of grapevines at different stages of development. Kliewer (1969) very recently determined the concentration of eight amino acids, total free amino acids, total nitrogen and free-amino-acid fraction nitrogen in the juices of 28 table varieties of grapes at early and late stages of fruit maturity. He found that 60–90% of the total nitrogen was accounted for by amino-acid-fraction nitrogen, and that 59–96% of this nitrogen was attributable to eight amino acids.

Arginine contributed the most nitrogen, accounting for 15–50% of the total. Depending on the maturity of the fruits, the predominant amino acid in the table varieties was usually arginine or proline. The concentration of the latter increased very rapidly during the later stages of fruit maturation. For reviews of the literature pertaining to amino acids in grapes and grape products, the reader is referred to Kliewer (1969) and Amerine et al. (1967).

The present report concerns the concentrations of eight of the predominant amino acids, total nitrogen and amino-acid-fraction nitrogen in fruits from 26 red and 23 white-wine varieties free from all known viruses.

EXPERIMENTAL

THE FRUITS of 26 red-wine varieties and 23 white-wine varieties of grapes (*Vitis vinifera* L.), all grown under the same climatic, cultural and soil conditions, were sampled in duplicate twice—once when the fruits were slightly to moderately ripe, and again when they were ripe to overripe—by the procedure described previously (Kliewer, 1967a). (The varieties "Salvador" and "Scarlet" have some American species in parentage.) The samples are referred to as early- and late-harvested fruits (Tables 1–5). The

Table 1—Total soluble solids, total nitrogen and amino-acid and non-amino-acid fraction nitrogen of red-wine varieties of grapes harvested at early or late stage of fruit maturity.

Variety	Date harvested, 1966		Total soluble solids, °Brix		Total nitrogen, mg/100 ml juice		Amino acid fraction nitrogen, mg/100 ml juice		Nonamino-acid-fraction nitrogen, mg/100 ml juice	
	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Aleatico	9-1	10-12	20.2	22.4	134.3	165.4	83.5	96.6	50.8	68.8
Alicante Bouschet	8-30	11-3	17.0	24.6	74.5	80.0	54.0	61.3	20.5	18.8
Cabernet-Sauvignon	8-30	9-30	21.0	25.5	62.5	86.6	49.0	65.6	13.5	11.0
Calzin	8-30	9-30	21.0	24.6	55.0	69.1	39.4	47.5	15.6	21.6
Carignane	9-1	11-3	18.7	27.3	50.0	116.1	33.5	85.4	16.5	30.7
Early Burgundy	8-30	9-20	24.0	26.1	80.0	87.2	57.0	64.0	23.0	23.2
Gamay	8-30	9-30	21.0	24.4	79.0	88.6	50.5	51.8	28.5	36.8
Gamay Beaujolais	8-19	9-20	18.4	25.2	114.5	161.4	73.0	93.1	41.5	68.3
Grenache	9-1	10-12	19.7	24.1	46.5	94.2	26.0	61.6	22.5	32.6
Grignolino	8-30	9-30	21.9	24.2	108.0	137.8	80.8	83.6	27.2	54.2
Malbec	8-30	10-12	20.2	24.6	109.0	155.6	72.5	96.8	26.5	58.8
Mataro	9-8	10-12	19.6	26.2	61.0	94.7	41.0	60.8	20.0	33.9
Merlot	8-30	9-30	22.3	24.2	75.6	97.0	50.3	64.0	15.3	33.0
Mission	9-1	10-12	19.5	23.5	69.0	123.0	40.0	78.6	29.0	44.4
Petit Bouschet	8-30	10-12	16.8	20.1	89.0	127.0	52.5	79.2	36.5	47.8
Petit Sirah	8-30	10-12	19.2	27.0	71.5	129.2	41.5	88.7	30.0	40.5
Pinot noir	8-19	9-20	20.0	27.1	126.0	213.6	77.0	143.1	49.0	70.5
Pinot Saint George	8-30	10-12	17.4	22.3	91.5	194.5	54.5	122.9	37.0	71.6
Royalty	9-1	10-12	21.0	23.8	62.5	137.1	44.5	80.1	18.0	57.0
Rubired	9-1	11-3	20.4	25.1	56.0	90.4	35.4	61.6	20.6	28.8
Salvador	9-1	9-30	21.6	25.4	110.0	149.1	70.1	108.0	39.9	41.1
Scarlet	9-1	10-12	16.9	22.3	69.5	108.6	44.1	72.0	25.4	36.6
Tinto Cão	9-1	11-3	19.6	24.3	119.0	256.2	81.2	163.4	37.8	92.8
Trousseau	8-19	9-20	17.9	26.5	111.1	144.8	71.3	104.0	39.8	40.8
Valdepeñas	8-19	9-30	19.5	23.8	123.2	189.9	81.2	105.9	42.0	84.0
Zinfandel	8-30	10-12	19.3	23.1	81.5	96.2	52.0	72.5	29.5	23.7

Table 2—Total soluble solids, total nitrogen and amino-acid and non-amino-acid fraction nitrogen of white-wine varieties of grapes harvested at early or late stage of fruit maturity.

Variety	Date harvested, 1966		Total soluble solids, °Brix		Total nitrogen, mg/100 ml juice		Amino-acid fraction nitrogen, mg/100 ml juice		Nonamino-acid fraction nitrogen, mg/100 ml juice	
	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Burger	8-30	11-3	16.6	22.6	90.0	109.0	65.0	75.6	25.0	33.4
Chardonnay	8-19	9-20	18.6	25.2	88.6	133.6	58.0	95.0	30.6	38.6
Chasselas doré	8-30	10-12	20.3	24.8	92.7	124.4	55.0	86.8	37.7	37.6
Chenin blanc	9-1	10-12	19.5	24.0	101.7	154.8	65.0	107.8	36.7	47.0
Emerald Riesling	9-1	9-30	21.5	25.9	57.5	74.6	36.0	50.0	21.5	24.6
Feher Szagos	9-8	10-12	16.6	20.2	99.0	127.7	69.5	91.7	29.5	36.0
Flora	8-30	9-20	23.9	27.1	171.0	189.0	110.0	131.0	61.0	58.0
Grey Riesling	8-30	9-20	23.5	24.1	149.5	183.1	112.0	129.5	37.5	53.6
Gewurztraminer	8-30	9-20	21.8	23.4	236.5	238.7	167.0	170.8	69.5	69.9
Green Hungarian	8-30	11-3	17.8	22.0	58.0	92.7	36.0	70.0	22.0	22.7
La Rienha	9-1	11-3	16.4	23.9	72.0	103.6	47.5	70.3	24.5	33.3
Malvasia bianca	8-30	9-30	21.8	27.4	85.5	147.5	60.0	98.0	25.5	49.5
Melon	8-30	9-20	24.0	24.6	104.4	115.5	72.2	81.0	32.2	34.5
Orange Muscat	8-19	9-20	19.5	25.6	72.1	94.0	49.5	69.2	22.6	24.8
Palomino	8-30	9-30	20.8	23.9	44.0	106.4	31.7	68.6	12.3	37.8
Peverella	9-1	10-12	19.5	25.2	108.0	123.2	62.0	83.5	46.0	39.7
Pinot blanc	8-30	9-20	18.3	23.8	92.5	120.1	55.3	82.6	37.2	37.5
Pinot gris	8-30	9-30	20.6	24.3	79.0	89.0	51.0	57.0	28.0	32.0
Red Veltliner	8-30	9-30	21.4	25.3	94.5	116.2	60.1	73.5	34.4	42.7
Sauvignon vert	9-1	10-12	19.6	24.7	77.5	135.8	52.5	86.4	25.0	49.4
Sauvignon blanc	9-1	9-20	22.7	24.9	86.6	98.9	54.5	71.0	32.1	27.9
Semillon	8-30	9-20	22.4	24.1	128.0	152.5	79.1	91.0	48.9	61.5
White Riesling	8-30	11-3	21.2	24.9	74.5	138.9	56.8	88.2	17.7	50.7

location and juice extraction procedure has been previously described (Kliewer, 1967a). Total dissolved solids (degree Brix, °B) in the juice were measured with an Abbé refractometer at 20°C.

A 10-ml portion of juice from each sample was applied to a column of Dowex 50W-X8 (H^+ form), and the amino acids and other positively charged substances were eluted from the column with NH_4OH and concentrated as described by Kliewer (1969). This fraction was used for further analysis of individual amino acids and is hereafter referred to as the amino acid fraction.

Arginine was determined by the Sakaguchi reaction as modified by Gilboe et al. (1956). Proline was determined by the acidic ninhydrin photometric method of Chinard (1952), which was later modified by Ough (1969). Alanine, γ-aminobutyric acid, aspartic acid, glutamic acid, serine and threonine were analyzed by paper chromatography (Nassar et al., 1966). Total nitrogen and amino-acid-fraction nitrogen were determined by the Kjeldahl method on 5-ml portions of the original juice sample and of the amino-acid-fraction extract, respectively. The nonamino-acid-fraction nitrogen is the difference between total nitrogen and amino-acid-fraction nitrogen.

RESULTS

BOTH TOTAL nitrogen and amino-acid-fraction nitrogen increased in the fruits

Table 3—Concentration of several free amino acids (μ moles per 100 ml juice) of red-wine varieties of grapes harvested at early or late stage of fruit maturity.

Variety	α-alanine		γ-amino-butyric acid		Arginine		Aspartic acid		Glutamic acid		Proline		Serine		Threonine		Sum of amino acids	
	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Alcatico	460	474	189	222	649	584	180	234	226	660	535	917	115	248	198	348	2552	3687
Alicante Bouschet	245	257	294	288	382	532	151	142	398	360	571	667	123	128	146	140	2410	2514
Cabernet-Sauvignon	335	244	130	176	256	190	89	112	92	95	1024	2650	128	90	120	112	2174	3669
Calzin	192	172	143	220	275	156	55	61	82	244	272	518	103	116	177	255	1299	1742
Carignane	355	412	184	410	188	227	88	92	190	287	363	2000	106	170	184	230	1658	3828
Early Burgundy	423	262	147	200	368	478	110	146	126	174	736	940	175	133	201	200	2286	2533
Gamay	187	560	61	200	349	413	38	26	121	241	339	583	58	144	88	98	1241	2265
Gamay Beaujolais	242	210	205	190	484	382	47	52	160	212	642	1086	120	110	125	140	2025	2382
Grenache	135	280	78	125	186	363	41	45	180	298	131	595	90	104	107	116	948	1926
Grignolino	280	320	234	220	389	408	58	72	166	245	1027	1667	108	142	152	190	2414	3264
Malbec	478	352	170	163	386	454	153	108	204	200	672	815	151	120	255	139	2469	2351
Mataro	240	290	120	216	228	310	71	87	160	342	278	1071	98	136	122	175	1317	2629
Merlot	306	271	138	228	198	105	65	82	125	134	1057	2506	130	122	85	112	2104	3560
Mission	211	265	115	143	136	351	70	76	147	298	265	1336	64	82	107	115	1115	2666
Petit Bouschet	255	287	148	230	228	483	60	72	174	267	276	952	127	135	103	111	1371	2537
Petit Sirah	205	298	136	205	195	374	72	69	179	293	282	1793	176	220	128	143	1373	3395
Pinot noir	285	385	150	280	450	1062	84	100	217	410	315	1076	202	285	173	250	1876	3848
Pinot Saint George	233	298	139	165	338	867	75	83	208	336	200	1016	146	188	117	129	1456	3081
Royalty	209	265	125	171	238	61	79	82	115	147	495	2017	118	109	110	102	1489	2954
Rubired	288	301	161	185	304	499	63	70	122	156	292	631	105	122	92	113	1427	3077
Salvador	739	1130	265	278	289	490	70	62	268	349	729	1589	146	125	134	150	2638	4173
Scarlet	520	980	205	256	243	250	59	73	244	205	89	262	98	117	105	92	1563	2235
Tinto Cão	359	460	167	295	499	827	70	92	196	370	351	1255	132	150	134	166	1908	3615
Trousseau	330	410	122	172	475	583	49	52	175	230	538	1168	112	115	92	98	1893	2828
Valdepeñas	305	440	148	237	385	677	57	70	143	295	402	1118	119	132	128	120	1686	3089
Zinfandel	147	195	187	233	261	314	88	72	105	102	818	1324	148	116	97	107	1851	2463

of every variety between early and late harvest (Tables 1 and 2). The nonamino-acid-fraction nitrogen also increased in most varieties with increase in fruit maturity. The concentration of total nitrogen in the juices ranged from 44 to 236 mg/100 ml in the lightly to moderately ripe fruits (early harvest), and from 69 to 256 mg/100 ml in the ripe to overripe fruits (late harvest, Tables 1 and 2). "Aleatico," "Flora," "Gamay Beaujolais," "Grey Riesling," "Gewurztraminer," "Pinot noir," "Tinto cão" and "Valdepeñas" were relatively high in nitrogen (greater than 160 mg *N*/100 ml) at the time of late harvest, while "Alicante Bouschet," "Calzin" and "Emerald Riesling" were relatively low in nitrogen (less than 80 mg *N*/100 ml.

The amino-acid-fraction nitrogen ranged from 26 to 167 mg *N*/100 ml juice in the early harvest to 48–171 mg/100 ml juice in the late harvest. It accounted for 53–76% of the total Kjeldahl nitrogen at both harvests with a mean of about 66% for all varieties (Tables 1 and 2). Total nitrogen and amino-acid-fraction nitrogen were usually greater in the white-wine varieties than in the red-wine varieties. The nonamino-acid-fraction nitrogen, which includes ammonia nitrogen, constituted 23–56% of the total nitrogen in the juices of the red- and white-wine varieties, with average concentrations of 31 and 43 mg *N*/100 ml juice for the early and late harvests, respectively.

The concentrations of the eight determined free amino acids varied greatly from one variety to another (Tables 3 and 4). Generally there was an increase in the levels of the acids with increasing ripeness, although there were some notable exceptions, especially for arginine. The change in the concentrations of proline and glutamic acid was especially pronounced in some varieties, with increases as great as two- to sixfold between the early and late harvests (Tables 3 and 4).

On the basis of the predominant amino acid present, the varieties were divided into three groups (Table 5). Proline was the main amino acid in 31 of the varieties at early harvest and in 45 varieties at late harvest. Arginine was the principal amino acid in 16 varieties at early harvest and in 3 varieties at late harvest. α-alanine was predominant in the variety Scarlet at both harvests, and also in the variety Salvador in the early harvest.

The percent of the total nitrogen accounted for by the eight amino acids (Tables 3 and 4) ranged from 29 to 66% and from 31 to 72% at the early and late harvests, respectively (Table 6). These amino acids also accounted for 47–93% and 52–96% of the amino-acid-fraction nitrogen at the early and late harvests, respectively. Arginine contributed by far the most nitrogen of the amino acids, accounting for 11–33% and 6–44%, respectively, of the total nitrogen in the juices of early- and late-harvested fruits, with a mean of about 22% for both harvests.

The data in Tables 3 and 4 indicate that the variety and degree of maturity of the fruits strikingly affect the concentration of free amino acids in grape berries. This is especially true for arginine and proline, in which the concentrations differed by as much as ten- to twelvefold among varieties and from two- to sixfold between the early- and late-harvested fruits of the same variety.

Proline and arginine were the predominant amino acids in all varieties at early and late harvests except for Salvador and Scarlet, in which α-alanine was present in largest amounts at the early harvest. Both of these varieties have some *V. labrusca* in their parentage, and this species may carry the gene responsible for the large amount of α-alanine. This postulation is supported by the finding that two table varieties, "Isabella" and "Niagara," both of which have *V. labrusca* in their parentage, also have a preponderance of α-alanine in their mature fruits (Kliewer, 1969).

Generally, α-alanine, glutamic acid, and α-aminobutyric acid were the next three most quantitatively important amino acids in the various varieties. Kliewer (1969) recently reported that arginine, proline, glutamic acid and α-alanine were the most prominent amino acids in 28 table varieties. Castor (1953) and Castor et al. (1956) measured the free amino acids in seven varieties grown in California and found proline, glutamic acid and arginine present in largest amounts. Lafon-Lafourcade et al. (1959) reported arginine, glutamic acid, proline, serine and threonine to be the major amino acids in the musts of France. Tercelj (1965) from Yugoslavia found a preponderance of arginine plus histidine in grape must, followed, in turn, by proline and glutamic acid. In five grape varieties investigated

Table 4—Concentration of several free amino acids (μ moles per 100 ml juice) of white-wine varieties of grapes harvested at early or late stage of fruit maturity.

Variety	α-alanine		γ-amino-butyric acid		Arginine		Aspartic acid		Glutamic acid		Proline		Serine		Threonine		Sum of amino acids determined	
	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Burger	460	492	190	220	534	418	120	70	258	215	738	1580	143	153	230	245	2673	3393
Chardonnay	600	660	166	295	257	536	88	120	208	438	993	2405	210	264	262	234	2784	4952
Chasselas doré	295	240	106	248	528	556	91	80	245	180	407	916	143	147	256	184	2071	2551
Chenin blanc	520	650	162	180	521	681	128	180	192	208	449	1071	214	262	265	312	2481	3544
Emerald Riesling	289	315	161	175	294	368	28	34	138	235	283	880	101	144	128	170	1422	2321
Feher Szagos	320	340	172	190	463	405	32	35	255	310	862	1167	243	252	150	180	2497	2879
Flora	182	350	121	420	670	1025	58	66	135	295	286	910	130	362	117	152	1699	3580
Grey Riesling	328	345	53	140	625	1038	105	114	182	197	618	761	192	160	210	195	2313	2950
Gewürztraminer	282	360	190	257	1211	1355	115	108	260	332	580	738	210	250	300	280	3148	3680
Green Hungarian	152	225	105	195	250	242	61	88	212	266	264	960	104	115	84	80	1232	2171
La Rienda	143	255	102	140	258	282	74	70	160	310	1212	1875	65	76	58	65	2072	3073
Malvasia bianca	305	350	148	192	360	574	35	42	171	218	618	1615	88	102	78	112	1803	3205
Melon	246	308	180	206	511	386	113	81	230	274	744	940	165	185	128	146	2317	2526
Orange Muscat	242	295	208	277	324	417	41	45	256	285	848	988	86	94	145	152	2150	2553
Palomino	205	290	180	272	132	557	65	70	285	330	203	750	180	258	156	210	1406	2737
Peverella	194	265	133	187	225	487	58	66	210	327	494	1310	108	136	102	153	1524	2931
Pinot blanc	350	368	202	244	321	718	32	51	250	372	350	964	194	212	155	190	1854	3119
Pinot gris	162	222	127	195	182	322	44	52	226	265	395	1354	128	162	135	118	1399	2690
Red Veltliner	130	166	175	189	483	631	53	44	221	247	354	668	68	77	104	115	1588	2137
Sauvignon vert	157	238	142	195	320	632	46	55	115	269	270	1414	74	91	97	108	1221	3002
Sauvignon blanc	179	285	120	177	413	657	55	67	137	162	594	839	103	120	95	113	1696	2420
Semillon	185	297	166	198	357	629	49	62	184	287	375	804	128	142	147	160	1591	2579
White Riesling	258	272	195	188	350	402	40	44	146	174	364	1547	155	170	108	130	1616	2927

in South Africa by Van Wyk et al. (1965), arginine, proline, alanine and valine were the predominant amino acids present.

Lafon-Lafourcade et al. (1962) and Kliewer (1968) investigated changes in the concentration of free amino acids in grapes during fruit ripening. They observed that the concentration of arginine usually increased rapidly during the early ripening period, and then often decreased as the fruits became ripe to overripe. During this latter period of fruit maturation, proline increased markedly, indicating that the synthesis of proline may occur at the expense of arginine during this phase of fruit development. Such changes in the concentrations of arginine and proline occurred in several varieties in the present study (Tables 3 and 4).

Table 5—Classification of red- and white-wine varieties of grapes according to the predominant free amino acid in the juice.

Proline		Arginine		α-Alanine	
Slightly to moderately ripe fruit	Very ripe to overripe fruit	Slightly to moderately ripe fruit	Very ripe to overripe fruit	Slightly to moderately ripe fruit	Very ripe to overripe fruit
Red varieties	Red varieties	Red varieties	White varieties	Red varieties	Red varieties
Alicante Bouschet	Aleatico	Aleatico	Flora	Salvador	Scarlet
Cabernet-Sauvignon	Alicante Bouschet	Calzin	Grey Riesling	Scarlet	
Carignane	Cabernet-Sauvignon	Gamay	Gewürztraminer		
Early Burgundy	Calzin	Grenache			
Gamay Beaujolais	Carignane	Pinot noir			
Grignolino	Early Burgundy	Pinot St. George			
Malbec	Gamay	Rubired			
Mataro	Gamay Beaujolais	Tinto Cão			
Merlot	Grenache				
Mission	Grignolino	White varieties			
Petit Bouschet	Malbec	Chasselas doré			
Petit Sirah	Mataro	Chenin blanc			
Royalty	Merlot	Emerald Riesling			
Trousseau	Mission	Flora			
Valdepeñas	Petit Bouschet	Grey Riesling			
Zinfandel	Petit Sirah	Gewürztraminer			
	Pinot noir	Red Veltliner			
		Sauvignon vert			
White varieties	Pinot St. George				
Burger	Royalty				
Chardonnay	Rubired				
Feher Szagos	Salvador				
Green Hungarian	Tinto Cão				
La Rienha	Trousseau				
Malvasia bianca	Valdepeñas				
Melon	Zinfandel				
Orange Muscat	White varieties				
Palomino	Burger				
Peverella	Chardonnay				
Pinot blanc	Chasselas doré				
Pinot gris	Chenin blanc				
Sauvignon blanc	Emerald Riesling				
Semillon	Feher Szagos				
White Riesling	Green Hungarian				
	La Rienha				
	Malvasia bianca				
	Melon				
	Orange Muscat				
	Palomino				
	Peverella				
	Pinot blanc				
	Pinot gris				
	Red Veltliner				
	Sauvignon vert				
	Sauvignon blanc				
	Semillon				
	White Riesling				

Boulter et al. (1963) found that when C^{14}-arginine was fed to bean seedlings, label was first found in proline and subsequently in ornithine and citrulline, indicating that proline may be synthesized by reversal of the ornithine cycle. Hennig (1955) and Nassar et al. (1966) have found both citrulline and ornithine in the juices of grapes, indicating that the ornithine cycle may exist in these fruits.

In addition to the scion and degree of fruit maturity, other factors such as rootstock, climate, mineral nutrition, crop level, trellising system and disease undoubtedly influence the concentrations of amino acids in grapes. However, since all data in this communication were obtained from fruits harvested from virus-free vines grown under the same climatic, cultural and soil conditions, the reported values should indicate relative differences among varieties when compared at the same degree of fruit maturity. The significance of the above environmental factors on the level of free amino acids in grapes must await further studies made under closely controlled conditions.

The amino acid composition of grape must may be of considerable economic importance in regard to the rate of fermentation by yeasts and bacteria. Peynaud et al (1961) reported that proline and threonine are difficult for yeast to assimilate, while arginine, glutamic acid and α-alanine are readily utilized sources of nitrogen. Moat et al. (1965) indicated that glutamic acid and aspartic acid are equal to ammonia as a nitrogen source for yeast growth. Ough et al. (1966) found that multiple correlation and regression analysis of fermentation rates was considerably superior when total nitrogen was used in the equations rather than ammonia nitrogen, thus indicating the importance of the amino-nitrogen fraction for fermentation. Peynaud et al. (1961) found that as grapes ripen, there is an increase in forms of nitrogen that are less easily utilized by yeast. This may explain why must of overripe grapes sometimes ferments slowly.

Comparison of the concentrations of the different free amino acids in the various varieties indicates that a close genetic relationship may exist among some varietal groups. This can be seen by comparing the levels of amino acids in the "Cabernet"-type varieties ("Carbernet-Sauvignon" and "Merlot") with the levels in those varieties that contain some *V. labrusca in the parentage.* Varieties in both of these groups are qualitatively and quantitatively similar in amino acids.

Total nitrogen and the amino acid fraction were, on the average, higher in the white-wine varieties than in the red-wine varieties, especially at the early har-

vest (Tables 1 and 2). This may have been due to the fact that usually fruits from white varieties are earlier maturing than fruit from red varieties. Consequently, the white varieties generally reached véraison earlier, and had a longer ripening period to accumulate nitrogenous substances.

Comparison of the concentrations of total nitrogen, amino-acid-fraction nitrogen (Tables 1 and 2) and individual amino acids (Tables 3 and 4) with concentrations of titratable acidity, tartrate and malate, determined on the same varieties in an earlier investigation (Kliewer et al., 1967a), failed to show any relationships. These results are in contrast to those of Peynaud et al. (1953), who reported a direct relationship between titratable acidity and amino nitrogen content of musts.

The amino acid fraction and the nonamino-acid-fraction nitrogen accounted for 53–76% and 23–56% of the total Kjeldahl nitrogen, respectively, of the various grape varieties. All nitrogeneous substances retained by Dowex 50 cation exchange resin are included in the amino acid fractions. Preliminary investigations indicated that, in addition to free amino acids, low-molecular-weight peptides were completely retained by the cation resin; and consequently these substances would be included as part of the amino-acid-fraction nitrogen. The eight amino acids in Tables 3 and 4 accounted for 47–96% of the amino-acid-fraction nitrogen. The nonamino acid fraction includes proteins, high-molecular-weight peptides, ammonia and nitrate nitrogen. The range in concentration of these substances in grapes has been reviewed previously (Kliewer, 1969).

Table 6—Percentage of total nitrogen and amino-acid-fraction nitrogen accounted for by eight amino acids.[1]

Variety	Percent of total N accounted for by 8 determined amino acids		Percent of amino-acid fraction N accounted for by 8 determined amino acids	
	Early	Late	Early	Late
Red Wine				
Aleatico	46.9	46.0	75.4	78.8
Alicante Bouschet	64.9	71.8	89.6	93.8
Cabernet-Sauvignon	65.9	68.5	84.0	90.5
Calzin	54.1	44.8	75.5	65.1
Carignane	62.2	54.4	92.8	73.9
Early Burgundy	59.3	63.7	83.3	86.8
Gamay	40.5	55.4	63.4	94.7
Gamay Beaujolais	42.5	30.6	66.7	53.1
Grenache	45.4	44.8	81.2	68.5
Grignolino	46.4	45.6	62.0	75.2
Malbec	46.6	33.4	70.0	53.7
Mataro	45.9	52.6	68.3	81.9
Merlot	50.0	53.0	75.1	80.4
Mission	30.9	33.3	53.3	52.2
Petit Bouschet	40.9	43.9	69.3	70.5
Petit Sirah	38.3	48.9	66.0	71.3
Pinot noir	35.9	46.1	58.7	68.8
Pinot St. George	37.8	40.9	63.4	64.7
Royalty	49.3	32.0	69.3	54.8
Rubired	58.5	55.3	92.5	81.2
Salvador	44.5	53.0	70.0	73.1
Scarlet	46.2	38.5	72.7	58.0
Tinto Cão	40.1	33.3	58.7	52.2
Trousseau	41.3	44.2	65.1	61.6
Valdepeñas	32.3	37.8	49.0	67.7
Zinfandel	45.2	49.6	70.9	65.8
White Wine				
Burger	66.5	59.7	92.1	86.1
Chardonnay	56.2	68.7	85.8	96.7
Chasselas doré	55.2	47.5	93.0	68.0
Chenin blanc	55.7	50.5	87.1	72.6
Emerald Riesling	56.1	64.3	89.6	95.9
Feher Szagos	54.9	44.9	78.3	62.5
Flora	30.4	49.3	47.2	71.1
Grey Riesling	39.2	46.4	52.4	65.6
Gewürztraminer	40.1	45.4	56.8	63.8
Green Hungarian	47.3	43.8	77.1	57.9
La Rienha	55.3	53.0	83.9	78.4
Malvasia bianca	47.2	46.8	67.3	70.4
Melon	51.5	44.7	74.6	63.7
Orange Muscat	60.5	56.6	88.3	76.9
Palomino	57.3	58.0	79.6	89.9
Peverella	28.5	49.9	49.7	73.6
Pinot blanc	42.5	61.5	71.3	89.4
Pinot gris	34.5	57.5	53.4	89.8
Red Veltliner	45.3	48.5	70.7	76.7
Sauvignon vert	39.4	50.5	58.2	79.4
Sauvignon blanc	47.4	62.2	79.4	86.6
Semillon	29.1	41.0	47.1	68.7
White Riesling	50.1	41.7	65.7	65.6

[1] Amino acids were those given in Tables 3 and 4.

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Pasteurization of Pacific Oysters by Radiation: Post-Mortem Changes in Nucleotides During Storage at 0–2°C

SUMMARY—*The concentration of nucleotides was lower in the adductor muscle of the oyster (1.64 μmoles/g) than in the remaining dark tissues of the oyster (2.75 μmoles/g). The concentration was less in the whole oyster meats (2.87 μmoles/g) than is usually found in fish muscle, or other marine invertebrates.*

In addition to the adenine nucleotides and inosine monophosphate, uridine triphosphate, guanosine triphosphate, guanosine diphosphate, guanosine monophosphate and guanosine diphosphate-mannose were found in the fresh oysters. Samples collected in summer had greater concentrations of nucleotides than similar winter samples. Inosine monophosphate formed rapidly from adenosine triphosphate during storage at 0°–2°C, while the turnover rate of inosine monophosphate was slow and reflected low 5'-nucleotidase activity.

Hypoxanthine, inosine, guanosine, guanine and uracil were formed during ice storage. The nucleotide breakdown in oysters was not changed by 2 mrads of radiation dose. Total nucleosides and free bases increased during storage of both unirradiated and irradiated samples. During the latter part of the storage period the concentrations of nucleosides and free bases were considerably greater in the irradiated samples. This difference probably is due to the utilization of these compounds by bacteria in the unirradiated samples. After 15 days of storage bacteria had increased to more than 10^6 organisms per g, while the counts for irradiated samples were very low (less than 10^3 per g).

INTRODUCTION

POST-MORTEM changes in muscle nucleotides are intimately associated with the events of rigor. Some products of their degradation are believed to contribute to typical flavors of flesh products (Jones, 1961; Wagner et al., 1963; Kuninaka et al., 1964; Shimazono, 1964).

The specific biochemical pathways of nucleotide breakdown and the accumulation of their products are of considerable interest in studies of post-mortem change. Some products of ATP catabolism have been proposed as indices of freshness for fish (Jones et al., 1964; Spinelli et al., 1964).

In recent years, a great deal of progress has been made toward explaining the specific reactions involved in the post-mortem nucleotide degradation in fish and other marine animals (Kassemsarn et al., 1963; Creelman et al., 1960; Tarr et al., 1962; Tarr et al., 1964; Tarr, 1955, 1958; Saito, 1961; Arai, 1966a, 1966b, 1966c). However, there is very little knowledge of the post-mortem nucleotide changes in oysters and a few other shellfish of commercial importance.

Oysters are rich in glycogen. This glycogen may serve as an energy source for regeneration of ATP during storage. In one study, Wylie et al. (1964) determined the composition of the nucleotide fraction of Pacific oysters (*C. gigas*) when they were frozen in liquid nitrogen immediately after shucking. These studies were limited in scope, as only a small proportion of the total nucleotides present was recovered. A more complete examination of oysters would provide valuable data on the changes during post-shucking storage, providing comparison with fish muscles, which have poorer glycogen stores.

The present study sought to provide data on the concentration of nucleotides in oysters. Other objectives were to determine effects of sexual development and post-mortem changes in the nucleotides of Pacific oysters (*C. gigas*) during ice storage and the effect of radiation pasteurization on these changes.

METHOD & MATERIALS

Oysters

All oysters used were obtained from a commercial culture in Burley Lagoon, near Purdy, Washington. Oysters from these waters are known to reach sexual maturity during the summer months but they seldom spawn. Oysters collected in May were used for the study of changes in nucleotide composition during ice storage. "Summer oysters" were collected in August and "winter oysters" in November. These were used to describe the effects of sexual maturity.

Sampling

The zero day samples were prepared by freezing the meats in liquid nitrogen immediately after they were shucked. These samples were transported to the laboratory in solid CO_2 and kept at −50°C until they were extracted for analysis. The adductor muscles from 10 oysters were separated from the rest of the tissue, and both portions were frozen in liquid nitrogen. These portions were subsequently treated as the previous samples.

For the storage experiment, whole shucked oysters were stored at 0–2°C. Samples were withdrawn periodically, frozen in liquid nitrogen, and kept at −50°C until ready for analysis.

Extraction of nucleotides, nucleosides, purine, and pyrimidine bases

The frozen oyster samples were combined with two vol of chilled 0.7 *N* perchloric acid. The mixture was blended at high speed in a Waring blendor for 60 sec. It was then centrifuged for 20 min at 20,000 × G in a Servall refrigerated centrifuge. The supernatant was quantitatively decanted and neutralized with 20% KOH to pH 6.8. The neutralized extract was centrifuged after 30 min of storage at −15°C to allow the potassium perchlorate ($KClO_4$) to crystallize. The resulting solution was somewhat turbid compared to the clear extracts obtained from fish muscle.

Determination of nucleotides

The neutralized extract, corresponding to 10–20 g of oyster, was analyzed by column chromatography, using Bio-Rad analytical grade AG1-X8 200-400 mesh formate resin (Calbiochem, Los Angeles, California) by the method of Jones et al. (1960). The resin was used directly as delivered, after the removal of fine particles. The reference nucleotides were obtained from Sigma Chemical Company, St. Louis, Missouri. The eluent was monitored continuously at 260 mμ, using a Vanguard automatic μv analyzer, Model 1056, and collected into 10-ml fractions. The fractions corresponding to one peak were pooled, adjusted to pH 2.0 with HCl, and their concentrations determined by reading against appropriate formate blanks (corrected for HCl addition) in a Hitachi-Perkin-Elmer Model 139 spectrophotometer.

Determination of nucleosides and free bases

The effluent from the formate column, which absorbed at 260 mμ when eluted with distilled water, was frozen immediately and retained for later analysis of nucleosides, purines and pyrimidines. These samples

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were lyophilized to reduce their volume. Aliquots corresponding to 5–10 g of oyster were analyzed by the method of Jones (1960), using the chloride analytical grade AG1-X8 200-400 mesh (Calbiochem, Los Angeles. California).

The concentration of the individual compounds was determined by pooling the fractions corresponding to one peak and reading directly vs a proper effluent blank, using a Model 139 Hitachi-Perkin-Elmer or a Beckman DB spectrophotometer. Inosine and guanosine eluted superimposed. Their concentrations were determined by reading this peak at 251 and 275 mμ and using simultaneous equations. The molecular absorbancy indices used were those reported by Beaven et al., (1955).

Methods used in the confirmation of nucleotides and nucleosides

The samples were concentrated under vacuum in a rotary evaporator to remove formic acid and ammonium formate. Alternatively, they were concentrated by lyophilizing under an infrared source (Hurlbert et al., 1954) for removal of formic acid or ammonium formate. The samples containing excessive amounts of ammonium formate were treated with activated carbon (Baddiley et al., (1956).

The UV spectra were determined with a Beckman DK or DB spectrophotometer and compared with published spectra (Circular OR-10, Pabst Laboratories). The spectra of nucleosides and bases were read against a corresponding effluent blank from the chloride ion exchange column. All nucleotide peaks obtained from the ion exchange columns were confirmed by chromatography on paper and thin-layer plates. Whatman No. 1 paper was used in the solvent systems described in Circular OR-10, Pabst Laboratories, and thin-layer plates, using a tertiary amylalcohol-formic acid-water solvent system (Randerath, 1963).

Identification of adenosine, inosine, and guanosine

The fractions absorbing at 260 mμ which were not retained by the formate or chloride columns were concentrated. The samples were spotted on Whatman No. 1 filter paper and developed, using a normal butanol-glacial acetic acid-water (100:22:50) solvent system (Ammann et al., 1964).

Adenosine was detected by spraying with a solution of 0.25 g of mercuric acetate dissolved in 100 ml of 95% ethanol containing 0.6 ml of glacial acetic acid, and a solution of 0.05 g diphenyl carbazone dissolved in 95% ethanol. (Ammann et al., 1964). This method was sensitive and effective for the detection of 10 μg of adenosine. In addition, the fractions were analyzed for adenine compounds by the colorimetric method of Davis et al. (1963).

The combined inosine and guanosine peaks eluted from the chloride column were hydrolyzed by the method of Kalckar et al., (1947). HCl was substituted for H_2SO_4. The acid was removed from the sample by placing it in an evaporating dish over pellets of NaOH in a vacuum desiccator. The hydrolyzed sample was chromatographed on the nucleoside column to separate the guanine and hypoxanthine products.

Chromatography of sugars

Paper chromatography for the identification of sugars was essentially that of Jermyn et al. (1949). The samples were spotted on Whatman No. 1 paper and developed, using an ethyl acetate-water-pyridine (2/2/1) solvent system. The chromatograms were dried overnight. The sugars were detected by spraying with a reagent prepared by dissolving 0.5 g of benzidine in 10 ml of acetic acid and adding 10 ml of 40% (w/v) aqueous trichloroacetic acid and 80 ml of ethanol (Bacon et al., 1951), and then heating the chromatogram for 5 min in an oven at 100°C.

Studies of the structure of the compound absorbing at 320 mμ

Amino groups were estimated by the ninhydrin method (Block et al., 1956). Peptide groups were estimated by the biuret method (Hawk et al., 1949). Phosphorus was determined by the method of Fiske et al. (1925) after hydrolysis of the samples (LePage, 1957).

The fraction containing the 320 mμ peak was hydrolyzed with 2 and 0.1 *N* HCl at 100°C for 15, 30, 60 and 240 min. The hydrolysates were analyzed for sugar as described above, after the HCl was removed by evaporation under vacuum.

RESULTS

A COMPOUND having a maximum absorbancy at 320 mμ (pH 2, 7 and 12) was eluted from the formate column (Jones et al., 1960) between fractions 1 and 50. The elution peak for this fraction varied. When the nucleosides increased (samples held in ice over 4 days) these peaks were superimposed. This compound was present in all the samples of oyster meats. Fractions containing this peak were isolated and rechromatographed on the formate column, using only distilled water for elution. In some samples it was possible to separate this compound from the nucleosides and bases, using this procedure. In these cases, the fractions containing the unidentified compound were combined and lyophilized to reduce the volume and were used for spectrophotometric study. Hydrolysis in 1.5 *N* HCl for 15 min at 100°C shifted the maximum absorbancy to 328 mμ. Hydrolysis in 2 *N* NaOH for 15 min at 100°C destroyed all absorption. However, these results could not always be duplicated.

Qualitative tests for phosphate and biuret were negative, and the ninhydrin test for amino groups was strongly positive. No amino sugars or other sugars could be detected by paper chromatography. The unknown compound could be dialyzed through 18/32 Visking tubing, suggesting a low molecular weight. Further testing is necessary before identification is possible.

GDPM was identified on the basis of the following criteria: It eluted from the nucleotide column similarly to commercially obtained GDPM. Maximum absorption at pH 2 was 255 mμ. When hydrolyzed for 15 min at 100°C in 0.01 *N* HCl (Cabib et al., 1954), it yielded GDP (column chromatography) and a spot at the position of mannose (paper chromatography).

Nucleosides and free bases eluted from the chloride column in fractions differing from those described for the same system by Jones (1960). Routinely, the peaks eluted 20 fractions (100 ml) later than described. This shifted pattern of elution did not affect the recovery or the resolution of the system. The inosine and guanosine peaks were superimposed and were hydrolyzed and rechromatographed, yielding hypoxanthine and guanine, thus confirming the presence of their corresponding nucleosides.

The fraction not retained by either the formate or chloride column was chromatographed on paper and no adenosine was found. Colorimetric determinations for adenine containing compounds were negative. There were no further attempts to identify compounds contained in this fraction.

DISCUSSION

IN MOST WESTERN countries only the skeletal muscle of fish and selected parts of marine mollusks are considered edible. The overall nucleotide composition and the accumulation of their breakdown products were studied in the whole oyster, since they are generally consumed in this form and these changes would affect their eating quality. Comparing post-mortem changes in oysters with similar events occurring in the edible portions of other marine animals, oysters present a more complex system, since their edible portions include organs, muscle, glands and stomach contents.

The concentrations of nucleotides found in oyster tissues are low as compared to fish muscle or to any of the several marine invertebrates reported by Arai (1966a). The values found are considerably higher than those reported by Wylie et al. (1964). The latter authors recognized that nucleotides are incompletely extracted by ethanol and acknowledge that there would be low recovery using their chromatographic technique.

The nucleotide concentrations found in summer seem to be somewhat higher than in winter (Table 1). This difference is due to the summer rise of energy-rich adenosine nucleotides, perhaps in the gonadal tissue. The adductor muscle tissue was lower in nucleotides than the corresponding dark tissues (Table 2).

No UDPG or any other nucleotide glucose derivative, known intermediates in glycogen synthesis, were found in any of the samples analyzed. This finding is

Table 1—Nucleotides in whole oyster meats—seasonal effects of sexual development (μmoles/g meat).

	Summer, Full sexual development	Winter, no sexual development
ATP	0.68	0.55
ADP	0.96	0.67
AMP	0.50	0.26
IMP	0.26	0.12
GTP	tr	0.06
GDP	tr	0.18
GMP	0.10	0.07
GDPM	0.34	0.32
UTP	0.03	0.10
Total	2.87	2.33

tr = trace.

Table 2—Nucleotides on oyster meats—distribution between adductor muscle and remaining tissues (μmoles/g meat).

	Whole	Adductor muscle	Remaining tissue
ATP	0.55	0.16	1.11
ADP	0.67	0.71	0.68
AMP	0.26	0.50	0.23
IMP	0.12	0.17	0.12
GTP	0.06	tr	0.17
GDP	0.18	tr	0.17
GMP	0.07	0.07	0.06
GDPM	0.32	0.03	0.21
UTP	0.10	ND	ND
Total	2.33	1.64	2.75

ND = not detected.
tr = trace.

similar to that reported by Wylie et al. (1964).

The only uridine nucleotide found was UTP. Neither UDP nor UMP, expected intermediates in the dephosphorylation of UTP, was found despite repeated attempts to identify these intermediates. Significant amounts of uracil detected in oyster meats stored in icc for even one day (Table 3) suggests that the complete degradation of UTP to uracil occurs very quickly. The concentrations of uracil during the latter part of storage exceed the levels expected from the original concentration of UTP. At present, there is no reasonable explanation for this increase.

The adenine nucleotides were dephosphorylated and deaminated rapidly to IMP. In contrast, IMP disappears very slowly from oyster tissues (Table 3). This indicates a slow action of the oyster 5′-nucleotidases when compared with the rates of IMP disappearance from fish muscle in ice.

This pattern of breakdown differs from that proposed by Arai (1966a,b,c) for several other marine invertebrates which show little or no accumulation of IMP due to the low adenylic acid deaminase activity in their muscles. Furthermore, no adenosine was found in the oyster meats at any time during storage. The formation of this nucleoside via the dephosphorylation of AMP does not seem to occur in oyster tissues.

The changes in nucleotide composition of oyster meats during ice storage (Table 3) were not affected significantly by irradiation at 2 mrads. This leads to the conclusion that neither the nucleotides themselves nor the enzyme systems responsible for their post-mortem catabo-

Table 3—Concentration of nucleotides and nucleosides (μmoles/g).

	Days in icc[1]							
Controls	0	1	2	4	7	15	20	35
Unirradiated								
ATP	0.68	tr	ND	ND	ND	ND	ND	ND
ADP	0.96	0.25	0.22	0.18	0.08	0.12	0.08	0.08
AMP	0.50	0.83	0.52	0.10	0.09	0.14	0.11	0.08
IMP	0.26	1.04	1.26	1.23	1.51	1.02	0.67	0.64
GTP	tr	ND	ND	ND	ND	ND	ND	ND
GDP	tr	0.05	tr	ND	ND	ND	ND	ND
GMP	0.10	0.24	0.15	0.11	0.10	0.10	tr	0.11
GDPM	0.34	ND	ND	ND	ND	ND	ND	ND
UTP	0.03	ND	ND	ND	ND	ND	ND	ND
Total nucleotides	2.87	2.41	2.15	1.62	1.78	1.38	0.86	0.91
Inosine	tr	0.03	tr	0.20	0.12	0.34	0.58	tr
Hypoxanthine	0.11	0.17	0.40	0.32	0.16	0.27	0.37	0.23
Guanosine	0.11	0.29	0.17	0.23	0.23	0.43	0.58	0.86
Guanine	tr	ND	ND	tr	tr	tr	ND	0.06
Uracil	0.04	0.11	0.04	0.22	0.13	0.09	0.07	0.12
Total nucleosides & free bases	0.26	0.60	0.61	0.97	0.64	1.13	1.60	1.27
Total	3.13	3.01	2.76	2.59	2.42	2.51	2.46	2.18
Recovery as % of initial concentration	100	96	88	83	77	80	75	70
Irradiated 2 Mr								
ATP	0.68	ND	ND	ND	ND	ND	ND	ND
ADP	0.96	0.27	0.14	0.30	0.15	0.14	0.14	0.07
AMP	0.50	1.19	0.60	0.15	0.16	0.18	0.11	0.08
IMP	0.26	1.30	1.38	1.34	1.41	0.99	0.57	0.39
GTP	tr	ND	ND	ND	ND	ND	ND	ND
GDP	tr	ND	ND	ND	ND	ND	ND	ND
GMP	0.10	0.10	0.12	0.09	0.08	0.10	tr	tr
GDPM	0.34	ND	ND	ND	ND	ND	ND	ND
UTP	0.03	ND	ND	ND	ND	ND	ND	ND
Total nucleotides	2.87	2.86	2.24	1.88	1.80	1.41	0.82	0.54
Inosine	tr	0.16	0.49	0.66	0.29	0.10	0.17	0.09
Hypoxanthine	0.11	0.02	0.16	0.18	0.62	0.24	0.33	0.67
Guanosine	0.11	0.29	0.38	0.20	0.53	0.87	1.34	1.16
Guanine	tr	tr	0.05	0.06	tr	tr	tr	tr
Uracil	0.04	tr	0.08	0.07	0.12	0.16	0.33	0.39
Total nucleosides & free bases	0.26	0.47	1.16	1.17	1.56	1.37	2.17	2.31
Total	3.13	3.33	3.40	3.05	3.36	2.78	2.99	2.85
Recovery as % of initial concentrations	100	106	108	98	107	89	96	91

[1] Pooled samples representing 3–5 shucked Oysters.
tr = trace; ND = not detected.

lism were affected by this level of radiation. Lower doses of radiation would not seem to affect these same systems. These findings are similar to results obtained with fish (Guardia et al., 1965).

The recovery of nucleotides, nucleosides and free bases originally present did not decrease for the irradiated samples throughout the storage period, but decreased steadily for the unirradiated samples (Table 3). There were no differences in the concentration of nucleotides themselves between the irradiated and unirradiated samples during 20 days' storage in ice. The decline in the total amount of nucleotides, nucleosides, and free bases in the unirradiated samples was probably due to utilization of nucleosides and free bases by bacteria, which were present in very high numbers (more than 10^6 per g) (Chung and Dollar, 1965, unpublished data, College of Fisheries, Univ. of Wash.).

Guanosine accumulated rapidly with simultaneous reduction in the amounts of hypoxanthine and inosine in both irradiated and unirradiated samples through 15 days of storage. The concentrations of guanosine exceeded the combined concentrations of all guanine-containing nucleotides in the freshly shucked oyster. Thus, based on stoichiometric relations, the formation of guanosine cannot be limited to the breakdown to guanine-containing nucleotides.

The absence of a proportional increase in other purine or pyrimidine-based compounds suggests that degradation of ribonucleic acid cannot be responsible for the increased levels of guanosine. Probably guanosine was formed from hypoxanthine-containing compounds through endogenous oxidation and amination. These systems are known to be present in mammalian tissues and have been suggested as responsible for the accumulation of guanine in ice-stored cod and plaice muscle, where equivalent concentrations of the corresponding guanine nucleotides were not found (Kassemsarn et al., 1963).

The variable hypoxanthine concentrations found during ice storage rule out the feasibility of using this compound as a quality index for either irradiated or unirradiated oysters.

Nishita et al., (1966) have reported the presence in abalone muscle of unidentified substances which show the absorption maximum at about 330 mμ. These substances eluted with the initial water effluent from the formate Dowex 1 column, as did the fraction from the oyster extract which absorbed strongly at 320 mμ. This interesting similarity deserves closer study.

The concentrations of IMP and GMP in oyster tissues during early storage were considerably above their reported threshold levels for organoleptic detection (Kuninaka et al., 1964; Shimazono, 1964). They could contribute significantly to the flavor of fresh oysters. Water solutions of GMP have been described by some organoleptic panel members as having an "oyster-like" flavor. Halibut muscle, in which significant amounts of GMP have been found, is sometimes said to have a "shellfish-like" taste.

ABBREVIATIONS USED

ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; AMP, Adenosine Monophosphate; UTP, Uridine Triphosphate; UDP, Uridine Diphosphate; UMP, Uridine Monophosphate; UDPG, Uridine Diphosphate Glucose; GTP, Guanosine Triphosphate; GDP, Guanosine Diphosphate; GMP; Guanosine Monophosphate; GDPM, Guanosine Diphosphate Mannose: IMP, Inosine Monophosphate.

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Ethylene Oxide Resistance of Microorganisms Important in Spoilage of Acid and High-Acid Foods

SUMMARY—*Resistance values were determined for spores of* Bacillus coagulans *(var. thermoacidurans), conidiospores of* Aspergillus niger, *vegetative cells of* Lactobacillus brevis, Leuconostoc mesenteroides, Hansenula anomala *and* Saccharomyces cerevisiae *(var. tetrasporus) exposed to a mixture of ethylene oxide (12%)and dichlorodifluoromethane (88%). Exposures were made of organisms deposited on glass and paper discs at 33% relative humidity, 30°C and a pressure to give 700 mg per liter of ethylene oxide. Spores of* B. coagulans *were also exposed at 40°, 50° and 60°C. The resistance parameter D was determined to characterize the resistance of each organism displaying logarithmic death. Values of z were determined for spores of* B. coagulans. *The most resistant organism was* B. coagulans. *The spores of this organism were approximately 1.8 to 8 times more resistant than the vegetative cells of* L. mesenteroides or L. brevis. *Vegetative cells of* H. anomala, S. cerevisiae *and conidiospores of* A. niger *displayed non-logarithmic death.* A. niger *was the most resistant of the three.*

INTRODUCTION

THE USE of vapor phase sterilants dates back to the late 1800's when such gases as formaldehyde and sulfur dioxide were used to fumigate sick rooms. This practice was discontinued due to an apparent lack of effectiveness and not until recently (over approximately the last 30 years) has renewed interest occurred in the use of vapor phase sterilization. This interest has risen primarily from the needs of the food, agricultural, medical and space industries for methods of sterilizing items that cannot be subjected to excessive heat, liquid chemical sterilization or radiation. Since sterilization with chemicals in the vapor phase may be carried out at relatively low temperatures and moisture levels and because most sterilants can diffuse through plastics, paper or fabric, and are easily removed, the use of this method offers advantages not afforded by other means of sterilization.

Of the many vapor sterilants, ethylene oxide meets many of the requirements for an ideal gas sterilant. The chief disadvantage of flammability is overcome by dilution with inert gases such as carbon dioxide, nitrogen and chlorofluorohydrocarbons.

Most of the work involved with defining ethylene oxide sterilization parameters has been with resistant spore forming bacteria such as *Bacillus subtilis and Clostridium botulinum*. This paper deals with resistance parameters for a select group of organisms of lower resistance. This group contains representatives of those most significant in the spoilage of either acid foods (pH 4.0–4.5) or most high-acid foods (pH less than 4.0), (Stumbo, 1965). Though the group has received far less attention than more resistant organisms, Kirby et al. (1936), Whelton et al. (1946) and Rauscher et al. (1957) reported the effectiveness of ethylene oxide against various yeasts, molds and vegetative cells of several species of bacteria. It was thought that further work to define more closely the resistance parameters of members of the group would greatly aid in the establishment of procedures for the sterilization of containers and equipment to be used in conjunction with aseptic packaging of foods with pH values below 4.5.

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MATERIALS & METHODS

Microorganisms

Selected for study were *Bacillus coagulans* (var. thermoacidurans) ATCC 8038, *Lactobacillus brevis* ATCC 83057, *Leuconostoc mesenteroides* ATCC 83060, *Aspergillus niger, Saccharomyces cerevisiae* (var. tetrasporus) and *Hansenula anomala.*

Preparation of test suspensions

B. coagulans. A 50-ml quantity of thermoacidurans broth in a cotton-plugged Erlenmeyer flask was inoculated and incubated for 18 hr at 45°C. Following incubation, a 10-ml quantity of the culture was aseptically removed and placed in a sterile screw-capped test tube, heated in a water bath at 80°C for 10 min and cooled rapidly to room temperature with cold tap water. 1 ml of the culture was then aseptically transferred to each of several screw-capped bottle slants (150 ml capacity containing 50 ml of thermoacidurans agar with 10 ppm added manganous sulfate) and spread evenly over the surfaces of the agar. The inoculated bottle slants were incubated at 45°C until a high percentage of mature spores (approximately 92%) was evident. (Approximately 48 hr was required to obtain maximum sporulation.) Growth was then removed from each bottle with 2 ml of cold sterile distilled water. Washings so obtained were combined as they were filtered through sterile cheese cloth into a sterile polyethylene centrifuge tube. The filtered culture was stored overnight at 4°C.

After refrigeration, the crude suspension was washed four times with cold sterile distilled water. It was centrifuged between washings at about 1000 *G* for 5 min. each time the supernatant being discarded and the cells resuspended in 20 ml of cold sterile distilled water. The viable count of the suspension was adjusted to approximately 10^9 spores per ml with sterile distilled water and the suspension stored at 4°C. To prepare a test spore suspension, 1 ml of the thoroughly agitated stock suspension was placed in 9 ml of sterile distilled water and heated for 10 min at 80°C in order to kill vegetative cells and activate the spores for germination. The test suspension contained approximately 10^8 spores per ml.

L. mesenteroides and L. brevis. For each organism, a 50-ml quantity of lactic broth in a 250-ml cotton-plugged Erlenmeyer flask was inoculated and incubated for 20 hr at 30°C. At the end of the incubation period the lactic broth culture was placed in a sterile 50 ml centrifuge tube. The cells were washed four times. Each time the suspension was centrifuged at about 7710 G for 5 min, the supernatant discarded and the cells resuspended in 10 ml of sterile M/10 phosphate buffer. After the final washing the cells were suspended in 50 ml of sterile M/10 phosphate buffer. The test suspension contained approximately 10^7 cells per ml. This procedure was repeated prior to each series of exposures.

A. niger conidiospores. 50 ml of tryptone glucose yeast extract broth in a 250-ml cotton-plugged Erlenmeyer flask was inoculated and incubated for 5 days at 30°C. The spent broth was then discarded leaving the mat of growth intact. 10 ml of a 0.01% solution of sodium lauryl sulfate were added and the flask agitated to separate the condiospores from the mat. The crude conidiospore suspension was then placed in a sterile 50 ml centrifuge tube and washed four times. Each time the suspension was centrifuged at about 7710 G for 5 min, the supernatant discarded and the cells resuspended in sterile distilled water. The final suspension was diluted to a concentration of approximately 10^7 cells per ml as determined using a Petroff-Hauser

counting chamber. This procedure was repeated prior to each series of exposures.

S. cerevisiae and H. anomala. Wort agar plates were streaked with a small amount of culture suspended in 1 ml of sterile distilled water. The inoculated plates were incubated at 30°C for 24 hr. After incubation, growth was removed from each plate with approximately 10 ml of cold sterile M/10 phosphate buffer and combined in a sterile 50 ml centrifuge tube. The yeast cells were washed four times. Each time the suspension was centrifuged at about 7710 G for 5 min, the supernatant discarded and the cells resuspended in 10 ml of cold sterile M/10 phosphate buffer. The suspension was then syringed vigorously five times through a number 23 hypodermic needle to break up clumps of cells. The resulting suspension was diluted with M/10 phosphate buffer to give approximately 10^7 cells per ml as determined using a Petroff-Hauser counting chamber. This procedure was repeated prior to each series of exposures.

Preparation of inoculated discs

Pyrex glass discs (1 cm diameter and 1 mm thick) and circular paper discs (1 cm diameter) placed on other of the glass discs served as the two carriers for the test suspensions. The discs were sterilized in Petri dishes and dried at 55°C. Each disc was then inoculated with 0.01 ml of the test suspension.

Exposure system

The apparatus and gases used for exposure were the same as described by LIU et al. (1968). The apparatus consisted of three stainless steel anaerobic jars contained in thermostatically controlled baths.

Exposure conditions

All organisms were exposed to a mixture of ethylene oxide (12%) and dichlorodifluoromethane (88%) at 33% R. H., 30°C and a pressure to give 700 mg of ethylene oxide per liter of exposure atmosphere. *B. coagulans* spores were also exposed at 40°, 50° and 60°C. The accuracy of exposure conditions were discussed by LIU et al. (1968).

Exposure procedures

Discs inoculated with test suspensions containing *S. cerevisiae, H. anomala, L. brevis* or *L. mesenteroides* were exposed immediately after inoculating. Those discs inoculated with spores of *B. coagulans* or *A. niger* were allowed to equilibrate at about 33% R.H. for 18 hr prior to exposing. A desiccator containing a beaker of saturated magnesium chloride solution served as the equilibrating chamber. Small beakers of saturated magnesium chloride solution were also placed inside each petri dish and the desiccator cooled to 4°C to prevent spore germination. The following procedure was used in exposing the inoculated discs:

1) The water and oil baths were adjusted to the desired temperature. Adjustment required at least 1 hr prior to the beginning of an exposure.

2) The heating tape, surrounding connecting lines and controlled by a thermal regulator, was turned on ½ hr prior to the beginning of an exposure.

Table 1—Summary of media and conditions used to determine viable count of survivors.

Media and Conditions	*B. coagulans*	*L. brevis* and *L. mesenteroides*	*H. anomala* and *S. cerevisiae*	*A. niger*
Media	Thermoacidurans agar	Lactic agar	Wort agar	Orange serum agar
Incubation time	40–48 hr	72 hr	48 hr	36 hr
Incubation temperature	45°C	30°C	30°C	30°C
Dilution fluid	Distilled H_2O, (10 ml blanks contained 1% Darvan No. 1)[1]	Lactic broth	0.1 *M* phosphate buffer	0.01% sodium lauryl sulfate

[1] Used as a dispersing agent.

3) When the baths were controlled at the desired temperatures, the entire system was evacuated to approximately 30 in. Hg with a vacuum pump.

4) Carbon dioxide was introduced into the system to a pressure of 15 psig. The system was again evacuated. This procedure was repeated three times to remove moisture that might have collected inside the apparatus.

5) The preconditioning chambers were then separately evacuated to approximately 30 in. Hg. The sterilant gas was introduced into these chambers until the pressure was 15 psig. The chambers were then sealed from the rest of the system.

6) The exposure chamber lid was removed and a petri dish containing the inoculated discs was placed inside the chamber on an inverted glass beaker serving as a stand. The Petri dish cover was then removed and the exposure chamber lid replaced.

7) The exposure chamber was evacuated to about 29 in. Hg.

8) Sufficient sterile distilled water was introduced into the exposure chamber, by means of a syringe through a rubber septum on the inlet line, to maintain a relative humidity of 30–35% throughout the exposure period. The relative humidity in the exposure chamber was checked by means of an electric hygrometer.

9)The sterilant gas was allowed to pass from the preconditioning chambers into the exposure chamber until a pressure was reached that corresponded to 700 mg of ethylene oxide per L under the conditions of the test. Timing of the exposure period was then started.

10) During the exposure period, the preconditioning chambers were refilled with the sterilant gas to a pressure of 15 psig.

11) At the end of the exposure period pressure was relieved by means of a water aspirator.

12) The exposure chamber lid was removed and the discs were taken out.

Determination of survivors

After exposure the discs were immediately placed in individual 10 ml dilution blanks. The dilution pour-plate technique was used to determine the number of survivors of all organisms. Media and conditions used for each organism are presented in Table 1. With the exception of *B. coagulans*, diluting and plating was conducted immediately after the discs were exposed. (The 10 ml dilution blanks containing discs from *B. coagulans* exposures were held overnight at 4°C prior to dilution and plating.)

All 10 ml dilution blanks into which the exposed paper discs were initially placed also contained three sterile glass discs. These blanks containing the discs were agitated (usually 15–30 sec) at maximum speed on a shaker until the paper discs were pulverized. This technique was used to release cells entrapped in the paper. All 10 ml dilution blanks into which the exposed glass discs (without paper discs) were initially placed were shaken at maximum speed for 15 sec. Dilutions were then made from the 10 ml blanks and plated in triplicate (using the appropriate agar) and the plates incubated at the designed temperatures and times. A sodium lauryl sulfate solution was used as the diluting fluid for discs containing *A. niger* conidiospores to aid in dispersing any cells in clumps.

RESULTS & DISCUSSION

SURVIVOR CURVES for organisms deposited on paper and glass discs and exposed to the sterilant gas are presented in Figures 1–7. Each curve represents an average of three trials (with the exception of *A. niger* which represents the average of two trials). During each exposure period three glass and three paper discs were exposed and the survivors on each diluted and plated in triplicate. Therefore, each point on the curves represents the average of 27 plate counts (with the exception of *A. niger* for which each point represents 18 plate counts).

D values (time required to destroy 90% of the cells present) were determined for those organisms generally displaying logarithmic death. The straight lines of best fit were determined by the least squares method and the resulting regression coefficients were used to determine the D values.

Phillips (1952) first reported that the usual large differences in resistance to heat sterilization between spores and vegetative cells of bacteria were not evident with ethylene oxide sterilization. In this study the resistance of *B. coagulans* spores was about 1.8 to 8 times greater than that of vegetative cells of *L. mesenteroides* or *L. brevis*. In contrast the heat resistance of *B. coagulans* spores may be

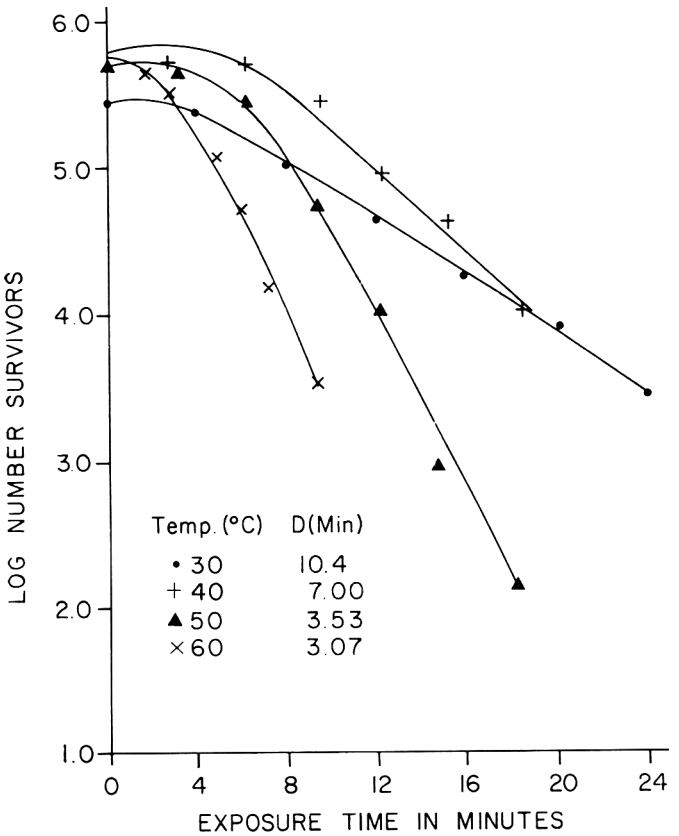

Fig. 1—Survivor curves for B. coagulans spores, on paper discs, exposed to the sterilant gas at the temperatures indicated.

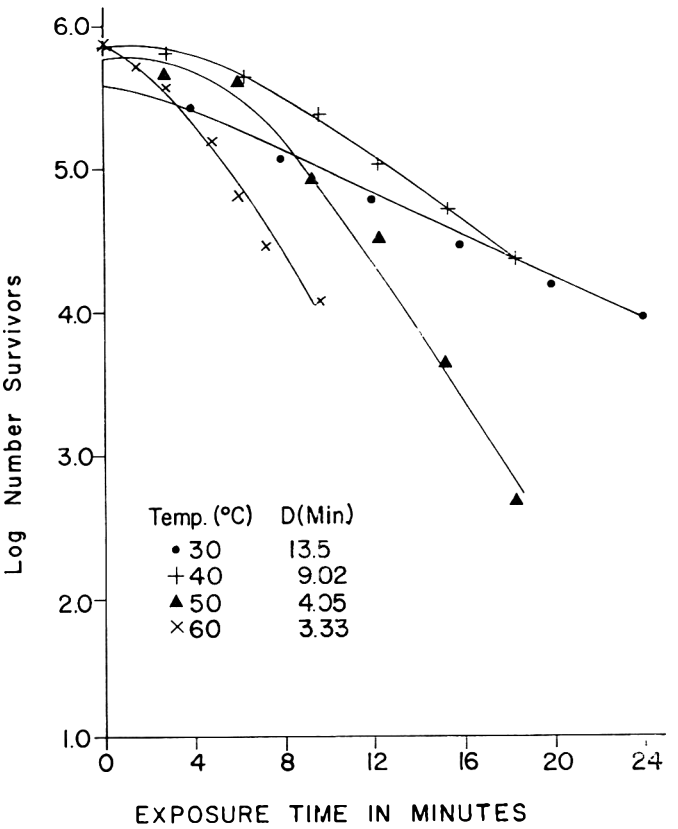

Fig. 2—Survivor curves for B. coagulans spores, on glass discs, exposed to the sterilant gas at the temperatures indicated.

as much as 200,000 times more than that of *L. brevis* or *L. mesenteroides*.

Survivor curves for *H. anomala*, *S.* cerevisiae and *A. niger* (Figs. 5–7) were not straight lines. Hiatt (1964) generalized the reasons for such departures as due either to heterogeneity of the microorganisms or to factors operating during the reaction causing death. It has been reported (Church et al., 1956) that the spores of some aerobic bacilli are heterogeneous with respect to ethylene oxide resistance. The shape of these three sets of curves indicate deviations were not due to heterogeneity of the organisms. If the organisms were heterogeneous with respect to ethylene oxide resistance the first portion of a curve would recede compara-

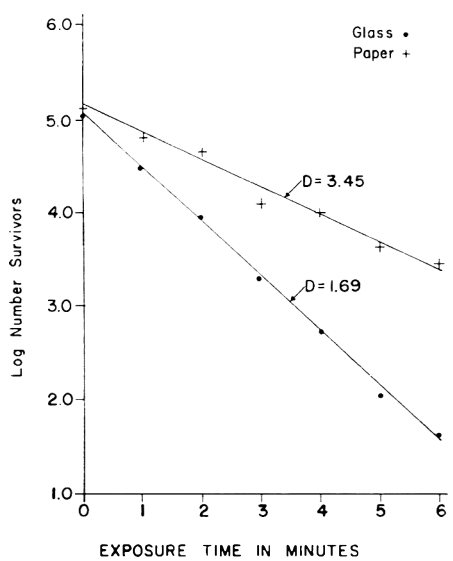

Fig. 3—Survivor curves for L. mesenteroides, on glass and paper discs, exposed to the sterilant gas at 30°C.

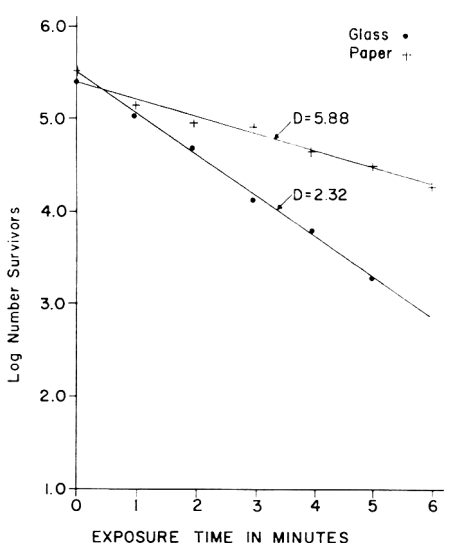

Fig. 4—Survivor curves for L. brevis, on glass and paper discs, exposed to the sterilant gas at 30°C.

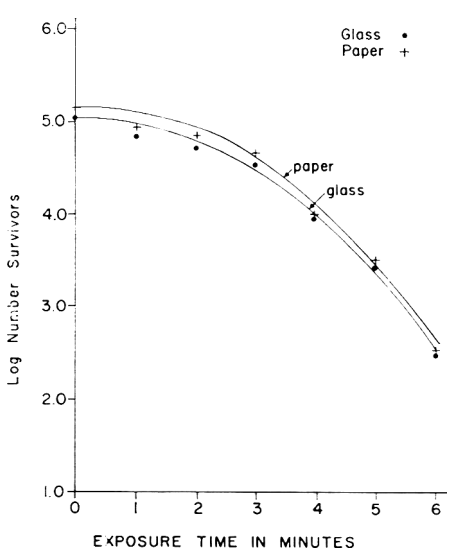

Fig. 5—Survivor curves for H. anomala, on glass and paper discs, exposed to the sterilant gas at 30°.

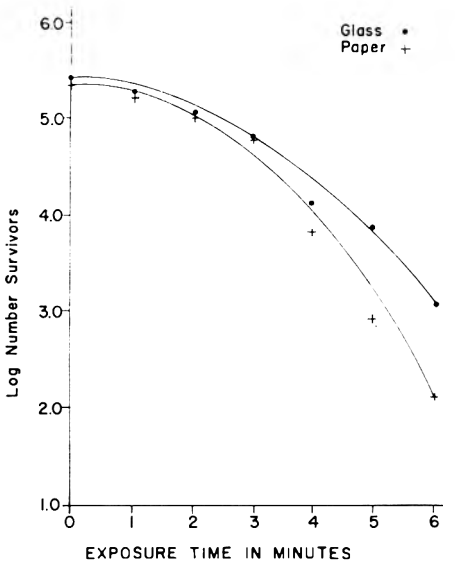

Fig. 6—Survivor curves for S. cerevisiae, on glass and paper discs, exposed to the sterilant gas at 30°C.

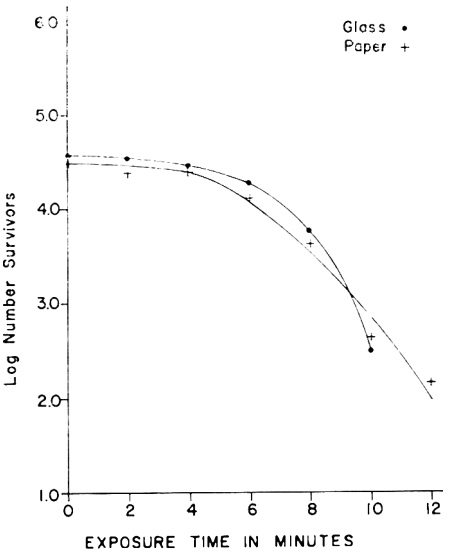

Fig. 7—Survivor curves for A. niger, on glass and paper discs, exposed to the sterilant gas at 30°C.

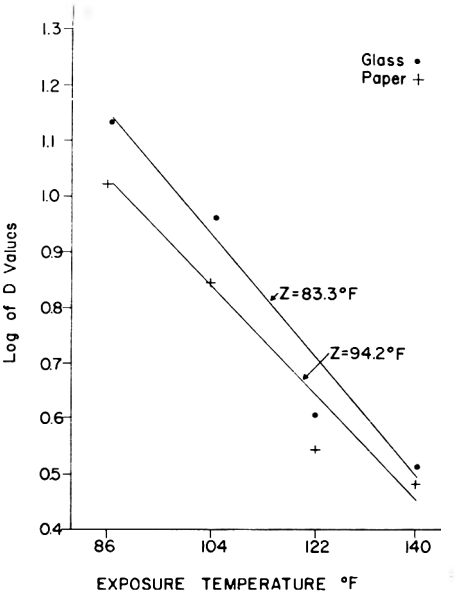

Fig. 8—Thermochemical destruction curves for B. coagulans spores on glass and paper, exposed to the sterilant gas.

tively rapidly, indicating death of the lower resistance cells, followed by a flatter portion of the curve indicating death of the higher resistance cells.

Several possibilites for non-logarithmic nature of the curves can be postulated from the data, e.g.: (1) a complex reaction, i.e., other than first order, is responsible for death of the organism; (2) cumulative damage to a sensitive site or sites within the cell was required to cause death of the organisms; (3) the lag or induction period evident in each of the curves indicated a sterilant gas penetration time to reach the sensitive site or sites within the cell. Additional data will be required to determine the true cause of the noted deviations from straight lines.

The type of carrier material has an effect on the resistance of *L. mesenteroides, L. brevis* and *B. coagulans.* The resistance of *B. coagulans* deposited on glass was greater. It has been theorized (Phillips et al., 1949) that when suspensions of microorganisms are desiccated, hard crusts are formed, thicker crusts being formed on impervious surfaces and thinner crusts on surfaces of absorbent materials. This would account for the greater resistance on glass of *B. coagulans,* as spores buried under thicker crusts would be more protected from the sterilant gas.

The opposite was evident with *L. brevis* and *L. mesenteroides,* i.e., cells deposited on paper had the greater resistance. Since these discs were not preconditioned, exposures of microorganisms were actually of 0.01 ml suspensions. Thus either or both of the following could account for such an effect (1) a greater concentration of sterilant gas was present in the small bubble on the glass due to its solubility, thereby resulting in greater killing or (2) the paper offered some protection for those cells below the surface resulting in a greater number of survivors for equal exposure times. No tendency toward greater resistance on either carrier was evident for those organisms displaying nonlogarithmic death.

Spores of *B. coagulans* were the most resistant of the organisms studied. Compared to spores of other bacteria tested under similar conditions, *B. coagulans* spores were more resistant than spores of P. A. 3679 (Haurand, 1966) but less resistant than spores of either *B. subtilis* (LIU, et al., 1968) or *C. botulinum,* Type A (Kuzminski, 1967). In order to assess the relative resistance of *B. coagulans* at various temperatures, D values were also determined at 40°, 50° and 60°C. The log of these D values were plotted against exposure temperatures to obtain the thermochemical destruction curves in Figure 8. The straight lines of best fit were again determined by the least squares method and the z values, or number of deg Fahrenheit required for these curves to transverse one log cycle, were determined from the regression coefficients.

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Inhibitory Effects of Pseudomonas on Selected Salmonella and Bacteria Isolated from Poultry

SUMMARY—*The inhibitory effects of* Pseudomonas *on selected* Salmonella *and poultry isolates were investigated. Two methods of demonstration of the inhibitory effects were used. A perpendicular streak technique was used as a preliminary screening procedure to determine relative degrees of inhibition exhibited by known strains of* Pseudomonas *against sensitive* Salmonella *and known organisms isolated from poultry. Spectrophotometric analysis was also used to measure inhibitory activity produced by different concentrations of filtrates from* Pseudomonas *cultures against sensitive organisms.*

Inhibition of sensitive organisms was more pronounced with agar plates than with cell density methods which employed broth. The production of pigment appeared to be related to the ability of different Pseudomonas *cultures to produce inhibition. Concentration of sensitive cells did not appear to be a limiting factor, since inhibition was demonstrable at both high and low levels of inocula.* Pseudomonas *strains were inhibitory to strains of* Salmonella, Staphylococcus, Escherichia *and* Streptococcus.

None of the inhibitory strains of Pseudomonas *isolated from poultry were mutually repressive. However, one strain of* Pseudomonas aeruginosa *not isolated from poultry did cause inhibition of growth of all of the* Pseudomonas *isolated from poultry. The public health significance of this work in relation to potential pathogens on processed poultry is discussed.*

INTRODUCTION

THE ROLE of bacterial competition and antagonism has been recognized in microbial populations found in natural environments but additional information is needed to support the hypotheses concerning these phenomena. In 1947, Waksman discussed mechanisms which may enable one bacterial strain to prevent the growth of others. Among these mechanisms are: competition for nutrients, toxic metabolites and the production of antibiotic substances.

Many studies have also been conducted on competitive growth of other microorganisms on the development of staphylococci (Oberhofer et al., 1961; Peterson et al., 1962; Graves et al., 1963; Troller et al., 1963a and 1963b; DiGiacinto et al., 1966; and Kao et al., 1966).

Kraft et al. (1966) reported on the effects of competitive growth of microorganisms and fluorescence development on inoculated poultry during refrigerated storage. Fluorescing bacteria attained large populations on chicken even when *Staphylococcus aureus* initially outnumbered the fluorescing spoilage organisms by a ratio of 100:1.

The ability of *Pseudomonas* spp. to produce antibacterial agents on poultry meat has not been investigated. The present work was undertaken to examine the effect of *Pseudomonas* on inhibition of *Salmonella* and other bacteria associated with processed poultry.

MATERIALS & METHODS

Organisms

Test organisms included: *Pseudomonas fluorescens* (F-21, 2 and 23a) isolated from poultry (Department of Food Technology, Iowa State University, Ames), *P. aeruginosa* (Pyo Gotze), and a strain of *E. coli* b 3000 obtained from Y. Hamon, Inst. Pasteur, Paris, France. Other organisms used were: *Streptococcus faecalis* (M-18), *S. faecium* (7), *Staphylococcus aureus* (196 and 20a), and the following *Salmonella* strains: *S. pullorum, S. bredeny, S. typhimurium, S. anatum, S. montevideo, S. thompson, S. lexington, S. infantis, S. give, S. tennessee* and *S. heidelberg*. With the exception of *S. anatum* and *Staph. aureus* 196, all of these organisms were from the departmental stock culture collection and previously isolated from poultry products. *S. anatum* was obtained from the Central Public Health Laboratory, London, England. *S. aureus* 196 was obtained from M. S. Bergdoll, University of Wisconsin, Madison.

Survey techniques

Three techniques for the demonstration of inhibition were employed. A perpendicular streak method was used for preliminary screening to assay for inhibitory effects of various *Pseudomonas* strains against other organisms. A streak, approximately 1 in. wide, of the suspected inhibitory organisms was made on brain heart infusion (BHI) agar (Difco) and the plates were incubated for 48 hr at 30°C. At this time, swabs of suspected sensitive organisms were made perpendicularly to the original streak. The plates were then incubated for an additional 24 hr at 30°C and the length of the resultant clear zones adjacent to the producer growth, indicating inhibition, were measured in centimeters with a vernier caliper.

A cell density procedure was also used to measure the inhibition of various concentrations of *Pseudomonas* filtrates against organisms previously found to be sensitive by the perpendicular streak survey technique. In each analysis, 0.1 ml of a 12 hr BHI broth culture of sensitive cells was introduced into tubes containing a combination of 0.1% peptone (Difco) buffer, 5 strength BHI broth (37 g/200 ml), and *Pseudomonas* filtrate. The final volume in each case was 10.0 ml per tube. Table 1 provides a description of the quantities of each component used in the spectrophotometric analyses.

The mixtures were read in Bausch and Lomb cuvettes (¾ in. × 6 in.) at 540 nm in a B and L Spectronic 20 colorimeter (Bausch and Lomb, Inc., Rochester, N.Y.). Plate counts were made on BHI agar to determine numbers of sensitive cells used as inocula. Counts were also made to determine relative degrees of growth of sensitive cells during exposure to *Pseudomonas* filtrate.

The inhibitory effects of *Pseudomonas* filtrates were demonstrated by an additional method. This involved the saturation of a

Table 1—Combinations of components used in spectrophotometric analyses.

Tube	Culture (ml)	BHI (ml)	Buffer (1 ml)	Buffer in control[1] (ml)	Filtrate (ml)	Filtrate (%)
1	0.1	1.9	—	0.1	8.0	80
2	0.1	1.9	1.0	1.1	7.0	70
3	0.1	1.9	2.0	2.1	6.0	60
4	0.1	1.9	3.0	3.1	5.0	50
5	0.1	1.9	4.0	4.1	4.0	40
6	0.1	1.9	5.0	5.1	3.0	30
7	0.1	1.9	6.0	6.1	2.0	20
8	0.1	1.9	7.0	7.1	1.0	10

[1] Control = tube minus inoculum.

sterile strip (1/2 in. × 4 in.) of filter paper (Whatman No. 40) with filtrate, placing it on a layer of BHI agar in the center of the plate and overlayering the strip again with BHI agar. Plates were stored for 3 days at 15°C to facilitate pigment diffusion through the medium. Sensitive organisms were then swabbed on the surface of the plates. These bacteria failed to show growth in the area of pigment diffusion after 24 hr at 30°C.

Production of filtrates

Pseudomonas strains were inoculated into the asparagine broth of Georgia et al. (1931) and incubated for approximately 48 hr at 30°C with shaking in a water bath (Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, N.J., set at 7). The cells were then centrifuged at 14,350 R.C.F. × *g* for 20 min and the supernatant was filtered in a Seitz apparatus with 0.1 μ pore size filter pads (Columbia Filter Co., Hawthorne, N.J.).

Table 2—Measurements (in cm) of zones of inhibition produced by Pseudomonas strains on various bacteria.[1]

Sensitive organisms	Producer Pseudomonas strains (cm)			
	Pyo Gotze	F-21	23a	2
E. coli b 3000	No growth	1.23	1.36	1.37
Strep. faecalis (M-18)	1.90	1.2	1.00	1.22
Strep. faecium (7)	0.54	1.13	1.25	1.32
Staph. aureus 196	1.78	1.11	1.26	1.36
Staph. aureus (20a)	2.85	1.76	1.77	1.83
Salmonella typhimurium	1.09	—	—	1.13
Salmonella pullorum	1.92	—	1.74	1.33
Salmone la heidelberg	1.93	1.02	1.16	
Salmonella give	1.59	1.23	1.31	
Salmonella tennessee	1.49	0.91	0.80	
Salmonella infantis	1.78	1.29	1.05	
Salmonella thompson	0.90	0.60	1.18	
Salmonella anatum	1.40	1.14	1.18	
Salmonella lexington	1.44	1.45	1.54	
Salmonella montevideo	1.15	1.14	1.18	

[1] With the exception of *P. aeruginosa* (Pyo Gotze), *E. coli* b 3000, and *Salmonella anatum*, all organisms were previously isolated from poultry products.

RESULTS & DISCUSSION

THROUGH THE USE of the streak technique, strains of *Pseudomonas* were shown to be inhibitory toward strains of *Salmonella*, *Staphylococcus*, *Streptococcus* and *Escherichia coli*. Figure 1 and Table 2 give results obtained in a typical experiment using the streak technique. The diffusion of pigment produced by the Pseudomonas strains through the agar medium appeared to play a role in the degree of inhibition demonstrable. Limited studies were then undertaken to determine the optimum time and temperature conditions necessary for maximum inhibition. After evaluating several combinations of time and temperature, it was found that for all of the *Pseudomonas* strains tested, incubation for 36–48 hr at 30°C provided maximum diffusion of pigment and resultant inhibition of sensitive organisms.

When *Pseudomonas* strain F-21, which produces pigment at 30°C, but not at 37°C, was surveyed against sensitive organisms, inhibition was not demonstrable at the higher temperature, but was clearly evident at the lower temperature.

Simultaneous inoculation of both inhibitory and sensitive organisms resulted in the absence of any visible inhibition. Further investigation in this area showed that the inhibitory *Pseudomonas* strains required an 18–24 hr growth advantage before any significant zones of inhibition were noticeable.

None of the inhibitory strains of *Pseudomonas* isolated from poultry were mu-

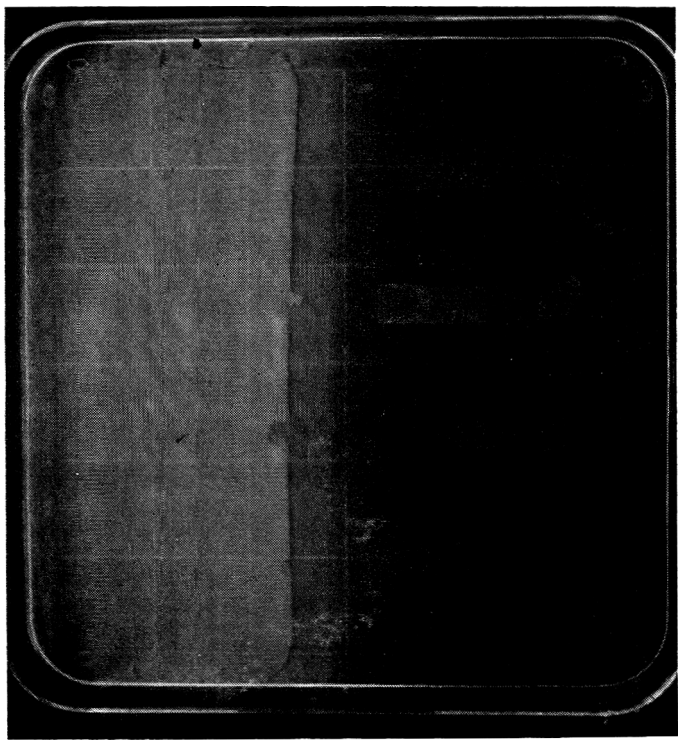

Fig. 1—Streak technique. (Fluorescent pigment diffusion under ultraviolet light). Inhibitory effects of Pseudomonas fluorescens *(F-21) on various bacteria, from top to bottom,* E. coli *b 3000,* Strep. faecalis *(M-18),* Staph. aureus *(20a), and* Salmonella pullorum.

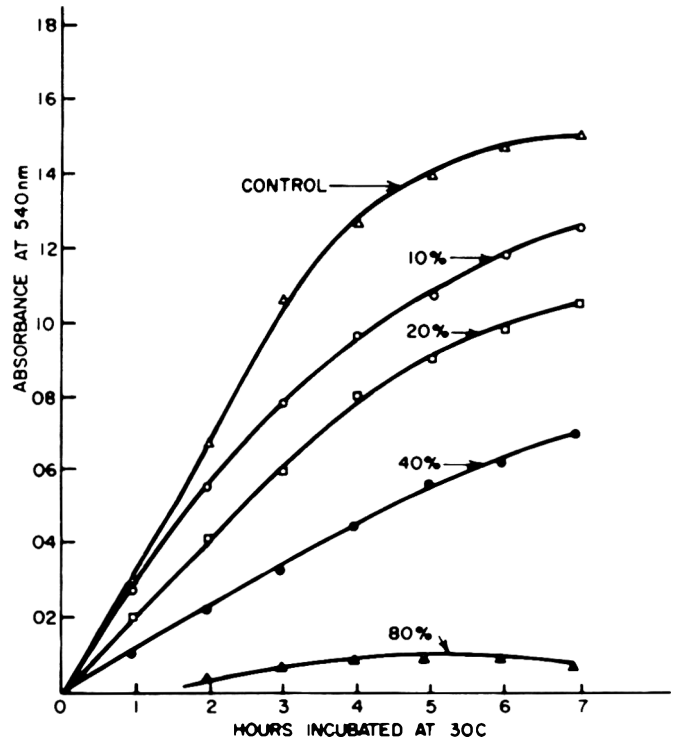

Fig. 2—Inhibition of E. coli *b 3000 by various concentrations (by volume) of* Pseudomonas aeruginosa *(Pyo Gotze) filtrate.*

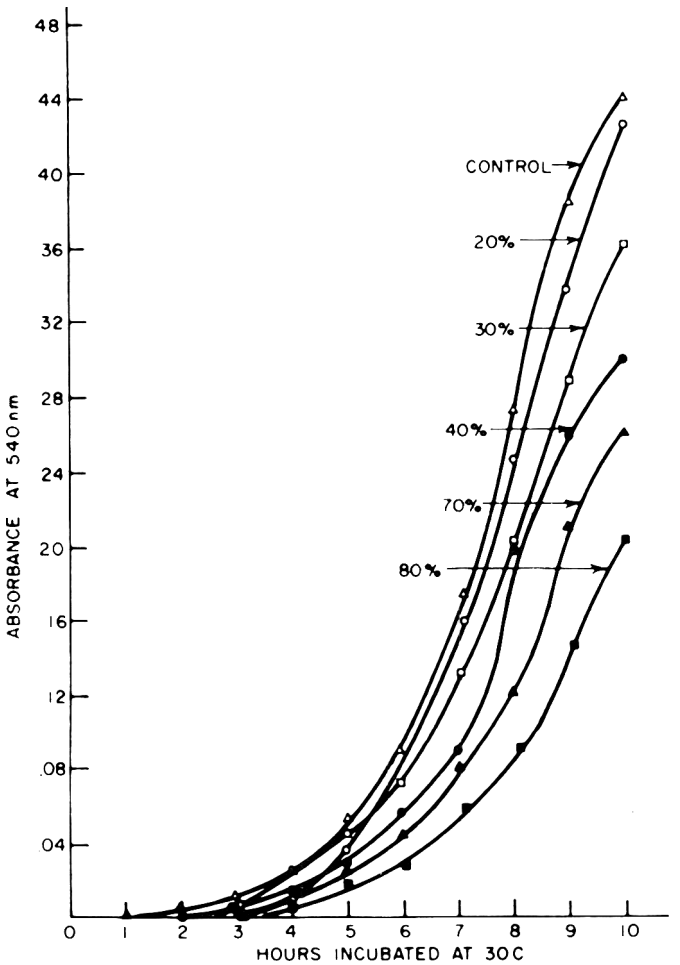

Fig. 3—*Inhibition of* Salmonella pullorum *by various concentrations (by volume) of* Pseudomonas fluorescens *(F-21) filtrate.*

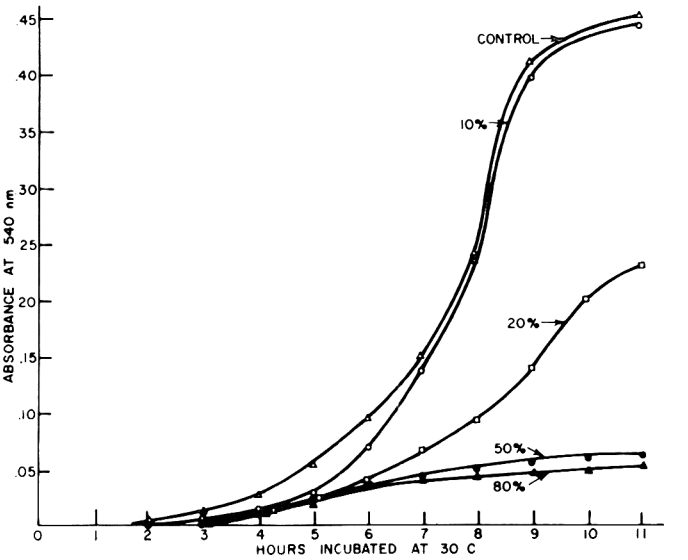

Fig. 4—*Inhibition of* Staphylococcus aureus *(20a) by various concentrations (by volume) of* Pseudomonas fluorescens *(2) filtrate.*

tually repressive. However, *Pseudomonas aeruginosa* strain Pyo Gotze not isolated from poultry did cause inhibition of growth of all of the *Pseudomonas* isolated from poultry.

The inhibitory activity measured by cell density methods employing *Pseudomonas* culture filtrates showed that, in most cases, a level greater than 30% filtrate (by volume) produced marked inhibition. Figures 2, 3 and 4 present curves for typical turbidimetric analyses.

The inocula ranged from 10^6 to 10^8 sensitive cells per ml. The various dilutions of filtrate were maintained at pH values of 7.2 to 7.4 in tubes 1 through 8, so pH did not appear to be a factor in the inhibition observed.

Inhibition was demonstrated by means of the cell density procedures, even after autoclaving (121°C for 15 min), with the filtrate from *P. aeruginosa* (Pyo Gotze). However, the degree of inhibition was not as pronounced as with the filtrate not autoclaved. It is anticipated that examination of other *Pseudomonas* filtrates will yield similar results. Additional work is needed to determine if the fluorescent pigment produced by pseudomonads is inhibitory per se. Purification and concentration procedures are desirable before the nature of the inhibitory substance may be exactly determined.

Preliminary studies of the nature of the component(s) responsible for inhibition indicate that the inhibitory material does not appear to be protein in nature; infrared and ultraviolet spectral analyses failed to demonstrate protein. Also, no precipitate was obtained upon treatment of the filtrate with ammonium sulfate (to saturation). Biuret determinations were also negative. Electrophoresis of concentrated (20×) filtrate showed migration toward the anode but no typical protein reaction upon staining overnight.

Further investigations of the properties of the filtrate are in progress.

The antibacterial activity exhibited by various *Pseudomonas* isolated from poultry and from other sources in this study may help in understanding the mechanisms whereby development of potential pathogens is suppressed. Additional work will be directed toward public health implications in poultry processing and utilization.

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Metmyoglobin Formation in Beef Stored in Carbon Dioxide Enriched and Oxygen Depleted Atmospheres

SUMMARY—A spectroreflectometric technique was used to determine the relative percentages of three myoglobin pigments, reduced myoglobin, oxymyoglobin and metmyoglobin at the surface of fresh beef. It was shown that, at constant humidity, the formation of metmyoglobin in beef was maximal at 6 ± 3 mm Hg of oxygen at 0°C and 7.5 ± 3 mm Hg at 7°C for semitendinosus muscles. Carbon dioxide concentrations of 10% and higher had negligible effect on the formation of metmyoglobin, provided the oxygen pressure was above about 5%. At high partial pressures of carbon dioxide, absorption of carbon dioxide increased and the pH of the surface decreased. In air, the formation of metmyoglobin varied widely from muscle to muscle.

INTRODUCTION

FOR TENDERIZING fresh beef by aging, it is necessary to hold the meat at temperatures above the freezing point for several days. Maximal tenderization is obtained at 0 to 2°C in about 14 days, shorter times being adequate at higher temperatures (Kuprianoff, 1964). At these temperatures bacterial spoilage is dependent on the initial population density. Extending storage life by using low relative humidities (Scott, 1936) leads to marked weight loss and discoloration. Haines (1933) and Scott (1938) extended the life of fresh meat by storage in selected atmospheres, low in oxygen and/or enriched with carbon dioxide. The present work was undertaken to determine if any undesirable color changes occurred in beef muscle stored at low oxygen and/or high carbon dioxide partial pressures, at a high relative humidity.

Any undesirable color changes will attain increased significance as the size of the joint decreases, thus if half or quarter carcasses are aged any surface discoloration will be relatively unimportant after butchering for retail consumption. Limitations on storage space often make this method of aging uneconomical and there is an increasing tendency to age in boneless, retail size cuts. Under these conditions surface discolorations are of major importance.

In sterile meat, of normal water content, the color is due mainly to the heme proteins myoglobin and hemoglobin. At a high relative humidity (99.3%), any discoloration of sterile, post-rigor meat is due to the oxidation of these pigments to the brown metmyoglobin and methemoglobin. (Myoglobin is the major colored protein in fresh beef and the color changes of myoglobin and hemoglobin are, to a first approximation, the same. Consequently the pigment states are generally analyzed in terms of myoglobin derivatives.)

Little objective work has been performed on the effect of carbon dioxide on color of fresh meat. Brooks (1933) found that up to 20% carbon dioxide (in air) had negligible effect on the oxidation of heme pigments in meat although 30% led to a slightly increased rate of oxidation, which he attributed to the reduced oxygen partial pressure. Brooks (1931, 1935) working with ox-blood (hemoglobin), and George et al. (1952a, 1952b) using pure horse heart myoglobin, found the rate of oxidation to be very dependent on the partial pressure of oxygen in the system. The rate was maximal at low partial pressure.

In meat the situation is more complex because (a) the oxidation is quasi-reversible as the enzymatic reduction of metmyoglobin to one of the reduced forms can occur (Stewart et al., 1965b), and (b) it is extremely difficult to determine, accurately and non-destructively, the pigment states at the meat surface. In the analysis of the pigment states in meat, extraction procedures are cumbersome; they destroy the sample analyzed and possibly cause changes in the relative proportions of the three pigments. For these reasons a spectroreflectometric method of analysis was preferred.

The three myoglobin pigments, both in meat and in solution, have an isosbestic point at 525 nm (Stewart et al., 1965b). Stewart et al. (1965a) estimated the percentage metmyoglobin at the surface of minced meat samples from the ratio of K/S values at 572 nm (an isosbestic point for reduced myoglobin and oxymyoglobin) to that at 525 nm. K/S is defined as

$$\frac{(1 - R\infty)^2}{2R\infty}$$ (Kubelka et al., 1931)

where $R\infty$ is the reflectivity of an opaque sample of such a thickness that there is no further change in reflectivity when the thickness is increased further.

This technique corrects for any difference in the total concentration of myoglobin pigments in the sample, but does not adequately allow for differences in the scattering (S) and absorption (K) coefficients of the meat matrix itself. Either one or both these coefficients will vary with pH, fat content, surface geometry and water content of the sample, as well as with the incident wavelength. The effects are such that the ratios of K/S at 572 nm to K/S at 525 nm are unlikely to be constant for "pigment free meat."

Snyder (1965) attempted to overcome the problem by adjusting all his spectral curves to a common reflectance (in absorbance units) of $R_A = 1.0$ at 525 nm, and estimating the metmyoglobin content by the reflectance at 572 nm. Unfortunately his plot of R_A at 572 nm against percent metmyoglobin, for known mixtures of oxymyoglobin and metmyoglobin in an aqueous suspension of dried milk was not linear.

Using these data Snyder et al. (1967) found that when K/S at 572 nm was plotted against metmyoglobin concentration the predicted linear relationship was obtained. They also found, for 18 beef rounds of known pigment state, that determinations of surface metmyoglobin concentration from the ratio of K/S values at 572 and 525 nm and from the K/S value at 572 nm after adjustment of R_A at 525 nm to 1.0 were equally accurate although, conceptually, they considered the ratio method to be preferable.

EXPERIMENTAL

Preparation of sterile samples

Discs of 14 mm radius and 15 ± 1 mm thickness were cut under aseptic conditions from a semitendinosus muscle which had been removed from the carcass directly after slaughter. Muscle was extensively flamed and aged for 2 days at 0°C in a closed, sterile container. These discs were used in the storage experiments. In initial experiments heme pigments in some of these discs were converted to 100 or 0% metmyoglobin by the action of ferricyanide (1%) and dithionite (20%) respectively. Other discs were minced and adjusted to various pH values

in the range 5.3 to 6.8 with M HCl and M NaOH before the pigments were converted to 100 or 0% metmyoglobin.

Gaseous atmospheres

A continuous flow of air or nitrogen (both containing 10% carbon dioxide), maintained at a relative humidity of 99.3% by bubbling through towers containing 0.2*M* sodium chloride solution, was passed through the container holding the samples. The whole system was sterilized before use. Microbial contaminants in incoming gases were removed by passing the gases through cotton wool plugs. Each system was stored in a room kept at the appropriate temperature (0 or 7°C). Air controls were stored under similar conditions. At least 9 samples were stored in each container.

In some experiments meat slices of radius 38 mm were stored in closed, sterile, plastic containers leaving a headspace of 18 ± 1 cc. Headspace samples were removed through sub-a-seals. A flushing arrangement through the seal allowed the gas composition in the headspace to be adjusted at will.

Spectral analysis, gas analysis and pH

The reflectance spectra of the meat samples were recorded against a magnesium oxide standard on a Hitachi Perkin-Elmer Spectrophotometer, Model 139, with reflectance attachment. The range scanned was 380 to 770 nm. After storage all samples were exposed to air (R.H. 99.3%) for 2–3 hr, at the storage temperature, so that any reduced myoglobin at the surface was oxygenated while avoiding any detectable change in the surface concentration of metmyoglobin. Spectra of the exposed meat surfaces were then recorded at 8 ± 1° C. Samples were contained in stainless steel cups of appropriate dimensions.

Gas analyses were performed at regular intervals using a 25V Fisher Gas Partitioner calibrated with purified gases. One tenth ml samples of gas, extracted with SGE gas tight syringes, were used. Average equilibrium concentrations are reported.

Surface pH was measured with a surface electrode and Radiometer Model TTT IC Titrator and pH meter.

Heme pigment concentration was measured according to the method of Hornsey (1956).

RESULTS & DISCUSSION

Determination of metmyoglobin at the surface

In preliminary experiments, minced semitendinosus samples, of known pigment state and different pH values were analyzed. In these the ratio of K/S at 572 nm to K/S at 525 nm, for 100% and 0% metmyoglobin varied and there was slightly better consistency in the K/S values at 572 nm when R_A at 525 nm was adjusted to 1.0 absorbancy units. Results obtained with several intact semitendinosus samples of known pigment state were consistent with those obtained on minced samples.

For 18 samples the K/S values at 572 nm, after adjustment of R_A to 1.0 at 525 nm, were respectively 2.41 ± 0.19 (range 2.15–2.62) and 6.05 ± 0.18 (range 5.80–6.30) for 100% and 0% metmyoglobin. Assuming the linear relationship between the adjusted K/S value at 572 nm and the percentage metmyoglobin, it was possible to calculate metmyoglobin as a percentage of the total surface pigments to within ±5%. The ratio of K/S at 572 nm to K/S at 525 nm yielded values of 1.450 ± 0.061 and 0.615 ± 0.050 respectively for 100% and 0% metmyoglobin, enabling the metmyoglobin at the surface to be calculated to within 6 or 7%.

As errors, determined by standard deviations, were greater using the ratio method the adjustment technique was used in the present study. The values obtained were independent of the area of illumination, whether the surface presented to the integrating sphere was flat, convex or concave.

Differences in spectra due to variations in total pigment concentrations are not eliminated by this technique, but calculations showed that differences by a factor of 2 in total concentration should lead to errors within the range of those found experimentally. Pigment concentrations were always within the range 4.6–6.9 mg per g of wet tissue for the different muscles studied.

Another error can arise from the fact that intramuscular fat has a characteristic spectrum. Thus it is unlikely that muscles with different fat contents can be compared by this technique, as the "pigment-free" meats will not yield the approximately parallel reflectance curves (in absorbancy units) that are necessary for this method to be valid. All the spectra determined, on meat of known pigment state, were found to be superimposable, to at least ±4% of R_A in the range 470–650 nm, and so it would appear that variations in the fat contents of the lean muscles studied were not of major importance.

In meat, oxymyoglobin and metmyoglobin have an isosbestic point at 474 nm (Stewart et al., 1965b) and all the above arguments were found to be valid, enabling the percent reduced myoglobin at the surface to be determined. The adjusted K/S values at 474 nm were 2.00 ± 0.09 (range 1.90–2.12) and 3.88 ± 0.10 (range 3.70–4.05) for 100% and 0% of the reduced pigment respectively. This enables percent of reduced myo-

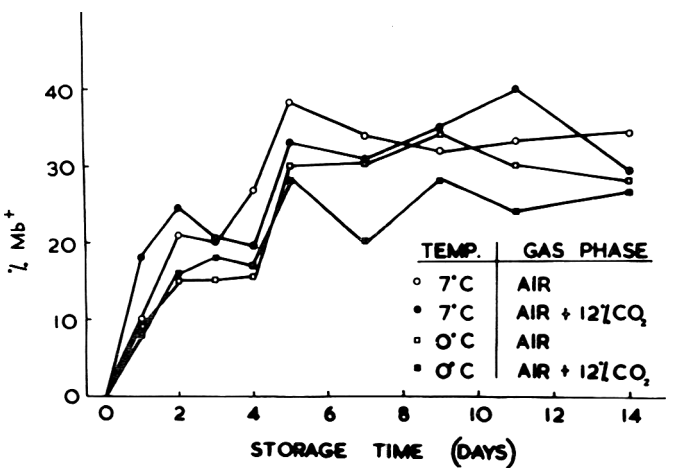

Fig. 1—The effect of 12.0 ± 0.5% CO_2 on the formation of metmyoglobin (Mb^+) at 0°C and 7°C, on a sterile muscle (pH 5.58) at a relative humidity of 99.3%.

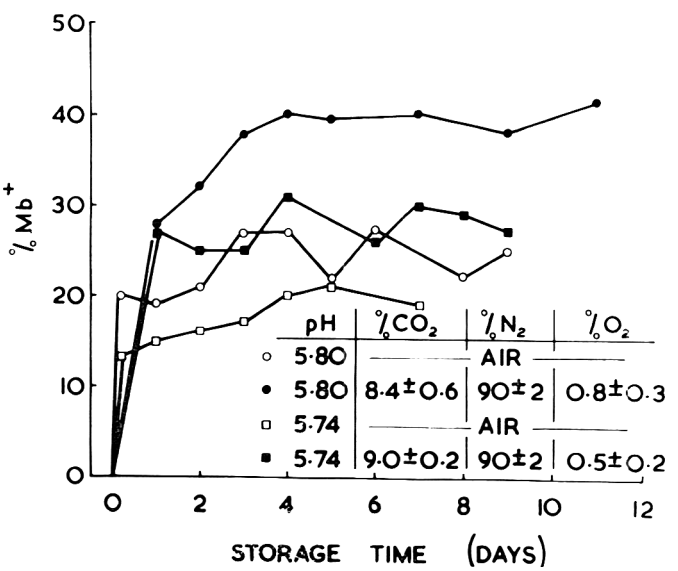

Fig. 2—The formation of Mb^+ at 7°C, on sterile muscles in various atmospheres at a relative humidity of 99.3%. Sampling was discontinued when unavoidable contamination occurred.

globin to be determined to an accuracy of about 5%.

Effect of storage on formation of metmyoglobin

Figure 1 shows the change in surface metmyoglobin concentration as a function of time for samples from the same muscle (pH 5.58) stored in air or 12% CO_2/air at 0 or 7°C and a R.H. of 99.3%. Figure 2 is a similar plot for samples of two muscles (pH 5.80 and 5.74) stored in the atmospheres indicated, at 7°C. At 0°C metmyoglobin formation in these samples was also greater at the lower oxygen partial pressures.

From Figure 1 it is seen that the presence of 12% carbon dioxide had negligible effect upon the formation of metmyoglobin. This was in accord with the observation of Brooks (1933). The increased oxidation found at the lower oxygen partial pressures (Fig. 2) was in general agreement with data obtained by Brooks (1935) and George et al. (1952b) with aqueous hemoglobin and myoglobin solutions.

Figures 1 and 2 both indicate that concentration of metmyoglobin was virtually constant after storage for 5 days.

When the freshly cut meat slices were sealed in the containers, concentration of carbon dioxide rose to between 10% and 15% while concentration of oxygen fell. Equilibrium was established within 48 hr. To ensure equilibrium, samples were stored for 12 ± 2 days, actual storage time governed by practical expediency. In a few instances pressure in the container was reduced by removing a measured volume of gas. In one experiment, muscle was sliced and packed in a nitrogen atmosphere so that the final atmosphere consisted solely of nitrogen and carbon dioxide.

The relationships between metmyoglobin concentration and partial pressure of oxygen, at 7 and 0°C, are summarized in Figures 3 and 4. All values are the means obtained for duplicate samples from the same slice.

Table 1—"Equilibrium" concentration of metmyoglobin for different muscles after storage in air for 12 ± 2 days.

Surface pH of the muscle	"Equilibrium" percent Mb^{+1} at the surface	
	7°C	0°C
1. 5.58	35 ± 4	31 ± 5
2. 5.60	22 ± 4	18 ± 4
3. 5.60	[2]	33.5 ± 5
4. 5.70	38 ± 5	31.5 ± 5
5. 5.74	20 ± 4	22 ± 4
6. 5.80	26 ± 4	31.5 ± 5
5.70	—	38.5 ± 4[3]
5.72	—	28 ± 3[3]
	Av. 28.2 ± 8	Av. 26.3 ± 6

[1] Mb^+ = metmyoglobin.
[2] Not measured—sample contaminated.
[3] Values not included in the average as these muscles were not used in the experiments reported in Figures 3 and 4.

The lower equilibrium concentrations of surface metmyoglobin on samples stored in air, were independent of pH (Table 1).

Brooks (1931) found that rate of oxidation of different samples of ox-blood, at 25°C and constant pH and ionic strength, varied greatly. The present results on meat (Table 1) show similar differences in that the equilibrium concentrations of metmyoglobin vary for different samples, the variation not appearing to be a function of pH. These differences may be explained by differences in rate of enzymatic reduction of metmyoglobin. Stewart et al. (1965b) have shown that the metmyoglobin reducing activity of different samples of ground beef can vary considerably under identical storage conditions.

The data reported by Stewart et al. (1965b) were obtained on metmyoglobin formed by ferricyanide oxidation and may not represent the true reduction of naturally formed metmyoglobin as ferrocyanide forms a complex with ferric heme pigments, this complex catalyzing the enzymatic reduction (Hegesh et al., 1967).

Recently several studies on the enzymatic reduction of ferric heme pigments have been reported and present evidence indicates that NADH plays a vital role (Hegesh et al., 1967), (Watts et al., 1966). Therefore the differences may represent different "NADH ferrihemoglobin and ferrimyoglobin reductase" activities in the muscle. No corrections have been applied to the experimentally determined metmyoglobin concentrations in Figures 3 and 4 to allow for the variations found in the air controls. If, however, corrections were made the fundamental character of plots was unchanged.

Figures 3 and 4 indicate that formation of metmyoglobin was maximal at a partial pressure of oxygen of 7.5 ± 3 mm Hg at 7°C and 6 ± 3 mm Hg at 0°C for the semitendinosus muscles studied. George et al. (1952b) found, for pure myoglobin solutions, that rate of autoxidation was maximal at about 1 mm Hg of oxygen at 30°C and pH 5.69. Brooks (1931) found rate to be maximal at 20 mm Hg of oxygen for ox-

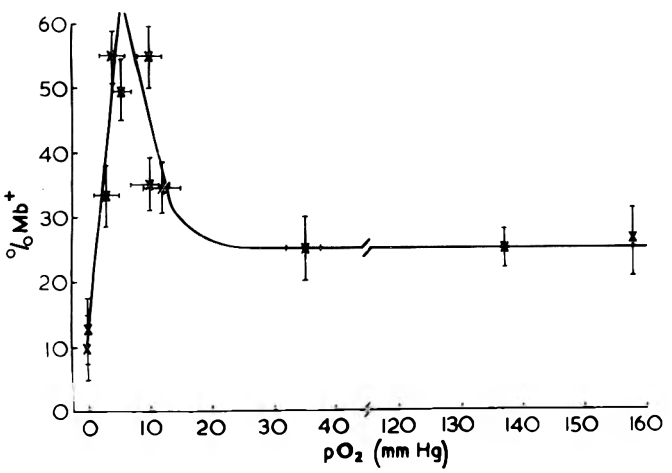

Fig. 3—The relationship between the partial pressure of oxygen and Mb^+ formation at the surface of sterile muscles after storage for 12 ± 2 days at 0°C.

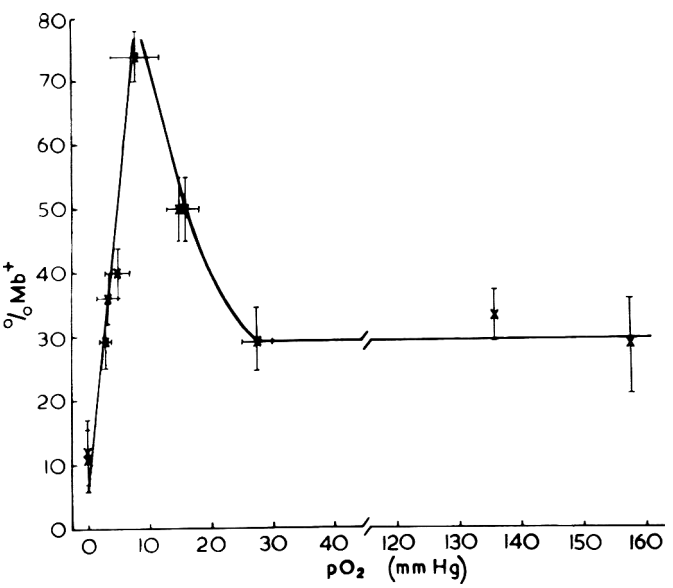

Fig. 4—The relationship between the partial pressure of oxygen and Mb^+ formation at the surface of sterile muscles after storage for 12 ± 2 days at 7°C.

Table 2—Effect of CO_2/air concentrations on the formation of metmyoglobin, during 14 days storage, at 0°C.

Pack no.	pCO_2 init. mm Hg	pO_2 init. mm Hg	pCO_2 final mm Hg	pO_2 final mm Hg	ΔpH[1]	Conc. Mb^+ %
1	79	106	63	86	0.03	26.0
2[2]	167	82	46	109	0	29.5
3	190	84	127	84	0.05	32.0
4	273	68	167	74	0.06	32.0
5	312	61	209	68	0.06	31.5
6	403	53	244	64	0.07	31.0
7	471	38	283	59	0.08	36.0
8	578	30	307	50	0.09	40.0
9	669	15	384	41	0.07	63.5 (2.5 hr) 52.0 (24 hr)
10	730	0	535	22	0.12	70.0 (2.5 hr) 51.0 (24 hr)

[1] ΔpH was the increase in surface pH of the samples upon removal from storage; the pH returned to its original value within 2 hr.
[2] Pack 2 leaked slowly with time.

Table 3—Effect of CO_2 pressure on the formation of metmyoglobin at 0°C.

pCO_2 mm Hg	pO_2 mm Hg	ΔpH[1]	"Eq. Conc." of Mb^+
0	152	—	38.5
76	135	0.06	42.0
190	104	0.05	39.0
380	76	0.06	43.0
510	56	0.10	43.0
650	110	0.27	39.5

[1] ΔpH is as per Table 2.

blood (hemoglobin) at 25°C and pH 5.69.

If the ratio of myoglobin to hemoglobin varied to a marked degree between the muscles studied, a comparison of results would not be valid when expressed as a function of the oxygen partial pressure. It was considered unlikely that such variations occurred. At low oxygen partial pressures, rates of both the autoxidation and enzymatic reductions are increased (Watts et al., 1966). The present results show that increase in rate of oxidation, at 0 and 7°C, is greater than any increase in the enzymatic reduction at the low oxygen pressures studied (Figs. 3 and 4).

This maximal formation of metmyoglobin occurs in all samples, independent of external oxygen pressure, provided this is above the critical value. However, with higher oxygen partial pressures the formation will occur below the surface, the depth at 0°C being about 5 mm in air and varying as the square root of the external oxygen pressure (Brooks, 1929). At the lower oxygen partial pressures the metmyoglobin layer will thus be nearer the surface until, at the critical partial pressure, it is at the surface.

When transferred from the oxygen depleted atmospheres to air, samples with high metmyoglobin contents tended to be reduced with time. This reduction was always very slow, the maximum reduction observed within 24 hr being from 72 to 63% at 7°C with no measurable reduction within this period at 0°C (less than 5% at 55% concentration).

Effect of increased carbon dioxide concentrations

Sterile samples were packed in sealed containers, in atmospheres of various carbon dioxide partial pressures at 0°C. Before packing the samples were left in air for 3–4 hr at 0°C to allow most of the physically bound carbon dioxide to be released. The volume of meat was 25 cc and the total volume 85 cc. In all cases the carbon dioxide concentrations fell due to absorption while the oxygen and nitrogen levels rose. Equilibrium was reached at 24–48 hr. The results are summarized in Table 2.

The values given for the percent of metmyoglobin quoted for the two higher concentrations of carbon dioxide are the values obtained after exposure to air for 2 hr, as reduction occurred over 24 hr (Table 2). All other values are averages of 4 readings, 2 on each of 2 samples, at 2.5 and 24 hr. These results indicate that at the higher carbon dioxide and lower oxygen partial pressures the formation of metmyoglobin increased, presumably due to the decreased oxygen pressures.

The reduction of metmyoglobin found in packs 9 and 10 on exposure to air was greater than any reduction that occurred after storage in 10% carbon dioxide/nitrogen mixtures. Although variations between muscles are to be expected the decrease in pH during storage in high carbon dioxide concentrations (Table 2) may also help to explain this, as autoxidation is accelerated at lower pH values (Brooks, 1931) while enzymatic reduction is retarded (Stewart et al., 1965b).

The initial nonequilibrium of the gas phase in these packs made interpretation of results difficult due to the interrelation between decreased pH and oxygen pressure. In further experiments at 0°C various equilibrium gas phases of carbon dioxide/oxygen were used. The oxygen pressure was always above the level necessary to cause increased metmyoglobin formation. Increasing the carbon dioxide pressure, at 0°C, had no effect on formation of metmyoglobin during 15 days storage even though the pH of the meat was markedly decreased (Table 3).

No measurable change occurred in the surface concentrations of metmyoglobin on re-exposure to air, at the relatively low concentrations of metmyoglobin used in this experiment.

In the first experiment, the meat in higher carbon dioxide atmospheres developed a greyish tinge, which masked the natural "redness" of the meat. This may have been due to decreased pH of the meat causing some of the sarcoplasmic proteins to undergo post-rigor isoelectric precipitation. In the second experiment, even though the muscle was of similar pH (5.72 and 5.70), the meat appeared "normal" at all the carbon dioxide pressures studied. In subsequent experiments this "greying" phenomenon has been observed in samples stored for 14 and 28 days in 60% carbon dioxide atmospheres, but no explanation can be offered for its occurrence in only certain muscles.

From the results described in the present paper it would appear that the storage of sterile beef in carbon dioxide enriched atmospheres leads to no increased metmyoglobin formation, provided the oxygen partial pressure is maintained above a limiting value of about 5%.

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Comparison of Carbonyl Compounds in Moldy and Non-moldy Cocoa Beans

SUMMARY—Carbonyl compounds in moldy and non-moldy cocoa beans were converted to dinitrophenylhydrazones and separated into monocarbonyl classes. Growth of mold was always accompanied by relatively large increases in carbonyl concentrations. Increases in total monocarbonyl values ranged from 20 to 500% and averaged almost 300% for the eight pairs of samples analyzed. Compared to non-moldy beans, moldy cocoa beans contained greater concentrations of methyl ketones, 2-enals and 2,4-dienals, but saturated aldehyde concentrations were quite often lower. TLC revealed the presence of C_3, C_4, C_6, C_{17} and several unidentified methyl ketones. Most of the ketones detected in moldy beans were also found in non-moldy beans but in lower concentrations. Qualitatively, the unsaturated aldehyde fractions varied considerably among samples. 2-Pentenal and 2,4-pentadienal appeared as prominent TLC spots and other 2-enals and 2,4-dienals were frequently observed in moldy beans. The only unsaturated aldehydes detected in non-moldy beans by TLC were 2-pentenal and 2,4-octadienal.

INTRODUCTION

CONDITIONS for mold attack in the tropics are favorable in damaged or improperly dried cocoa beans. It would be expected that beans are normally highly infested with mold spores and with an increase in moisture these spores may germinate and cause undesirable changes to occur.

Boyd, et al. (1965) in a study of the monocarbonyls of chocolate suggested that mold activity might have contributed significantly to the observed variation in methyl ketone content of cocoa beans. Unfortunately, these investigators did not have an opportunity to collect supporting data by analyzing moldy cocoa beans. It was envisioned that if Boyd's suggestion could be verified, a way might be opened for the development of an objective chemical procedure to replace the current subjective "cut test" for determining mold infestation. At present the cut test involves cutting the cocoa bean longitudinally and visually observing for mold.

The research reported in this paper concerns the differences in total carbonyls, total monocarbonyls and relatively concentrations of the different monocarbonyl classes in moldy and non-moldy cocoa beans. The data collected were based on the methods of Schwartz, et al. (1963) as modified for chocolate products by Boyd, et al. (1965). The techniques involved the conversion of carbonyls to 2,4-dinitrophenylhydrazones (DNP-hydrazones) followed by separation into the following classes: methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals. Thin layer chromatography procedures of Schwartz, et al. (1968) were used to identify and assess the complexity of each class of monocarbonyls.

EXPERIMENTAL

Samples

Several samples of moldy cocoa beans, supplied by chocolate manufacturers, yielded unusually high carbonyl values. Since many variables affect carbonyl concentrations (Boyd, 1965), it was decided that the best experimental approach was to develop mold on beans under controlled laboratory conditions. This made possible comparison of carbonyl values before and after growth of mold, thereby minimizing the effect of many interfering variables.

Aspergillus and *Penicillium* were the two main types of mold isolated from cocoa beans and were used to inoculate non-moldy beans for control-molded samples. The *Aspergillus* and *Penicillium* spores were removed from moldy cocoa beans with sterile water and then inoculated onto cocoa beans using a sterile, platinum loop. The inoculated cocoa beans were placed in a 1 qt polyethylene container with a small mat of filter paper (1 in. × 1 in. × 1/4 in.) saturated with sterile water to induce mold growth. The beans were stored at approximately 24°C to facilitate mold growth and simulate tropical conditions. Cocoa beans from several of the major producing countries were included in the study.

Moldy beans were also obtained directly from the tropics through the cooperation of the Turrialba Experiment Station, Costa Rica. A batch of fermented Matima beans had been split and one portion dried under normal conditions while the other half was purposely kept from drying for several days to allow mold to grow. These samples made it possible to determine the changes which take place as a result of mold growth during the fermenting and drying stages.

Solvents

All solvents were freshly distilled and rendered carbonyl free (Schwartz, et al.,

Table 1—Concentrations of total carbonyls and total monocarbonyls of moldy and non-moldy cocoa beans.

	Micromoles per 30 g of beans					
	Total carbonyls			Total monocarbonyls		
Samples	Non-moldy	Moldy	Increase in μM	Non-moldy	Moldy	Increase in μM
Bahia A	34.8	44.8	10.0	2.9	11.7	8.8
Tabasco	31.0	60.0	29.0	4.1	21.1	17.0
Caracas	32.0	52.0	20.0	7.5	17.2	9.7
Sanchez	43.2	64.2	21.0	8.5	10.2	1.7
Accra A	26.7	47.8	21.1	1.8	10.4	8.6
Bahia B	32.0	60.3	18.3	5.8	12.1	6.3
Accra B	27.7	45.5	17.8	8.3	11.2	2.9
Matima	42.0	57.1	15.1	16.6	23.3	6.7

1961). The use of freshly prepared solvents was found to be absolutely essential.

Recovery of carbonyls as DNP-hydrazones

30 g of shelled, finely-pulverized beans were ground in a mortar with Celite 545 and 4 to 8 ml of water, depending upon the initial moisture content of the beans (moldy beans were characteristically higher in moisture). The mixture was then packed firmly in a glass column and the carbonyls and fat were eluted with 300 ml of hexane. Recovery procedures were almost identical to those of Boyd, et al. (1965).

The effluent from the extraction column was allowed to drip directly onto a Celite column bed impregnated with DNP-hydrazine, H_3PO_4 and H_2O (Schwartz, et al., 1963) to convert monocarbonyls to DNP-hydrazones. The column was rinsed with 200 ml of hexane to yield 500 ml of effluent. A DNP-hydrazone concentration (total carbonyls) was obtained by finding the absorbance of the solution compared to hexane at 340 mμ in a Beckman DBG Spectrophotometer and converting the reading to micromoles (μM) using E = 22,500 (Jones, et al., 1956).

The DNP-hydrazones were freed of lipid material by passing the hexane solution through a Celite 545-Sea Sorb 43 column as described by Boyd, et al. (1965). Because of the greater carbonyl content, 20 g of column packing was used for the moldy samples whereas only 12 g was needed for the control samples. The column was essentially fat free after flushing with 250 ml of hexane. Adsorbed hydrazones of monocarbonyls were eluted with 150 ml of 3:1 chloroform: nitromethane and after evaporation of the solvent under N_2 the residue was dissolved in 5–10 ml of hexane.

Removal of ketoglycerides and class separation

Monocarbonyl derivatives were separated from ketoglyceride DNP-hydrazones on a column of weak alumina (Schwartz, et al., 1963). 20 g of alumina was used for carbonyls from moldy samples and 10 g was employed for the non-moldy control. Monocarbonyls were eluted with 120 ml of hexane followed by 120 ml of benzene. The monocarbonyl solution was taken to dryness under N_2 and then adjusted to a 250 ml volume with hexane after which a quantitative value for monocarbonyls was obtained at 350 mμ.

The solvent was again evaporated and the monocarbonyl residue was dissolved in 10 ml of hexane and applied to a column containing 12 g or 16 g of 1:1 Celite 545 (heated for 24 hr at 150°C) and Sea Sorb 43 which had been slurried in hexane and packed with light air pressure. A thin column ($^1/_2$ in. × 18 in.) was used to favor good separation of the monocarbonyl classes. Effluent was collected in 10 ml aliquots using an LKB fraction collector. Separation of the four DNP-hydrazone classes (methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals) was obtained using the following sequence of solvents: 100 ml quantities of 20, 40, 60 and 80% chloroform in hexane; 150 ml of 100% chloroform; 100 ml quantities of 0.5, 1, 2, 5 and 10% methanol in chloroform; and finally, 150 ml of 35% nitromethane in chloroform.

Individual tubes from the fraction collector were taken to dryness under N_2 and then adjusted to a 10 ml volume with chloroform. Each tube was checked spectrophotometrically for purity and class authenticity on the basis of the following absorption maxima (mμ): methyl ketones, 365; saturated aldehydes; 355; 2-enals, 373; 2,4-dienals, 390. The tubes were pooled and quantitative values calculated for each class with application of the proper molar extinction coefficient (Jones, et al., 1956).

Separation of individual components within a class

The separation of the individual methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals was according to the procedures described by Schwartz, et al. (1968), with modifications. Approximately 6.3 ml of polyethylene glycol 400, 35 ml 1% KOH in methanol and 7.5 g of Micro Cel T-38 (heated 100°C for 24 hr) were vigorously mixed together before the chromatographic plates were prepared. The TLC plates (0.25 mm thick) were air dried for 5–10 min and then placed in a desiccator over ascarite.

The carbonyl fraction, dissolved in benzene, was spotted on the TLC plates and separated using the following solvent systems: methyl ketones, hexane saturated with polyethylene glycol 400 or 94% hexane and 6% benzene saturated with polyethylene glycol 400; saturated aldehydes, 85% hexane and 15% benzene saturated with polyethylene glycol 400; 2-enals and 2,4-dienals, using either 65% hexane and 35% benzene or equal volumes of each saturated with polyethylene glycol 400.

The different classes of hydrazones emitted characteristic color on KOH treated plates: rose-red for 2,4-dienal, pinkish-red for 2-enals, tan for saturated aldehydes and brown for methyl ketones. With these color differences it was possible to assess the purity of a carbonyl class.

To obtain further evidence concerning the identity of individual compounds, TLC preparative chromatography was used to obtain enough material for additional testing. The preparative plates were similar to those previously described except that pure methanol replaced the methanolic 1% KOH solution. Each DNP-hydrazone band from a developed plate was scraped and placed in a pasteur disposable pipette over $^1/_2$ in. of alumina. The DNP-hydrazone was extracted from the TLC absorbent by elution with benzene, and after concentration under N_2, the derivative was spotted along side of and in mixture with (co-chromatography) the suspected known on KOH plates.

For methyl ketones, if an unknown did not separate from a known reference derivative by co-chromatography then additional confirmatory evidence was obtained by spotting the two compounds, individually and as a mixture, on $AgNO_3$ plates (Schwartz, et al., 1968).

Normal partition systems described in the literature for separating a series of reacted ketones will usually separate only up to tridecanone (C_{13}). To resolve the higher molecular weight methyl ketones, reverse phase partition chromatography was attempted. Because of solubility problems associated with higher molecular weight ketones, separating by reverse phase chromatography was not totally effective. However, despite its inadequacies, clues concerning the identity of some members could still be obtained.

The plates for reverse phase chromatography were prepared using a mixture of 15 g of Micro Cel T-38 in 90 ml of hexane and 5 ml of Nujol (a brand of heavy mineral oil). Since the plate was to be placed upside down in the tank during an equilibration period (1 hr) it was necessary to scrape that portion which would be immersed in the solvent. After equilibration the plate was turned around and allowed to develop in acetonitrile:water 85:15.

RESULTS & DISCUSSION

THE CONCENTRATIONS of total carbonyls in several varieties of moldy cocoa beans compared to non-moldy controls are recorded in Table 1. In every instance, growth of mold resulted in striking increases in carbonyl values. "Total carbonyls" represents the combined contributions of ketoglycerides, monocarbonyls and those compounds retained on the defatting column such as keto acids and dicarbonyls. For the eight samples recorded in Table 1, the average for total carbonyls of the moldy beans was 54 μM which was 20 μM or 59% greater than in the control beans. Total monocarbonyls for moldy beans averaged 14.6 μM which was double the average for non-moldy

control beans.

In normal cocoa beans most of the total carbonyl value is contributed by ketoglycerides (Boyd, et al., 1965). However, it is likely that other compounds are primarily responsible for the great increase in total carbonyls in moldy beans. Referring to Table 1, it is readily apparent that the contribution of monocarbonyls to the increase in total carbonyls varied markedly among the samples analyzed.

For example, in Bahia A 88% of the 10 μM increase was contributed by monocarbonyls, whereas in Sanchez beans monocarbonyls contributed less than 10% of the difference in total carbonyls between moldy and non-moldy beans. It is reasonable to expect that such factors as pH of the cocoa bean, shell adherence to the cotyledon and the proportion of damaged beans in a sample could influence the activity of the mold and the compounds they produce.

Interestingly, the smallest increase in monocarbonyls occurred in Sanchez beans, a variety which usually does not undergo a fermentation. That the shell can function as a protective barrier against mold attack is suggested from the data showing greater increases in monocarbonyl values for Bahia A and Accra A in comparison to Bahia B and Accra B. In the "A" samples visual inspection indicated that the shells were not as firmly bound to the cotyledons as was the case with the "B" samples.

Table 2—Concentration of monocarbonyl classes in raw moldy and non-moldy cocoa beans.

Samples	Micromoles per 30 g of beans			
	Methyl ketones	Saturated aldehydes	2-enal	2,4-dienal
Moldy Bahia A	9.44	1.77	.138	.388
Non-moldy Bahia A	2.15	.70	.115	.001
Moldy Bahia B	10.73	.72	.360	.290
Non-moldy Bahia B	5.55	.21	.061	.028
Moldy Accra A	9.82	.02	.450	.125
Non-moldy Accra A	1.55	.17	.110	.020
Moldy Accra B	9.65	.83	.450	.265
Non-moldy Accra B	6.43	1.30	.180	.400

Quantitative data from the class separation of the monocarbonyls into methyl ketone, saturated aldehyde, 2-enal and 2,4-dienal portions are recorded in Table 2. Quantitatively, the most pronounced change among the monocarbonyls occurred in the methyl ketone fraction. In every experiment the development of mold on cocoa beans was accompanied by an increase in methyl ketone concentration. For the four pairs of samples reported in Table 2, the increases were 3.2, 5.2, 7.2 and 8.3 μM per 30 g of beans. The greatest quantitative change in methyl ketone concentration occurred in Bahia A and Accra A which, as mentioned previously, appeared to have shells that were not as firmly attached as in the "B" beans.

Methyl ketone synthesis probably occurred via the same three enzymatic mechanisms involved in methyl ketone formation in mold ripened cheese as described by Hawke, et al. (1966): (a) liberation of free fatty acids from the triglycerides of fat by lipase; (b) oxidation of the free fatty acids to β-keto acids; and, (c) decarboxylation of β-keto acids to methyl ketones. Both cocoa beans and Roquefort cheese have high concentrations of fat which is an excellent source of energy for the mold.

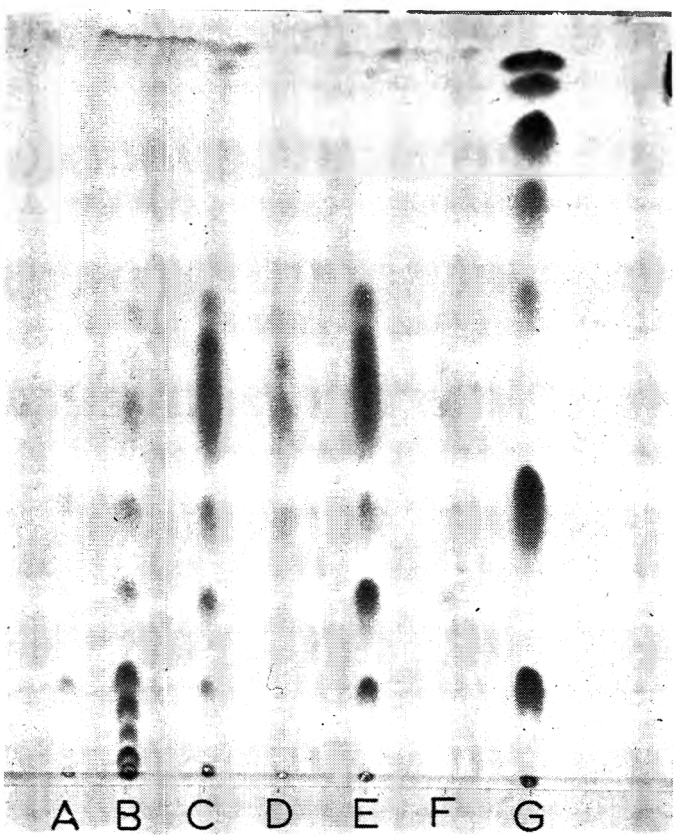

Fig. 1—Thin-layer chromatoplate of 2,4-dinitrophenylhydrazone methyl ketones from moldy and non-moldy cocoa beans. (A) non-moldy Bahia A, (B) moldy Bahia A, (C) moldy Bahia B, (D) non-moldy Bahia B, (E) moldy Accra, (F) non-moldy Accra, (G) C_3, C_5, C_7, C_9, C_{11}, C_{13}, C_{15} methyl ketones.

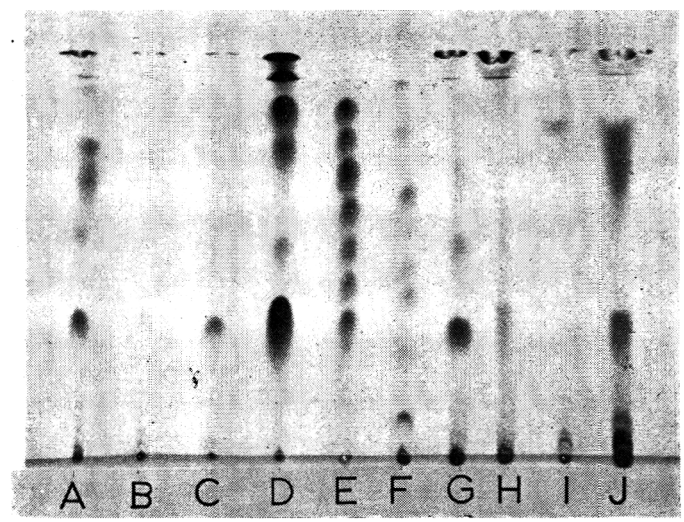

Fig. 2—Thin-layer chromatoplate of 2,4-dinitrophenylhydrazones of saturated aldehydes, 2-enals and 2,4-dienals from moldy and non-moldy Bahia cocoa beans. Non-moldy: (A) saturated aldehydes, (B) 2-enals, (C) 2,4-dienals. Standards: (D) saturated aldehydes C_3, $_5$, $_7$, $_9$, $_{11}$, $_{16}$, (E) 2-enals C_5-C_{11}, (F) 2,4-dienals C_5, $_7$, $_9$, $_{10}$, $_{11}$, $_{12}$, $_{14}$, $_{16}$. Moldy beans: (G) saturated aldehydes, (H) 2-enals, (I and J) 2,4-dienals. The first portion of the dienal fraction eluted from the class separation column is represented by (I). Very little DNP hydrazone was found in (I) and is evidence that a good separation of the enal and dienal fractions was obtained.

The origin of the methyl ketones found in cocoa beans which did not show visible evidence of mold growth cannot be readily explained. The use of the term non-moldy is perhaps misleading since some mold growth inevitably occurs during fermentation of cocoa beans. While slight mold infection may not be visually detectable in beans as received by the chocolate manufacturer, there may have been enough activity to account for the methyl ketones chemically detected in "non-moldly" cocoa beans.

The effect of mold infection on the concentration of saturated aldehydes in cocoa beans was found to be less predictable than was the case for methyl ketones. For the samples reported in Table 2, mold activity resulted in an increase in concentration of saturated aldehydes in the Bahia beans whereas a decrease was observed for the two Accra samples. Other data collected during the investigation but not reported herein indicate that saturated aldehyde concentration is more likely to decrease when cocoa beans become moldy.

The unsaturated aldehyde concentration almost always increased when mold was allowed to develop on cocoa beans. Since the 2-enals and 2,4-dienals are potent aroma emitting compounds, even a small increase in their concentration could have a significant effect on flavor.

The increase in 2-enals and 2,4-dienals most likely involved the autoxidation of unsaturated fatty acids. Cocoa fat contains 1.2–2.8% linoleic acid and 0.1–1.0% linolenic acid and upon oxidation will yield 2-enals, 2,4-dienals and other degradation products. As reported by Dillard, et al. (1961), lipoxidase has great catalytic activity for the peroxidation of unsaturated fatty acids.

Figure 1 is a reproduction of a typical thin-layer chromatogram of methyl ketone fractions from equal amounts of moldy and non-moldy cocoa beans. Most of the ketones present in moldy cocoa beans were also found in non-moldy beans but in lower concentrations. In two of the three samples shown in Figure 1 (C and E), ketones with retention times near pentan-2-one and heptan-2-one were present in relatively large amounts. This pattern was most typical of the numerous samples analyzed in the investigations. However, exceptions were encountered (Sample B, Figure 1). This sample was dominated by methyl ketones not present in other samples.

The moldy sample Accra (E) was used as source material for the identification of individual methyl ketones. Identification was confirmed by co-chromatographing on $AgNO_3$ or KOH treated Micro Cel T-38 after recovering purified ketones separated on preparative plates. Positive identification by these procedures was obtained for the following methyl ketones; acetone, butanone, hexanone and heptadecanone. The two spots just above hexanone on the TLC plates were most likely branched chain ketones.

Figure 2 shows a typical TLC separation of the saturated aldehyde, 2-enal and 2,4-dienal fractions. As previously discussed, spectrophotometric measurements revealed that, invariably, the concentration of saturated aldehydes in moldy beans was less than in normal bean samples. This is readily apparent by visual comparison of the saturated aldehyde separation of control (A) and moldy (G) beans in Figure 2. In the moldy bean sample only three saturated aldehydes were present in sufficient concentrations to be revealed by TLC. Two of the compounds had Rf values corresponding to acetaldehyde and pentanal. The non-moldy control also showed the presence of the above mentioned aldehydes and in addition contained several others up to C_{16} with the greatest concentration being in the region corresponding to pentanal and heptanal. It is to be remembered that the beans examined in this investigation had not been roasted, a treatment which leads to the formation of significant amounts of aldehyde via the Strecker degradation of amino acids (Bailey, et al., 1962). Boyd, et al. (1965) in a study of roasted beans found eight to ten saturated aldehydes with the greatest concentration being in the butanal to hexanal region on TLC plates.

In Figure 2 the control bean sample is shown as having a much lower concentration of 2-enals than the moldy beans. The 2-enal present in greatest concentration was 2-pentenal which could originate from the autoxidation of linolenic acid. Faint sopts corresponding to 2-hexenal and 2-heptenal were also evident and a considerable amount of material migrated to the solvent front indicating the presence of 2-enals greater than undecenal. Difficulty was encountered with streaking of the 2-enal fraction on the plate and this precluded a more extensive identification of these aldehydes.

As was the case with the 2-enal fractions, moldy beans almost always contained a greater concentration of 2,4-dienals than did the control beans. The only spot appearing in the 2,4-dienal fraction of non-moldy beans corresponded to 2,4-octadienal which was tentatively identified by Boyd, et al. (1965). Moldy beans also contained this 2,4-dienal and, in addition, several other compounds were present in appreciable concentration. Using preparative plates and co-chromatographing with the standard, 2,4-pentadienal was identified. This was the only 2,4-dienal present in sufficient concentration to make possible this more rigorous identification. Other 2,4-dienals were detected and were concentrated on the thin-layer plate between the position occupied by C_{12} and C_{14} 2,4-dienal standards.

While the methyl ketone fractions of most moldy cocoa bean samples separated by TLC were qualitatively very similar, such was not the case with the 2-enal and 2,4-dienal fractions. Considerable variation was observed in respect to the compounds present and their relative concentrations. The bean sample used for illustrative purposes in Figure 2 shows more 2,4-dienal than 2-enal. However, in other samples the 2-enals were present in greatest concentration and in others the two unsaturated aldehyde fractions were almost identical with respect to amounts present. In one sample, 2,4-pentadienal was the only dienal detected. The reasons for the considerable variation in the unsaturated aldehyde fraction are unknown.

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Anthocyanin Pigments in Tinto Cão Grapes

SUMMARY—The anthocyanin pigments present in the skins of Tinto cão grapes were extracted with 0.1% HCl in methanol. The pigments were purified by Dowex 50 W-X4 cation exchange resin, and separated into individual pigments by two-dimensional paper chromatography with n-butanol–acetic acid–water (4:1:5, v/v) and acetic acid–water–HCl (15:82:3, v/v) as solvent systems. Partial acid hydrolysis revealed the number of sugar molecules in each pigment. Acyl components and sugar moieties were identified through acid hydrolysis and spectral measurement.

The R_f measurement of the pigments and their hydrolysis products, together with the alkaline degradation of the aglycone, confirmed the chemical structures of the anthocyanins as malvidin 3-monoglucoside, peonidin 3-monoglucoside, cyanidin 3-monoglucoside, petunidin 3-monoglucoside, petunidin 3-monoglucoside acylated with caffeic acid, malvidin 3-monoglucoside acylated with caffeic acid, malvidin 3-monoglucoside acylated with p-coumaric acid, peonidin 3-monoglucoside acylated with p-coumaric acid, and cyanidin 3-monoglucoside acylated with caffeic acid.

Malvidin and peonidin were not present in the original sample. They were formed during the extraction and purification procedures. Malvidin 3-monoglucoside and malvidin 3-monoglucoside acylated with p-coumaric acid were the dominant anthocyanins present in Tinto-cao grapes.

INTRODUCTION

THE ANTHOCYANIN pigments contribute to the visual appeal of many fruits, juices, jellies and wines. Some grape varieties are richer in anthocyanins than others, and are used for amelioration of color in wines and grape concentrates.

30 *Vitis vinifera* varieties of grapes were examined by Ribèreau-Gayon et al. (1955). They concluded that the pigments were principally monoglucosides with only traces of diglucosides. Ribèreau-Gayon et al. (1958) showed that diglucosides were completely absent in *V. vinifera* varieties. Rankine et al. (1958) surveyed anthocyanins in 42 *V. vinifera* varieties and found the same pigment as presenting the major amount in all the varieties with some variation in concentration of other pigments.

An additional 125 varieties of *V. vinifera* grapes were examined by Albach et al. (1959). They reported 8 pigments, and malvidin 3-monoglucoside was present in greatest amount. Other *V. vinifera* varieties such as Flame Tokay, Emperor, Red Malaga and Cabernet-Sauvignon have been studied by Akiyoshi et al. (1963) and Somaatmadja et al. (1963); six anthocyanins were found in these varieties as monoglucosides. Their results are in agreement with the conclusion given by Ribereau-Gayon et al. (1955; 1958). Reuther (1961) reported that diglucosides were characteristic of *V. riparia* and *V. rupestris*.

In these species, diglucosides served as a dominant genetic marker, while monoglucoside was a genetic marker in *V. vinifera* varieties. Albach et al. (1963) reported that both caffeic and *p*-coumaric acids were present as acyl components of anthocyanidin monoglucosides. Chlorogenic acid was found by Somaatmadja et al. (1963) to be an acyl component of anthocyanin in Cabernet-Sauvignon grapes. Smith et al. (1965) investigated the anthocyanin pigments in the hybrid grape variety Rubired. Chen et al. (1967) investigated anthocyanins in Royalty grape, a hybrid from crossing Alicante Ganzin and Trousseau varieties.

The Tinto cão variety, which was originated in Portugal, has been chosen as a breeding stock because of its vigor and quality characteristics for port wine. The chemistry of the anthocyanins in this variety is not known.

This work describes the identification of anthocyanin pigments in Tinto cão grapes.

EXPERIMENTAL

Grapes

Ripe Tinto cão grapes were supplied by Professor H. P. Olmo and A. Koyama of the Viticulture and Enology Department of the University of California. They were harvested from 10-year-old vines grown in the University Vineyard at Davis. The berries were stored at 0°C for 48 hr, washed twice with tap water, stemmed, sealed in plastic bags and then deep frozen at −18°C. Analysis of the mature Tinto cão grapes revealed the following characteristics: weight per 100 berries, 157.1 g; Brix at 20°C, 19.8; pH, 4.05; total acidity, 0.89% as tartaric acid.

Extraction and purification of pigments

500 g of grapes were thawed. The skin portion was macerated in a Waring blendor with 200 ml of 0.1% (v/v) conc. HCl in absolute methanol for 5 min under a nitrogen atmosphere. The macerate was filtered in a Buchner funnel through Whatman No. 1 paper. The residue was extracted four times with the same solvent. A sufficient amount of Dowex 50W-X4 cation exchange resin in the hydrogen form was added to the combined extract with occasional stirring. Shortly after the adsorption, the supernatant was decanted, and the resin was washed several times with distilled water to remove free sugars, and then with absolute methanol to remove organic compounds other than anthocyanins.

The pigments were eluted from the resin with a total of 6 l of acidified methanol. The concentration of HCl in the methanol was increased gradually from 0.1 to 1% (v/v).

The combined eluate was concentrated in a flash evaporator under vacuum to almost dryness and then redissolved in a minimum amount of 0.1% HCl in methanol. The crude extract was stored at 0°F in the dark under a nitrogen atmosphere.

Two-dimensional paper chromatography

The two-dimensional paper chromatographic technique was used for separation of the anthocyanins. 200 Whatman No. 3 MM papers (67 × 46.5cm) were used to separate the pigments. The paper was developed with BAW solvent in the short direction and AWH in the long one. The like spots were cut from the almost-dried paper and eluted several times in a beaker with the same solvent used in extraction. The eluate was concentrated to a small volume in a flash evaporator and stored under nitrogen in the cold room until needed for identification.

Some of the pigments did not separate satisfactorily, especially the acylated ones. So some of the pigments from the two dimensional chromatograms were separated on the paper again with BAW solvent to get sufficient purified sample for spectral measurements and other studies.

The solvent systems used for separation and identification of the pigments are listed in Table 1.

For separation of anthocyanins, freshly prepared *n*-butanol-acetic acid-water (BAW, 4:1:5 v/v) was used. For R_f measurement, the solvent mixture was allowed to stand for 3 days after shaking vigorously.

Chromogenic reagents

The chromogenic reagent for the sugar spots on the paper chromatogram was an

Table 1—Solvent systems for paper chromatography of anthocyanins, sugars and phenolic acids.

Abbreviation	Composition	Ratio (v/v)	Layer used	Compound detected	Time, hr
BAW	*n*-butanol:HOAc:H_2O	4:1:5	Upper	Anthocyanin	17
				Aglycone	17
				Sugar	17(R_f)
				Acid	16
AWH	HOAc:H_2O:conc. HCl	15:82:3	Miscible	Anthocyanin	10
				Aglycone	10
1% HCl	conc. HCl:H_2O	3:97	Miscible	Anthocyanin	4
Forestal	HOAc:H_2O:conc. HCl	30:10:3	Miscible	Aglycone	15
Formic	Formic acid:conc. HCl:H_2O	5:2:3	Miscible	Aglycone	6
Et-HOAc-W	Ethylacetate:HOAc:H_2O	3:1:3	Miscible	Sugar	15(R_g)
Bu-Py-W	*n*-butanol:pyridine:H_2O	Upper	Sugar	Sugar	36(T_g)
2% HOAc	HOAc:H_2O	2:98	Miscible	Acid	3

R_f = Distance of substance from origin/distance of solvent front from origin.
R_g = Distance of substance from origin/distance of glucose from origin.

Table 2—Color characteristics and aluminum chloride reaction of anthocyanins from Tinto cão grapes.

Pigment	Visible color	Color under UV radiation	Color change with $AlCl_3$
1	Magenta	Dull mauve	–
2	Orange pink	Dull magenta	–
3	Faint magenta	Purplish pink	+
4	Faint magenta	Light purple	+
5	Faint magenta	Purple	+
6	Orange red	Dull red	–
7	Magenta	Dull mauve	–
8	Orange pink	Dull mauve	–
9	Faint magenta	Purplish pink	+
10	Magenta	Dull mauve	–
11	Orange pink	Pink	–

alcoholic solution of aniline hydrogen phthalate (Partridge, 1948, 1949). DPNA (diazotized p-nitroaniline) was used as the chromogenic reagent for the phenolic compounds as described by Swain (1953). The reagent was freshly prepared in an ice bath by mixing 0.5% (v/v) *p*-nitroaniline in 2 *N* HCl, 5% sodium nitrite, and 20% sodium acetate in a ratio of 1:10:30.

Absorption spectra

The absorption spectra of the pigments in methanol, both in the visible and ultraviolet region, were recorded with a Beckman DB recording spectrophotometer, using absolute methanol as a blank. It is important to avoid too much acid in the sample (Geissman, 1955). Spectral shift after adding 3 drops of 5% $AlCl_3$ in ethanol was also measured shortly after adding the reagent.

Partial acid hydrolysis

Partial acid hydrolysis of individual anthocyanins was done with 1 *N* HCl after the method of Abe et al. (1956). The detail of this method was described by Chen et al. (1967).

Identification of the acyl components

When the partial acid hydrolysis was completed, the remaining mixture in the flask was cooled and extracted several times with anhydrous ether. The ether extract was spotted on Whatman No. 1 papers, along with authentic samples of caffeic, *p*-coumaric, ferulic and chlorogenic acids. The papers were chromatographed in a descending direction with BAW for 16 hr and with 2% acetic acid for 3 hr. If the sample used for hydrolysis contained two or more pigments, two-dimensional chromatography of the ether extract was applied, using BAW as the first solvent and 2% acetic acid as the second. The dried chromatogram was examined under long wave ultraviolet radiation before and after exposure to ammonia as described by Swain (1953). The DPNA chromogenic reagent was used to identify the individual phenolic acids. (Lynn et al., 1964).

Sugar moieties of anthocyanins

Sufficient Dowex 50W-X4 cation exchange resin in the hydrogen form and Dowex 1-X8 anion exchange resin in the acetate form were added to the hydrolysate after extraction with ether. These resins remove the aglycone and HCl. The clear solution was spotted on Whatman No. 1 papers along with authentic samples of glucose, arabinose, galactose, xylose and rhamnose. The chromatograms were developed with BAW for 17 and 36 hr respectively, Bu-Py-W for 36 hr and Et-HOAC-W for 15 hr. The sugar spots were visualized by spraying with aniline-hydrogen phthalate reagent and heating at 105°C for 5 min (Partridge, 1948, 1949). The R_f and R_g values of the sugar moiety were compared with those of known sugars (Luh et al., 1965; Chen et al., 1967).

Identification of anthocyanidins

The resin containing the anthocyanidin was washed with distilled water, eluted with methanol containing 3% conc. HCl (v/v), and then concentrated in a flash evaporator. The anthocyanidin was chromatographed on Whatman No. 1 papers with BAW for 17 hr, Forestal solvent for 15 hr, and Formic solvent for 6 hr, respectively.

Alkaline degradation of anthocyanidins

The barium hydroxide degradation method described by Hsia et al. (1965) was used to study the chemical structures of the anthocyanidins.

Photodensitometric measurement of pigment

Photodensitometric measurements of the pigments on Whatman 3 MM paper strips cut from a two dimensional chromatogram were recorded with a Photovolt Densitometer Model 525 (Photovolt Corporation, 111 Broadway, New York 10, N.Y.). A green filter was used. The areas under the peaks were measured with a planimeter.

RESULTS

Two-dimensional chromatography

Figure 1 is a two-dimensional paper chromatogram of anthocyanin pigments in Tinto cão grapes. Spots outlined in solid line were the major pigments while those in dotted lines were present in small amounts. Pigments 10 and 11 did not appear on all the chromatograms; they appeared only when a higher concentration of acid was used in the extraction. Thus pigments 10 and 11 might be degradation products of acylated pigments. According to Dodds et al. (1955) the bonds between acyl groups and sugar moieties are labile. They were readily hydrolyzed to yield deacylated pigment.

Pigments 3 and 4 were present only in small amounts. 200 sheets of chromatographic papers were used to get enough amounts for identification. Pigments 5, 6 and 9 were also minor pigments. It was extremely difficult to get a sufficient quantity in purified form for chemical identification. They were easily contaminated with pigments 7 and 8. On the two-dimensional paper chromatogram, pigments 1 and 2, 7 and 8, 10 and 11 were adjacent, thus the second separation with BAW solvent system was needed in order to get the purified sample for chemical identification.

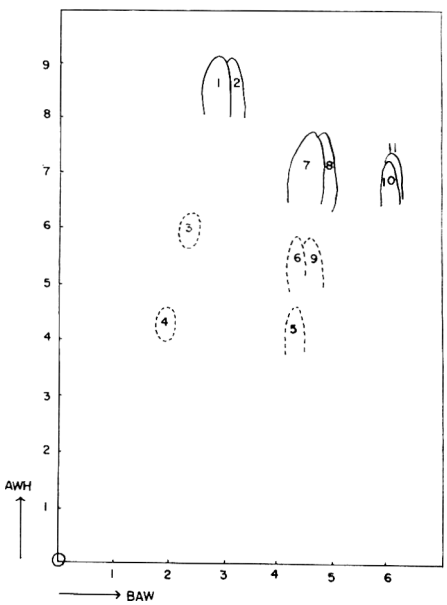

Fig. 1—Two-dimensional chromatogram of anthocyanins from Tinto cão grapes.

Table 3—R_f values of anthocyanin pigments from Tinto cão grapes.

Pigment	Identification	R_f values of anthocyanins at 20°C BAW	1% HCl	AWH
1	Malvidin 3G	0.40	0.06	0.33
2	Peonidin 3G	0.41	0.09	0.33
3	Cyanidin 3G	0.40	0.07	0.26
4	Petunidin 3G	0.37	0.04	0.22
5	Petunidin 3G —caffeic acid	0.60	—	0.21
6	Malvidin 3G —caffeic acid	0.63	—	0.24
7	Malvidin 3G —*p*-coumaric acid	0.65	0.03	0.30
8	Peonidin 3G —*p*-coumaric acid	0.66	0.04	0.30
9	Cyanidin 3G —caffeic acid	0.65	—	0.24
10	Malvidin	0.72		
11	Peonidin	0.73		
Reported (Harborne, 1958a)				
Malvidin 3G		0.38	0.06	0.29
Peonidin 3G		0.41	0.09	0.33
Cyanidin 3G		0.38	0.04	0.26
Petunidin 3G		0.35	0.04	0.22

Aluminum chloride reaction and properties of anthocyanins

Table 2 lists the visible color of the individual pigments, their color under ultraviolet radiation, and their ability to change to a blue color when sprayed with $AlCl_3$ reagent. Some of the anthocyanins show color shades different from others. It is important to differentiate them as a clue to the type of aglycone in the pigments. Examination under ultraviolet radiation was also of value. The ability of some anthocyanins to fluoresce provide a means to their identification.

It appears that there was no C_5-glycosidated anthocyanins in Tinto cão grapes. Presence of sugar moiety at the 5-position of the ring A, would show intense fluorescence under ultraviolet radiation. None of the pigments in this variety shows such a phenomenon. Pigments 3, 4, 5 and 9 turn blue when sprayed with $AlCl_3$ reagent. These pigments belong to the group of anthocyanins having two hydroxyl groups at the ortho position such as cyanindin, delphinidin and petunidin (Harborne, 1958a). The other pigments did not show a positive color change. They may have pelargonidin, peonidin, or malvidin as aglycones.

Table 3 lists the R_f values of the anthocyanin pigments from Tinto cão grapes in three solvent systems: BAW, 1% HCl, and AWH, together with those reported by Harborne (1958a).

Photodensitometric measurement

Table 4 lists the relative amounts of individual anthocyanin pigments in Tinto cão grapes. Pigments 1 (malvidin 3 G) and 7 (malvidin 3 G acylated with *p*-coumaric acid) were present in larger amounts than others.

Table 4—Relative density of anthocyanin pigments in Tinto cão grapes.

Pigment	Identification	Percentage
1	Malvidin 3G	52.95
2	Peonidin 3G	0.31
3	Cyanidin 3G	0.96
4	Petunidin 3G	0.47
5	Petunidin 3G— caffeic acid	
6	Malvidin 3G— caffeic acid	
7	Malvidin 3G— *p*-coumaric acid	43.19
8	Peonidin 3G— *p*-coumaric acid	0.42
9	Cyanidin 3G— caffeic acid	
5 + 6 + 9	Petunidin 3G— caffeic acid, Malvidin 3G— caffeic acid, Cyanidin 3G— caffeic acid	1.70
Total		100.00

Absorption spectra of the anthocyanin pigments

The absorption spectra of the individual pigments were measured in absolute methanol. The shift in absorption peaks after adding $AlCl_3$ was also studied. Table 5 lists the absorption maxima of the individual anthocyanins in the visible and ultraviolet regions. The ratio of the optical density at 440 mμ to that at the maximum were also reported. Harborne (1958b) reported that O.D. 440 mμ/O.D. max of 5-substituted anthocyanin was approximately half that of the corresponding anthocyanin in which the 5-hydroxyl group is free. The ratio provides a good means of distinguishing between 3- and 3,5-diglucosides.

The presence or absence of acyl component could also be read from the ratio of the optical density at the absorption peak in the ultraviolet region to that in the visible region. The anthocyanins acylated with *p*-coumaric show two peaks in the ultraviolet region at 289 and 310 mμ, those acylated with caffeic acid at 330 mμ. From the absorption maxima together with the $O.D._{(440\ m\mu)}/O.D._{max.}$ ratios, pigment 1 was identified as malvidin 3-monoglucoside, pigment 2 as peonidin 3-monoglucoside, pigment 3 as cyanidin 3-monoglucoside and pigment 4 as petunidin 3-monoglucoside. The absorption peak of pigment 3 shifted from 530 to 564 mμ and that of pigment 4 from 538 to 562 mμ when $AlCl_3$ was added.

From the absorption maxima and ratios of O.D. 440/mμ/O.D. max pigment 7 was identified as malvidin 3-monoglucoside and pigment 8 as peonidin 3-monoglucoside. The ratio max $_{(uv)}$/max. (visible) seemed to differ somewhat from those reported in the literature. The absorption maxima at 305 mμ correspond to those for *p*-coumaric acid. Thus pigments 7 and 8 were acylated with *p*-coumaric acid.

Partial acid hydrolysis

The partial acid hydrolysis technique was applied to determine the number of sugar molecules in the individual pigments. Sugar residues are generally attached to hydroxyl groups at the 3-position or at the 3- and 5-positions of the A ring. Those pigments with the 5-OH in ring A linked with sugar exhibit fluorescent characteristics under ultraviolet radiation. A 3-monoglycoside differs from 3,5-diglycoside in the number of intermediate products on partial acid hydrolysis.

Partial acid dehydrolysis was done only

Table 5—Absorption maxima of anthocyanin pigments in methanol from Tinto cão grapes.

Pigment	Identification	λmax (mμ)	O.D. λ440 mμ / O.D. λmax	O.D. max (uv) / O.D. λmax	$AlCl_3$ Shift
1	Malvidin 3G	535	18.4%	—	0
2	Peonidin 3G	520	28.1	—	0
3	Cyanidin 3G	530	21.8	—	+34mμ
4	Petunidin 3G	538	20.2	—	+24mμ
7	Malvidin 3G— *p*-coumaric acid	280, 305, 534	16.2	83%	0
8	Peonidin 3G— *p*-coumaric acid	280, 303, 524	27.7	—	0
Reported (Harborne 1958b)					
Malvidin 3G	—	535	18	—	0
Malvidin 3,5G	—	535	12	—	0
Peonidin 3G	—	523	26	—	0
Peonidin 3,5G	—	523	13	—	0
Cyanidin 3G	—	525	22	—	+18mμ
Cyanidin 3,5G	—	522	13	—	
Petunidin 3G	—	535	18	—	+24mμ
Petunidin 3,5G	—	533	10	—	
Malvidin 3G— *p*-coumaric acid	—	282, 308, 535	—	71%	0

Table 6—R_f and color characteristics of the acyl component of anthocyanin pigments from Tinto cão grapes.

Pigment	R_f		Color				Identification
	BAW	2% HOAc	Under UV	UV + NH_3	with DPNA	DPNA + NH_3	
1	—	—	—	—	—	—	No acid
2	—	—	—	—	—	—	No acid
3	—	—	—	—	—	—	No acid
4	—	—	—	—	—	—	No acid
5, 6, 9	0.80	0.31	Blue F	Blue F[1]	Tan	Brown	Caffeic acid
7, 8	0.85	0.41	—	Blue F	Light orange	Purple	*p*-coumaric acid
10, 11	—	—	—	—	—	—	No acid
Total extract	0.81	0.34	Blue F	Blue F	Tan	Tan	Caffeic acid
	0.87	0.44	—	Blue F	Light orange	Purple	*p*-coumaric acid
Reported							
p-coumaric acid	0.86	0.44	—	Blue F	Light orange	Purple	
Caffeic acid	0.80	0.33	Blue F	Blue F	Tan	Brown	
Ferulic acid	0.82	0.39	Blue F	Blue F	Red	Purplish blue	
Chlorogenic acid	0.58	0.62	Blue F	Blue F	Tan	Tan	

[1] Fluorescence.

on pigments 1, 2, 3, 4, 7 and 8. They were isolated in sufficient quantities for acid hydrolysis work.

Pigments 1, 2 and 4 were monoglycosides. They gave rise to only one hydrolysis product, namely the aglycone after the sugar residue was removed. Since the original pigments did not fluoresce under UV radiation, it was reasonable to conclude that pigments 1, 2 and 4 were 3-monoglycosides.

Pigments 7 and 8 did not fluoresce under UV radiation. From their R_f values, it appears that they could not be 3,5-diglycosides. Besides the aglycone, there were 2 spots going together. According to Harborne (1958a), this was the most characteristic feature of acylated pigment, in that the deacylated pigments had a higher R_f value in aqueous solvents than the original acylated ones. Attempts had been made to spray the paper with DPNA to look for the acyl component. With the acid, which would be identified later, pigments 7 and 8 were acylated derivatives of 3-monoglycosides. The fact that the deacylated pigment came out earlier than the aglycone showed that the acyl linkage to the anthocyanin is more labile than the sugar linkage.

Pigment 3 did not fluoresce. It could neither be an acylated pigment nor a diglycoside. The aglycone of pigment 3 turned blue when sprayed with the $AlCl_3$ reagent. Spraying with DPNA was also tried, but it failed to show any acid. So it appears that pigment 3 was a non-acylated 3-monoglycoside.

Sugar moiety in the anthocyanin pigments

The R_f and R_g values of sugar moieties of the individual pigments were determined in three solvent systems. Glucose was identified as the sugar moiety of pigments 1 to 9 by cochromatography with an authentic sample. Arabinose was also found on almost every chromatogram. Harborne et al. (1957) reported that arabinose was an artifact resulting from the action on paper of mineral acid present in the solvent system. This explains the presence of arabinose on every chromatogram whenever the pigment was purified by paper chromatography.

To confirm this point, acid hydrolysis of total pigment extract which had not been chromatographed on paper was carried out. No arabinose was found in the hydrolysate. It was concluded that glucose was the only sugar present in the anthocyanins of Tinto cão grapes. The results are in agreement with the observations made on Royalty grapes (Chen et al., 1967).

In the case of acid hydrolysates of pigments 10 and 11, there was no sugar appearing on the chromatogram. This confirmed the observation in a previous section that they were degradation products of pigments 1 and 2, or pigments 7 and 8.

Table 7—R_f values of anthocyidins from Tinto cão grape anthocyanins.

Pigment	R_f			Identification
	BAW	Forestal	Formic	
1	0.58	0.59	0.27	Malvidin
2	0.69	0.63	0.29	Peonidin
3	0.68	0.50	0.21	Cyanidin
4	0.55	0.48	0.20	Petunidin
7	0.58	0.60	0.27	Malvidin
8	0.69	0.63	0.30	Peonidin
Reported				
Malvidin	0.58	0.60	0.27	
Peonidin	0.71	0.63	0.30	
Cyanidin	0.68	0.49	0.22	
Petunidin	0.52	0.46	0.20	

Acyl components of anthocyanins

Diazotized *p*-nitroaniline (DPNA) is an excellent spray reagent for identifying phenolic acids. The R_f values and color characteristics of the acyl groups in the anthocyanins are shown in Table 6. There were no acyl groups in pigments 1, 2, 3 and 4. *p*-Coumaric acid was the acyl component of pigments 7 and 8, and caffeic acid was that of pigments 5, 6 and 9. Since there was no acyl group and sugar in samples 10 and 11, it is reasonable to conclude that they were aglycones derived from pigments 1, 2, 7 and 8.

Identification of anthocyanidins

Anthocyanidins are less stable than anthocyanins at higher pH values. It is advisable to wash the chromatographic paper first with diluted hydrochloric acid if the chromatograms are to be developed with the BAW solvent. The other two solvent systems gave satisfactory results. Table 7 shows the R_f values of aglycones derived from anthocyanins in Tinto cão grapes.

Alkaline degradation of anthocyanidins

The identification of the alkaline degradation products serves to confirm the chemical structure of the anthocyanin pigments. None of the degradation products shows fluorescence under UV radiation. Phloroglucinol was the only one that fluoresced after exposure to ammonia vapor. The R_f values and color characteristics of the spots and spray reagents are shown in Table 8.

The aglycone of pigments 1 and 7 was malvidin; pigments 2 and 8, peonidin; pigment 3, cyanidin; and pigment 4, petunidin. Similar results were obtained when separate samples of pigments 7 and 8 were degraded with $Ba(OH)_2$. From the R_f values of pigments 5, 6 and 9, together with those of the alkaline degradation products on a two-dimensional chromatograms, it was concluded that pigment 9 was the acylated derivative of cyanidin 3-monoglucoside, and pigment 5 was the acylated derivative of petunidin 3-monoglucoside.

Pigment 10 was shown to be malvidin; and pigment 11, peonidin. They did not appear on every chromatogram but only in samples with excessive acidity. They did not contain sugar and yielded no acyl groups on acid hydrolysis. Thus it is rea-

Table 8—R_f values and color characteristics of the alkaline degradation products of aglycones from Tinto cão grapes.

Pigment	R_f		Color				Identification
	BAW	2% HOAc	UV	UV + NH_3	DPNA	DPNA + NH_3	
1	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.85	0.54	—	—	Orange	Blue	Syringic acid
2	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.86	0.57	—	—	Yellow	Bright purple	Vanillic acid
3	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.81	0.54	—	—	Tan	Dull purple	Protocatechuic acid
4	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.78	0.50	—	—	Orange pink	Red purple	3-O-methylgallic acid
5, 6, 9	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.81	0.54	—	—	Tan	Dull purple	Protocatechuic acid
	0.78	0.50	—	—	Orange pink	Red purple	3-O-methylgallic acid
	0.85	0.54	—	—	Orange	Blue	Syringic acid
7, 8	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.85	0.54	—	—	Orange	Blue	Syringic acid
	0.86	0.57	—	—	Yellow	Bright purple	Vanillic acid
10, 11	0.69	0.62	—	Blue F	Orange	Deep orange	Phloroglucinol
	0.85	0.54	—	—	Orange	Blue	Syringic acid
	0.86	0.57	—	—	Yellow	Bright purple	Vanillic acid
Reported							
Phloroglucinol	0.70	0.62	—	Blue F	Orange	Deep orange	
Syringic acid	0.83	0.53	—	—	Orange	Blue	
Vanillic acid	0.88	0.56	—	—	Yellow	Bright purple	
Protocatechuic acid	0.80	0.54	—	—	Tan	Dull purple	
3-O-methylgallic acid	0.79	0.50	—	—	Orange pink	Red purple	

sonable to assume that pigment 10 was an artifact derived from pigments 1 and 7, and pigment 11 an artifact from pigment 2 and 8.

DISCUSSION

THE GENETIC relationship among *V. vinifera* varieties with regard to anthocyanin pigments in grapes has been discussed by Ribèreau-Gayon et al. (1958). It was concluded that *V. vinifera* varieties contain only monoglycosides of anthocyanidins. Diglycosides of anthocyanidins are present only in *V. viparia* and *V. rupestris*. From investigation of the pigments of grape skins from two hybrid seedling grapes (crosses of *V. vinifera* and *V. riparia*) Reuther (1961) concluded that the anthocyanins could be divided into the monoglucosides as a genetic marker in hybrids for *V. vinifera*, and diglycosides as genetic markers for *V. riparia*.

The present study indicates that the Tinto cão grapes contain monoglucosides of malvidin, peonidin, cyanidin and petunidin. The presence of *p*-coumaric and caffeic acyl groups in the pigments is also indicated. It appears that Tinto cão belongs to the *V. vinifera* variety.

Tinto cão is considered one of the most ancient grape varieties in the Douro port wine region of Portugal (Olmo et al., 1962). It yields high quality wine and adds great color stability to the port wine blend. The Tinto cão grapevine has high tolerance to powdery mildew and red spider. Its great vigor and minimum care requirement were utilized to produce the new variety, Rubired, by crossing with Alicante Ganzin (Olmo et al., 1962). The Rubired variety is rich in anthocyanin pigments and can be used to improve the color of grape concentrates and red wines. The chemistry of anthocyanin pigments in Alicante Ganzin grapes from which the Rubired variety was derived is yet to be investigated.

The anthocyanin pigments in Tinto cão grapes were adsorbed on a Dowex 50W-X4 cation exchange column from which the interfering impurities were effectively removed by washing with water and methanol. Purification of the individual pigments rests largely on two-dimensional paper chromatographic separation with BAW and AWH solvent systems. Through acid hydrolysis, the sugar moiety and the acyl group in the pigments were identified. By the R_f values of the pigments and their hydrolysis products in various solvent systems, together with the alkali degradation of the aglycones, the chemical structures of the anthocyanins in Tinto cão grapes were identified as Malvidin 3-monoglucoside (#1), Peonidin 3-monoglucoside (#2), Cyanidin 3-monoglucoside (#3), Petunidin 3-monoglucoside (#4), Petunidin 3-monoglucoside acylated with caffeic acid (#5), Malvidin 3-monoglucoside acylated with caffeic acid (#6), Malvidin 3-monoglucoside acylated with *p*-coumaric acid (#7), Peonidin 3-monoglucoside acylated with *p*-coumaric acid (#8), Cyanidin 3-monoglucoside acylated with caffeic acid (#9), Malvidin (#10), and Peonidin (#11).

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Respiration of Potato Mitochondria and Whole Tubers and Relation to Sugar Accumulation

SUMMARY—We have made a quantitative assessment of the changes in reducing and non-reducing sugar contents and respiration in stored White Rose potato tubers as a function of temperature of storage. The increase in sugar content with decrease in storage temperature and the decrease in sugars with increase in temperature is accounted for in only a minor way by the corresponding decrease or increase in respiratory activity of the tubers. The maximal possible contribution of respiratory activity to the changes in sugar content varied from less than 1% to 13% depending on the particular storage conditions. The temperature-dependence of respiratory activity of potato mitochondria was compared to that of whole tubers, and a quantitative comparison was made of the potential respiratory activity of the mitochondria to respiration of the whole tubers. The potential mitochondrial activity is such that respiration of whole tubers would not be limited by this factor at any temperature studied although it is recognized that the effect of mitochondrial activity could be an indirect one. Unlike whole tubers, mitochondria displayed a temperature response typical of an enzymic reaction with an apparent energy of activation of 14,000 cal/mole with succinate as substrate. The temperature-dependence of potato mitochondrial respiration is typical of that found for other plant mitochondrial systems but differs markedly from that of mitochondria of mammals and poikilotherms.

INTRODUCTION

IT IS OFTEN desirable to store potato tubers for extended periods of time with minimal losses due to sprouting, infection and loss of water. The undesirable effects can be inhibited by the use of low temperatures. However, if the potatoes are to be used for certain processed products, such as potato chips or French fries, the accumulation of sugars at the low temperatures may lead to excessive browning during processing. The build-up of sugars in potato tubers at low temperatures has been extensively documented, and the early literature has been reviewed by James (1953).

The accumulation of sugars at low temperatures is accomplished at the expense of starch. When tubers are removed from low temperature storage and placed at higher temperatures, the amount of sugar decreases. Several attempts have been made to elucidate the mechanism of the increase in sugar at low temperatures.

When temperature of storage is lowered, the respiratory rate of post-harvest plant tissue is lowered. The question arises, therefore, as to whether the increase in sugars in potato tubers at low temperatures is due to the lowering of the respiratory rate or to other factors related to the rate of production of the sugars. Tishel et al. (1966) studied this problem with halved White Rose potatoes and showed that the amount of glucose accumulated at 2°C after four days of storage was greater than could be expected from the build-up of sugars if respiration were completely eliminated by the low temperature. In this way, they showed that no more than 44% of the sugar build-up could possibly be due to change in respiration.

Since respiration is not eliminated at this temperature (2°C), the contribution of the slowing down of respiration to the increase in sugar would actually be less than this value. In a similar way, the decrease in sugars in potatoes placed at high temperatures is in excess of the CO_2 produced by respiration (James, 1953). Because of this and because there was no significant growth, the excess sugar is presumed to have reconverted to starch.

When we began this study in 1965, the work of Tishel et al. (1966) had not been published, and there was no report which dealt with the problem of the extent of involvement the decrease in respiration at low temperatures had with the increase in sugars. As was stated above, the work of Tishel et al. (1966) only set an upper limit and employed halved tubers.

We have studied the changes in sugars in White Rose potato tubers stored at various temperatures. We have compared these changes with respiration to determine the possible extent of involvement of respiration in the process of sugar accumulation. We have also examined the effect of temperature on isolated potato mitochondria to determine how the respiratory activity of the subcellular particles responsible for respiration compares to that of the whole tubers.

EXPERIMENTAL

Materials

Potatoes of the White Rose variety were purchased through a local retail outlet. Only sound tubers weighing between 180 and 250 g were used. The potatoes were held for 2 weeks at 55° to 60°F and a relative humidity of 80–90% to improve or develop new periderm (the curing process). After this preliminary period of storage, the tubers were divided at random into four lots of 40 lb each and placed at 34°, 40°, 55°, and 70°F.

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Potatoes were removed at the time intervals indicated below for respiratory studies and chemical analyses.

In general, mitochondria were prepared from tubers stored at 55°F. The only exception was when we tested to see if mitochondria from tubers stored at 34° or 40°F were damaged during the period of storage.

Sodium succinate, bovine serum albumin (BSA) Fraction V, cytochrome c, Type VI from horse heart, and hexokinase Type III from yeast were products of Sigma Chemical Co. ADP was purchased from P-L Biochemicals, Inc. and labeled phosphoric acid (P^{32}), carrier-free, was obtained from New England Nuclear Corp. All other reagents were the purest commercially obtainable.

Methods

Determination of Respiratory Activity of Tubers. Carbon dioxide production of whole tubers was determined by absorption of the gas in sodium hydroxide, precipitation of the carbonate with barium chloride, and titration of excess hydroxide with hydrochloric acid.

Sugar Analyses. Approximately 1.5 lb of potatoes were chosen at random from a given batch peeled and pulped. The sugars were extracted in boiling ethanol, the extracts filtered and the ethanol removed by heating. The aqueous extract was clarified with neutral lead acetate and the excess lead removed with sodium oxalate. After filtration, the clarified aqueous extracts were analyzed for reducing and non-reducing sugars (the latter after acid hydrolysis) by the Munson-Walker technique. All procedures were carried out according to methods described in the A.O.A.C. (1960). Throughout this report we make the assumption that the predominant non-reducing sugar is sucrose (Schwimmer et al., 1954), and we use the terms sucrose and non-reducing sugar interchangeably.

Determination of pH. The pH was measured directly on the pulped potatoes using the glass electrode of a Radiometer pH meter, Model 28.

Isolation of Mitochondria. The mitochondria were isolated by a procedure similar to that used by Verleur (1965). Potatoes previously stored at 55°F were chilled for about 2 hr at 0°–4°C. All subsequent procedures were carried out at this temperature. The central core of the potato was used for extraction of mitochondria after very thin slicing. Tissue samples of 150 g were homogenized in 300 ml of medium containing a final concentration of 0.5*M* mannitol: 0.01*M* phosphate, pH 7.2: 1 mM ethylenediaminotetraacetate (EDTA): and 0.1% bovine serum albumin (BSA). The mixture was blended in a Waring blendor at low speed for 23 sec and at full speed for 7 sec.

After homogenization, the mixture was filtered through cheesecloth: the pH of the filtrate was maintained at 7.0 to 7.2 by the dropwise addition of 5*N* NaOH solution using a Radiometer pH meter, Model 28, to monitor the system. The filtrate was centrifuged at 1000 G for 10 min and the sediment discarded. The supernatant fraction was then centrifuged at 12,100 G for 20 min. The yellow sediment was resuspended in the same medium as described above. Cysteine was added to the homogenizing medium immediately before use to a final concentration of 2 mM, and to the washing medium at a concentration of 1 mM. The suspension was recentrifuged at 12,000 G for 10 min and the final sediment resuspended in a known volume of the washing medium (usually 7.5 ml) to give the mitochondrial suspension used in the experiments.

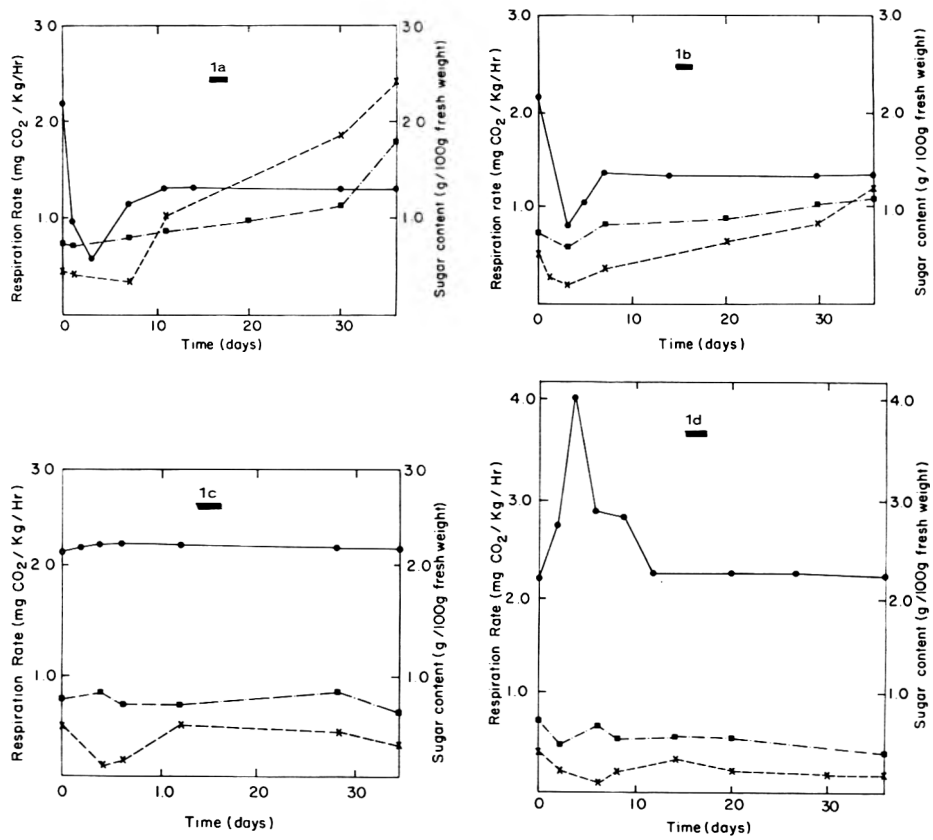

Fig. 1—Changes in reducing and non-reducing sugars and respiration with time for potatoes stored at different temperatures: 34°F (a); 40°F (b); 55°F (c); 70°F (d). Respiration, —●—●—; sucrose; · x · · · · x · ·; reducing surgars, – —■— – – —■—.

Mitochondrial Respiration

Mitochondrial respiration was measured manometrically (Umbreit et al., 1959) using succinate as substrate in a Gilson Differential Respirometer. The reaction medium contained mannitol, 0.5*M*; potassium phosphate buffer, pH 7.2, 0.01*M*; EDTA, 0.5 m*M*; cytochrome c, 6×10^{-6}*M*; succinate, 4 m*M*; ADP, 0.1 m*M*; $MgCl_2$, 10 m*M*; and bovine serum albumin (BSA), 0.1%. Generally 1 ml of the mitochondrial suspension was used per flask (total volume of 3 ml), and the readings were taken at 10 min intervals for 1 hr after equilibration and mixing of the flask contents.

Phosphorylation

Esterification of inorganic phosphate was determined by the procedure of Lindberg et al. (1960) as modified by Penniall (1966). Hexokinase and glucose were used as a trapping system for the ATP formed (Slater, 1967). A Nuclear-Chicago Model 7200 liquid scintillation counter was used for counting the radioactivity. A 0.2 ml aliquot of sample to be counted was blown into a counting vial containing 10.8 ml of scintillation solution (6 g of 2,5-diphenyloxazole (PPO) and 0.1 g of p-*bis*2-(5-phenyloxazolyl)-benzene (POPOP) in 1 L of toluene) and 4 ml of absolute ethanol (Rapkin, 1963).

Determination of Protein

Protein in the mitochondrial fractions were determined according to the procedure of Lowry et al. (1951).

RESULTS

THE SUMMATION of the changes in reducing and non-reducing sugars and respiration for each batch of potatoes stored at the different temperatures after curing at 55°F is given in Figure 1 (a–d). These data are similar to those observed by other workers, and since it was not the purpose of this work to merely repeat previous experiments, we shall not dwell on these results. One interesting point to be pointed out, however is the initial drop in respiration when the potatoes were first moved from the curing temperature (55°F) to 34° or 40°F. This initial decrease brings the respiratory activity of the tubers to a lower value than that found after equilibrium is established and probably represents a period of adjustment of the tubers to the cold temperature. James (1953) reported that downward changes of temperature showed only a drop to the new level without this temporary acceleration. The temporary

acceleration has its counterpart in an excessive increase in respiration when the tubers are moved from 55° to 70°F.

Our results with pH parallel those of other workers. The initial pH of the cured potatoes was 6.2. This rapidly decreased to 6 within 24 hr after removal of the tubers to storage at 34° or 40°F where they remained approximately constant throughout the storage period of 36 days. Tubers stored at 55°F maintained their initial pH of 6.2 while those at 70°F slowly increased in pH to 6.3.

In Figure 2 (a–c) are the changes observed in sugar contents and respiratory activities when potatoes stored at the lower temperatures were placed at 70°F. There is a general decrease in sugar concentration, the decrease being greater the lower the previous temperature of storage, viz., the higher the sugar content when placed at 70°F. The initial burst in respiration when the tubers are placed at the higher temperature is apparent as is the fact that the extent of this respiratory burst is related to the previous temperature of storage, decreasing with increasing temperature. These results confirm those reported earlier (Craft, 1963). The pH of tubers previously held at 34°F increased from 6 to 6.4 during storage at 70°F, that of tubers from 40°F storage from 6 to 6.2, and from tubers at 55°F the pH remained constant at 6.2.

Although many investigators have studied the changes in sugars and respiration of potato tubers in storage at various temperatures and there have been suggestions as to the possible relation between changes in sugars and respiration, the exact relation between the two is not at all clear. The basic question which we were attempting to answer was whether the increase in sugar content of potatoes stored at low temperatures is due to the decrease in respiration caused by lowering the temperature. And, conversely, whether the decrease in sugar content at high temperature is caused by the increase in respiration. We felt it necessary to get exact quantitative data on sugars and respiration to fully answer this question.

To determine exactly how much of the sugar lost or accumulated in the tubers could be accounted for by the respiratory changes, the following method was used. Each group of potatoes gave a particular respiratory pattern when moved to a different temperature from the 55°F at which they were cured. When these changes in respiration are plotted against time, the area between the graphs for any two storage temperatures is a measure of the difference in total respiration between the two which is a function of the temperature of storage. This is illustrated graphically in Figure 3 where respiratory patterns are plotted for three temperatures, 34°, 55°, and 70°F. The uppermost curve is that for 70°F, the center line for 55°F, and the lower line for 34°F. The checkered area represents, therefore, the difference in total respiration over the 36-day storage period between tubers stored at 34° and 55°F, the area shaded with slanted lines the difference between 55° and 70°F, and the total of both the difference between 34° and 70°F. The graph for 40°F storage was omitted for the sake of clarity, but it too could be graphically represented and the differences in respiration between any two storage temperatures determined.

Since all potatoes had initially the same sugar content, differences in levels of sugars at the end of the storage period between tubers stored at different temperatures can be compared to the differences in total respiration between the tubers. In this way it is possible to determine whether changes in respiration with temperature can account for the differences in sugar contents or at least the maximal contribution that differences in respiration could make towards differences in sugar concentrations. We assume in these calculations that 6 moles of CO_2 are equivalent to 1 mole of reducing monosaccharide.

In Table 1, the maximal percentage

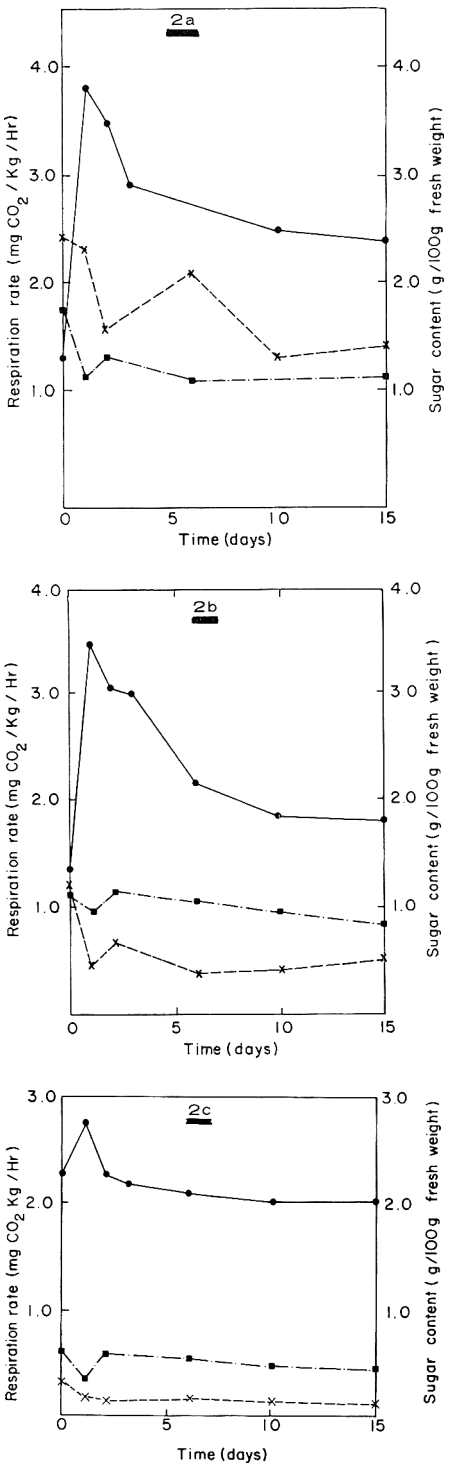

Fig. 2—Changes in reducing and non-reducing sugars and respiration when potatoes previously stored at low temperatures are moved to 70°F. Previous storage temperature: 34°F (a); 40°F (b); 55°F (c). Respiration, —●—●—; sucrose, - x - - - x - -; reducing sugars; – —■— — — ■ —

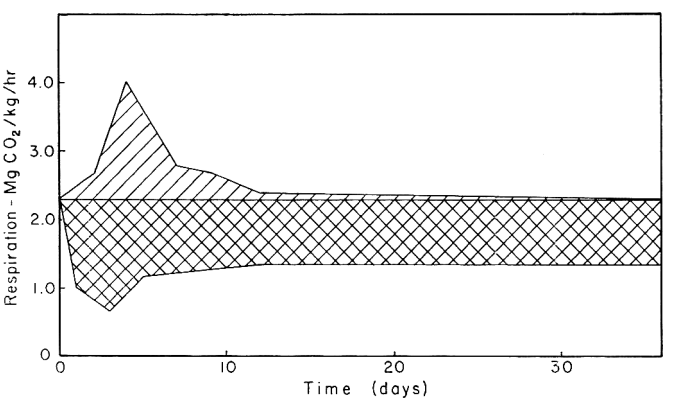

Fig. 3—Respiration with time as a function of temperature of the stored tubers. The area between any two curves represents the difference in total respiration between tubers stored at those temperatures. Top curve—70°F; middle curve—55°F; bottom curve—34°F.

Table 1—Possible maximal contributions of differences in respiration to changes in sugar contents of stored White Rose potatoes.

Differential temperatures in storage	% Maximal sugar change due to respiratory differential[1]	
	Reducing sugars	Total sugars
34–40	0.4	0.1
34–55	4.7	1.7
34–70	4.8	1.8
40–55	11.0	3.7
40–70	9.6	3.8
55–70	13.0	4.0

[1] The comparison of the increase or decrease of sugar content to decrease or increase in respiration was made on the basis of both reducing and the total of reducing and non-reducing sugars.

contribution of change in respiration to change (increase or decrease) in sugar content between any two temperatures of storage is given. In comparing a lower to higher temperature, it always represents an increase in sugar content and a decrease in total respiratory activity. Calculations based on total sugars are also included in the Table. These data indicate that under our experimental conditions, respiratory changes have a relatively minor role in determining the sugar content of potato tubers.

When the tubers were placed at 70°F after the initial storage for 36 days at the lower temperatures, they showed an increase in respiration inversely proportional to the previous storage temperature. Since sugar content at time of transfer was also inversely proportional to previous storage temperature, respiration was compared to the sugar content of the tubers at the time of transfer as shown

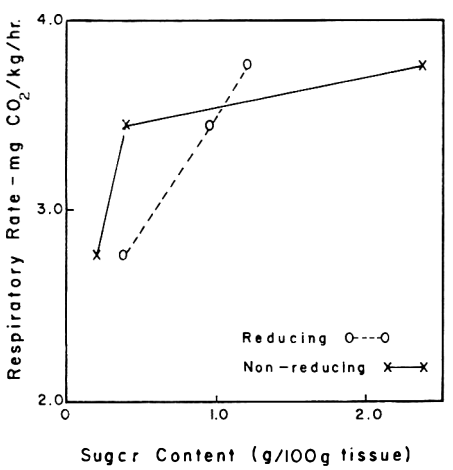

Fig. 4—Respiratory rate of potato tubers at 70°F after previous storage at low temperature vs. sugar content of the tubers at time of removal from the low temperature. The highest respiratory rate corresponds to the lowest temperature of storage (34°F), the intermediate to 40°F, and the lowest rate to the highest temperature (55°F).

Table 2—Effect of temperature on respiratory activity of tubers and mitochondria and P/O ratio of mitochondria.

Temperature (°F)	Tubers respiration (mg CO_2/kg/hr)		Mitochondria respiration	
	Steady-state[1]	Maximal[2]	µl O_2/hr/mg protein N	P/O
34	1.3	3.8	48	0.9
40	1.3	3.5	55	1.0
55	2.3	2.8	108	1.0
70	2.3	- -	208	0.9
86	—	—	520	1.0

[1] The steady-state activities were determined at the indicated temperature after a constant rate was achieved.

[2] The maximal activities were all determined at 70°F, 24 hr after being placed at that temperature from the previous storage temperature as indicated in the left-hand column.

in Figure 4. The relation between reducing sugars and respiratory activity is linear, that with sucrose is not, but respiration nevertheless increases with increasing sucrose content. However, the linear relationship between respiration and content of reducing sugars in Figure 4 may be coincidental since only three conditions were examined. Also, the rates of respiration and sugar contents are changing fast, and the data in Figure 4 represent sugar contents at the time of change of storage temperature while the respiratory rates were obtained 24 hr later.

One might with caution suggest that under certain specified conditions the amount of sugar in the tubers will influence respiratory activity. One cannot ignore the possibility that increase or decrease in respiration and the opposing decrease or increase in sugar contents are manifestations of other effects, i.e., neither is the cause of the other but both are effects of some other cause. Also, a simple direct relationship between respiration and sugar content is negated because respiration returns to normal after moving the tubers to 70°F from a lower temperature long before the sugar content does. In other words, the same level of respiratory activity was observed at two different levels of sugar depending on whether the potatoes had undergone previous storage at low temperature.

The respiratory activities and ratios of moles of phosphate esterified to atoms of oxygen consumed (P/O) were determined at several temperatures for mitochondria isolated from tubers stored at 55°F, and the results are presented in Table 2. When an Arrhenius plot of the data is made, the apparent energy of activation for this reaction with succinate as substrate is approximately 14,000 calories. The corresponding Q_{10} (°C) values were around 2 at the lower temperatures and somewhat higher (to about 2.5) at the higher temperatures studied. We observed no change in P/O ratios at temperatures from 34° to 86°F; the values were constant at about 1. Apparently there is no uncoupling effect of temperature *per se* over the range studied, and the increase in respiration at higher temperatures is in no way a result of uncoupling. Beevers et al. (1964) observed a constancy of P/O ratios with corn mitochondria over the range from 20° to 40°C (68°–104°F) although above this temperature there was a rapid drop.

In Table 2 we have also summarized the respiratory activities of the whole White Rose tubers used in our study. In the "steady-state" column are presented the values after a relatively constant level of activity has been reached at each temperature of storage. The "maximal rate" column gives the rates of respiration obtained at 70°F one day after the tubers were moved from the temperature indicated; in other words, the data in this column varied because of previous storage temperature.

The values found by us were approximately one-half of those reported by Tishel et al. (1966) for whole White Rose potatoes. This difference is possibly due in part to a different stage of maturity and what were probably significant differences in handling and prior storage. The constant steady-state values at the two lower and the two higher temperatures indicate that the rate of respiration is not controlled by a single reaction and that many factors are probably involved. It was reported by Hopkins (1924) many years ago that potato tubers exhibited a minimal rate of respiration at 37.4°F and there was an increase in respiration at 32°F.

To compare the respiration of whole tubers to mitochondrial respiration, we converted the respiratory activities of the potato mitochondria to the same basis as that of the tubers. To do this we made the assumption that 1 mole of O_2 consumed by the mitochondria was equivalent to 1 mole of CO_2 given off by the whole tubers. This is based on the assumption that O_2 uptake by the whole tubers is a measure of mitochondrial activity in the tubers and on the observed fact that CO_2 evolution and O_2 uptake of whole tubers are identical (Craft, 1963).

The activity of the mitochondria is then expressed on the basis of the weight of tissue from which they were extracted and compared to the data for whole tubers (Table 2). This comparison is shown in Table 3.

Figures in Table 3 indicate that the respiration of whole tubers should not be limited by a lack of mitochondrial activity at any of the temperatures studied. This includes even the maximal respiration seen during the "respiratory burst" of

Table 3—Quantitative comparison of respiration of whole tubers and mitochondria.

Temperature of assay (°F)	Respiration (mg CO_2 per kg per hr)	
	Whole tubers	Mitochondria[1]
34	1.3	2.5
40	1.3	2.8
55	2.3	5.5
70	2.3	10.6
70	3.8[2]	—

[1] The respiratory activity of the mitochondria is calculated on the basis of the weight of the potato from which the mitochondria were derived and utilizing succinate as oxidizable substrate. Assay conditions and methods of calculation are given in the text.

[2] This value represents the peak of the respiratory burst after potatoes which had been stored at 34°F were moved to storage at 70°F. This was the maximal respiratory rate which we observed.

cold-stored tubers moved to a warm temperature. We believe that measurement of mitochondrial respiration with succinate represents a true indication of mitochondrial potential since Van Dam (1966) has shown that reducing equivalents from DPNH compete with those from succinate in the respiratory chain. Presumably, the presentation of a variety of substrates to the mitochondria would have little effect on total respiratory activity.

Another possible problem which must be considered with respect to this quantitative assessment is that of the state of the mitochondria. Mitochondria which have been uncoupled (the oxidation of substrate is "uncoupled" from phosphate esterification) exhibit higher respiratory rates. We do not have data relating to respiratory control ratios (the ratio of mitochondrial activity in the presence of ADP to that in its absence) which is the best measure of uncoupling. But the significant P/O ratios which we did observe indicate that coupling is reasonably good and we certainly are not dealing with a completely uncoupled system. Uncoupling plant mitochondria with dinitrophenol, which produces very low P/O ratios (Childress et al., 1965), will give an increase in respiration of only 20 to 100%, (for example, see Abdul-Baki et al., 1965; Childress et al., 1965; Lance et al., 1965; Wiskich, 1966; and Wiskich et al., 1964).

If we reduce our observed mitochondrial respiratory levels on the basis that we might have this degree of uncoupling, there would still be an excess of mitochondrial activity over that which corresponds to the activity of whole tubers. In other words assuming a 100% increase in mitochondrial respiration due to uncoupling, we would divide the mitochondrial activities in the second column of Table 3 by 2 to obtain the true activity. Since our P/O ratios were approximately 1, it is unlikely that we have this degree of uncoupling. Further, no estimate has been made for the amount of mitochondria lost during preparation. We have assumed 100% retention which is obviously not correct. Adjustments for any losses would increase the yield of mitochondrial activity per kg of tissue and further support the proposition that mitochondrial potential activity does not limit whole tuber respiration.

One further point was checked. All mitochondrial samples were obtained from potatoes stored at one temperature (55°F). This was done to eliminate possible variations due to temperature of storage of the tubers on mitochondrial activity and to make sure that differences observed were solely due to differences in temperature of assay. However, in our study of respiration and sugar accumulation, it was necessary to store the tubers at constant low temperatures; hence we felt it necessary to determine whether such low temperature storage had any effect on the properties of the mitochondria. Lieberman et al. (1958) have reported that P/O ratios of sweet potato mitochondria declined with time when chilling temperatures were employed, and Lyons et al. (1964) have suggested that chilling injury in cold-sensitive plants might be caused by changes in the relatively inflexible membranes of the mitochondria of these species.

Our results with mitochondria prepared from tubers stored at 34°, 40°, or 55°F for 15 weeks showed no significant differences in respiratory activities with succinate as substrate or in P/O ratios among these conditions of temperature. Craft (1966) found similar results with potato mitochondria in that O_2 consumption in a normal medium and P/O ratios were not affected by storage of the tubers at 0°C for 3 months when compared to tubers stored at 12.8°C. He also found that mitochondrial reduction of the dye, 2,3,5,-triphenyltetrazolium chloride, with succinate as substrate was not affected by previous low temperature storage of the tubers. However, dye reduction was greater with mitochondria stored at the lower temperature with either citrate or α-ketoglutarate as substrate, and oxygen consumption by mitochondria from cold-stored tubers was inhibited to a lesser extent by hypertonic concentrations of KCl than that from tubers stored at 12.8°C. The significance of these results is not clear.

DISCUSSION

POTATO TUBERS accumulate sugars at low temperatures. This is a general characteristic of many plant tissues and is possibly related to a protective action against frost damage (Pressey et al., 1966). We studied the question of how much the slowing down of respiration could contribute to the accumulation of sugars and conclude that the decrease in respiration is not a major factor in the change in reducing (or total) sugars with decreasing temperature. Also, the increase in respiration when tubers are moved to 70°F is only a small fraction of the decrease in sugars occurring under the same conditions. Tishel et al. (1966) have shown that with halved White Rose potatoes total elimination of respiration would only account for 44% of the increase in glucose after 4 days of storage at 2°C. By measuring respiration continuously at both the high and low temperatures we have obtained exact quantitative data and have shown that under several conditions of storage temperature with whole White Rose tubers the possible maximal value is much lower, ranging from less than 1% to approximately 13% depending on the temperatures at which the comparisons are made.

Surprisingly little work has been done concerning the temperature-dependent activity of mitochondria. Newell (1966) has shown that the respiratory activity of mitochondria prepared from several poikilotherms varied in an unusual way. As the temperature increased there was a slow increase in respiratory rate ($Q = 1.3$) until a certain "break-point" was reached whence there was a very sharp increase in respiration. This was later followed at still higher temperatures by a decrease. The "break-point" temperature varied from 10° to 35°C depending on the species, and it was suggested that the "break-point" temperature corresponded to the maximal temperature that the species was likely to encounter.

In a similar way Newell et al. (1966) showed that the activity of mammalian mitochondria was roughly constant ($Q_{10} = 1.0$ to 1.1) over the range of temperature from 5° to 35°C after which a sharp increase occurred. They suggested that the lack of response at the lower temperatures was due to a physical phenomenon, probably a restriction on substrate penetration to the active sites. At the higher temperatures changes occur in the mitochondrial membranes which remove this restriction and the effect of temperature then becomes that expected for a chemical reaction. In both of these reports it made no difference whether succinate, pyruvate, or malate was used as the oxidizable substrate.

Contrary to these findings, Beevers et al. (1964) found a continuous increase of respiratory activity over the temperature range of 10° to 45°C for mitochondria prepared from corn shoots or roots. Above this temperature there was a rather rapid decline. These authors observed a Q_{10} value of about 2. Klickoff (1966) reported the mitochondrial respiratory activity of high and low altitude plants over

a range of temperatures from 20° to 40°C. He found that mitochondria of plants from higher altitudes (lower environmental temperatures) had higher oxidative rates at lower temperatures than did those of plants from lower altitudes. Although the absolute respiratory values varied with the species and the Q_{10} values differed somewhat, all samples exhibited an increase over the range of temperature studied. Q_{10} values varied from 2 to 3.

Our results are similar to these studies with plant mitochondria both as regards specific Q_{10} values and, more importantly, the general nature of the reaction in that a continuous and relatively constant change occurs over a wide temperature range. It is possible that there is a fundamental difference between plant and animal mitochondria which could be related to adaptability to temperatures and ability to carry out metabolic functions at various temperatures. These results showing a greater temperature-sensitivity of plant mitochondria might be a manifestation of lower order of control of internal environment by plants as compared to animals.

However, one must use caution in any interpretation involving plant mitochondria. The well-known difficulties of obtaining plant mitochondria without excessive inactivation is an indication of the severe effects caused by such plant constituents as organic acids and tannins. If the suggestion of Newell et al. (1966) is correct that physical barriers to substrate penetration are responsible for the low Q_{10} values of animal mitochondria at low temperatures, then destruction of these physical barriers of plant mitochondria during isolation could have produced the observed results. The problem of whether plant mitochondria have this different temperature-dependence than animal mitochondria is important and research toward solving it is greatly needed.

We conclude that it is highly probable that there is sufficient mitochondria with sufficient potential activity in potatoes so as not to limit respiration of whole tubers. The patterns of respiration as a function of temperature of tubers and mitochondria are not alike, and we must conclude that there is no simple, direct relationship between mitochondrial and whole tuber respiration. However, we must be careful not to conclude that the differential temperature response of mitochondria is not a governing force on the response of whole tubers to temperature. Relatively small changes in substrate or cofactor concentrations or in environmental conditions such as pH or cation concentrations, which could be a reflection of mitochondrial response to temperature, could have major effects on other metabolic systems which might control sugar concentrations. A mitochondrial response to temperature could thus indirectly affect whole tuber respiration.

The increase of respiratory activity with temperature which we observed with our potato mitochondria (Q_{10} of 2 to 3) is similar to that found for other plant mitochondria (Beevers et al., 1964; Klickoff, 1966). In addition, the apparent energy of activation of mitochondrial respiration with succinate (14,000 cal) is similar to the value of 12,000 cal found for fresh and aged thin disks (0.75 mm) and fresh thick disks (up to 3 mm) of potato tubers where O_2 diffusion is not rate-limiting. Also, the diffusion of O_2 is not rate-limiting in respiration of whole tubers stored in air (MacDonald, 1967). This close similarity between the response of mitochondria and cut tissue (the so-called wound respiration) to temperature deserves further study since it points out the possibility that wound respiration may be limited by potential mitochondrial activity.

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Sodium Chloride Effect on Autoxidation of the Lard Component of a Gel

SUMMARY—Stable gels composed of lard, sodium carbomethoxy cellulose and water were used for the examination of factors involved in the pro- and antioxidant activities of sodium chloride, other inorganic salts, heme compounds, meat fractions and other additives. Autoxidation processes were evaluated by peroxide and monocarbonyl determinations. The solid translucent gels, in which additives had been incorporated, were stored frozen, freeze-dried or allowed to oxidize without physical change. The hydrated gels were well aerated in preparation and oxidized in the dark at a convenient pace at 20°C. When the gel was freeze-dried, a sponge-like structure was obtained which, after an induction period, autoxidized rapidly. Freezer-stored gels autoxidized at a rate roughly similar to freezer-stored meat. Sodium chloride had a direct pro-oxidant action on the lard of freezer-stored and dehydrated gels. Hydrated gels containing NaCl when stored at 20°C had an inhibiting autoxidation pattern somewhat similar to the quantitative influence of NaCl on pH. Ethylene-diaminetetraacetate (EDTA) had a powerful antioxidant influence. Sodium chloride accelerated heme catalysis regardless of the presence of antioxidant or chelator. Interesting differences in monocarbonyl patterns and monocarbonyl/peroxide ratios as influenced by additives and moisture content of the gels were observed.

INTRODUCTION

THE MECHANISM of the pro-oxidant effect of NaCl on triglycerides in meat has not been completely elucidated. Background and earlier research efforts on this anomalous meat preservation effect (Mabrouk et al., 1960; Watts, 1962; Castell et al., 1965) have been recently reviewed (Ellis et al., 1968a). Ellis et al. (1968) probed factors involved in the pro-oxidant action on pork cuts and mixtures of backfat and lean. It was found that NaCl, either acting directly or indepedently and/or by sensitizing catalysts in the meat, seemed to have the effect of changing some of the autoxidation characteristics of the triglycerides.

The main problems appear at this time to concentrate to the following objectives: (1) Investigation of the existence of an independent oxidative effect of NaCl on fats; (2) investigation of the effect of NaCl on powerful catalysts such as heme pigments; and (3) observation of the characteristics of fat autoxidation promoted by the heme pigments in terms of hydroperoxide breakdown, specificity of fatty acid attack and monocarbonyl compound patterns.

For such a study a rapid means of fat autoxidation was required which would permit the isolation of factors responsible for glyceride fatty acid breakdown in meat. Bishov et al. (1960) used sodium carbomethoxy cellulose to considerable advantage in the preparation of stable emulsions for study of model systems under dehydrated conditions. This model system was utilized in this investigation.

EXPERIMENTAL

Materials

Sodium carbomethoxy cellulose gum (CMC-7H3SF) was donated by Hercules Powder Co.

Hemoglobin (Hb), myoglobin (Mb), cytochrome C, and tetrasodium ethylenediaminetetraacetate (EDTA) were obtained from Nutritional Biochemicals Corp.

Lard was rendered as described by Gaddis et al. (1966). Four lots were used in the experiments discussed in this paper. The number of separate experiments carried out amounted to 64.

Solid translucent gels were made up with combinations of 1:2:40 by weight of CMC, lard and water. The following sequence was used in preparing the gels: Water was placed in a Waring blendor and additives, such as NaCl or Hb, were combined with the water; the lard was added and the mixture was blended briefly. CMC was added and the components were blended thoroughly for 1 min. The resulting gel was used in the hydrated form or freeze-dried. The freeze-dried products were ground in the Waring blendor before storage. Controls were run in each experiment and experiments repeated several times to check reproducibility.

Oxidation of the freeze-dried and hydrated gels was followed by determination of peroxide values (PV) by the method of Kenaston et al. (1955). PV's were calculated as meq/1,000 g lard. Samples of fat for iodometric measurement of PV were obtained by extraction of the gel with chloroform.

Monocarbonyls were determined as 2,4-dinitrophenylhydrazone derivatives. Fat and oxidation products were extracted from the oxidized gels with hexane. An aliquot of the hexane extract, representing 5.0 g of lard, was reacted with 2,4-dinitrophenylhydrazone on the Schwartz column (Schwartz et al., 1963). The resulting carbonyl derivatives were separated from the lard and fractionated to simple monocarbonyl derivatives as described by Schwartz et al. (1963). Milder vacuum distillation methods (Gaddis et al., 1966) could not be used because of the tenacity of the gel which made quantitative removal of the free volatile carbonyls uncertain. However, the method employed was considered suitable for comparative purposes.

Recovery experiments of known monocarbonyl compounds indicated salvage of micro quantities in the range of 85–90%. Monocarbonyl hydrazones were separated into alkanal, alk-2-enal, and alk-2,4-dienal classes by the method of Gaddis et al. (1959). This data enabled determination of proportions of classes of monocarbonyl compounds. Each class was separated into individual compounds and the compounds were estimated as described by Ellis et al. (1959).

RESULTS & DISCUSSION

Hydrated gels

The hydrated gels autoxidized rapidly at 20°C. Storage in the dark was necessary to obtain a moderate and experimentally convenient rate of oxidation. Table 1 indicates the precision obtained with 23 control samples of hydrated gel representing four different lard lots. The unit of time employed was one day intervals. The greatest variability in time was ±1.17 days at PV 40 with an overall average of ±0.77. Precision was very good during the induction period and in the early stages of autoxidation. For samples set up simultaneously, the pre-

Table 1—Precision of gel method: Expressed in variability in Peroxide values; average deviation from mean.

Days of storage	Peroxide values
5	±0.31
10	±0.43
20	±0.85
30	±1.0
40	±1.17
50	±0.82
60	±0.82

cision is believed much closer than indicated in Table 1. The relative ease of lard autoxidation under these conditions is attributed to an ample distribution and even contact with air throughout the gel samples.

The incorporation of NaCl in hydrated gels with storage at 20°C resulted in retarded autoxidation. At PV's of 20, 40 and 60 controls had times of 4, 8 and 11 days; 2.3% NaCl, 9, 12 and 14 days; 4.5% NaCl, 8, 13 and 16 days; and 10.8% NaCl, 11, 15 and 19 days. This indicates that increased amounts of NaCl produced a progressively greater inhibition of autoxidation rates. This does not hold up for higher concentrations of NaCl.

A second experiment with greater amounts of NaCl up to saturation indicated a leveling off in the inhibition at concentrations of from 8 to 10% NaCl. The saturated NaCl did not accelerate autoxidation. This does not agree with the report of Chang et al. (1950) in which higher concentrations of NaCl accelerated fat oxidation.

Mabrouk et al. (1960) in studies of oxygen absorption by phosphate-buffered NaCl emulsions of methyl linoleate observed progressive inhibition related to salt concentration. Their explanation of this was a decrease in oxygen availability by NaCl. Salt had this depressing effect even though it lowers the pH (Coleman, 1951; Castell et al., 1965). Mabrouk et al. (1960) observed an increased oxidation rate of the linoleate with decreasing pH values as regulated by buffers.

A powerful influence by NaCl in decreasing the pH value of the gels has been observed in this work. As shown in Figure 1, pH of the hydrated gel decreased with increased NaCl up to about 8% concentration, after which values leveled off. The acidifying effect of NaCl could not be readily controlled by the use of 1.10*M* phosphate buffer (pH 6.7).

In meat, a decrease in pH generally accelerates lipid oxidation, and this may occur even in emulsions of linoleate (Mabrouk et al., 1960). Castell et al. (1965) have observed an effect of NaCl on the lipid oxidation of fish muscle which was believed independent of the pH. However, in the hydrated gel the NaCl inhibited the oxidation. Potassium chloride in 2.9% concentration inhibited autoxidation. A gel containing 2.9% KCl had a peroxide value of 20 at 11 days of storage compared with 8 days for 2.3% NaCl and 5 days for the control. Amounts of KCl and NaCl used here were molar equivalents.

Addition of micro amounts of Hb, Mb and cytochrome C to the hydrated gels resulted in acceleration of autoxidation at 20°C. An example is the effect of 9 mg Mb/20 g lard or 0.002% Mb in the gel. This gel had PV 20 at 3 days, PV 40 at 6 days, and PV 60 at 9 days. The control required 4, 8 and 10 days to reach PV's of 20, 40 and 60, respectively. Addition of 2.3% NaCl to 0.002% Mb resulted in greater acceleration of oxidation of oxidation. The gel reached PV 20 at 2 days, PV 40 at 5 days and PV 60 at 7 days. KCl also accelerated the pro-oxidative effect of the heme pigments.

Moderate amounts of NaCl accelerated autoxidation catalyzed by heme pigments, but high NaCl quantities caused a depression or leveling off. A control gel had PV 20 at 8 days, PV 40 at 18 days and PV 60 at 22 days. A gel containing 0.005% Hb had PV 20 at 3 days, PV 40 at 16 days and PV 60 at 19 days. Addition of 2.3% salt to 0.005% Hb gel resulted in somewhat greater oxidation with PV 20 at 3 days, PV 40 at 13 days and PV 60 at 18 days.

Increasing NaCl to 10.4% inhibited the 0.005% Hb catalysis. This gel had PV 20 at 3 days, PV 40 at 17 days and PV 60 at 21 days. The above relationship was observed in repeated experiments. Voleman (1951) showed NaCl accelerated oxidation of Hb and Mb to the ferric form. The heme pigments were used as received from the chemical house and were possibly in the oxidized (met) form.

Ferric chloride ($FeCl_3 \cdot 6H_2O$) had a pro-oxidative effect on the lard in the hydrated gel and this increased with concentration. In this series of experiments 0.047% $FeCl_3 \cdot 6H_2O$ had practically the same pro-oxidative effect as that of 0.0063% Mb. However, addition of 0.0047% and 0.012% $FeCl_3 \cdot 6H_2O$ had no significant effect on the oxidation promoted by 0.0063% Mb. There was little effect by 0.0023%, 0.0047% and 0.012% $FeCl_3 \cdot 6H_2O$ on the retarding influence of 2.3% NaCl. It is interesting to compare this with the report of Moskovits et al. (1960) which showed a powerful pro-oxidant effect of $FeCl_3$ and NaCl alone and in combination on sausage.

When added in microgram amounts, small increases in heme pigments produced a more rapid rate of autoxidation. However, relatively large amounts of Hb resulted in an inhibition or slowing of the rate of autoxidation to much less than that of the uncatalyzed control. The results of an experiment of this type are shown in Figure 3. A very interesting observation here was that combination of the inhibitory amount of Hb with NaCl produced an increase in the rate of autoxidation. The amounts of reactants used in this particular experiment still did not restore the oxidation rate to as great as that of the uncatalyzed control. Sodium chloride appears therefore to

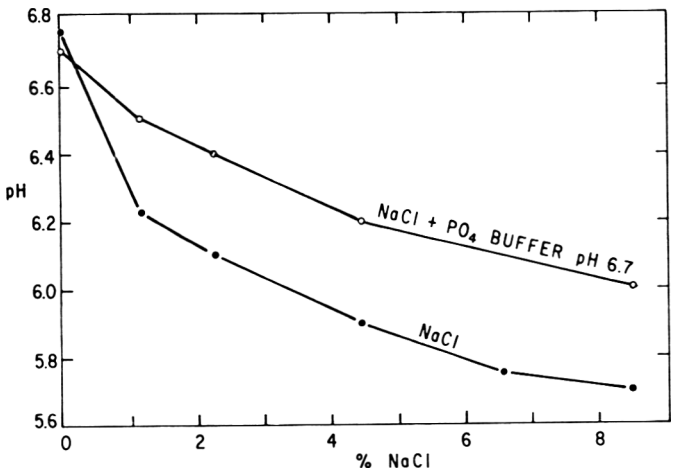

Fig. 1—Influence of NaCl concentration on pH of hydrated gel.

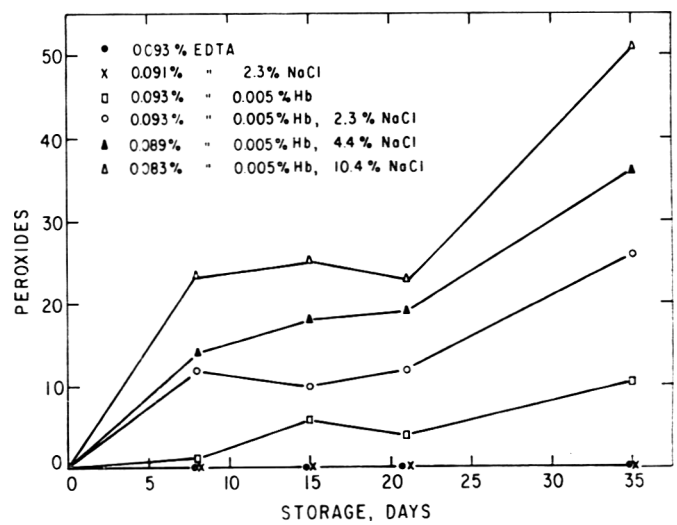

Fig. 2—Effect of EDTA on Hb and NaCl pro-oxidant activities on a hydrated gel.

have the power of reducing the effect of oxidation inhibiting amounts of Hb or heme pigments.

Banks et al. (1961) reported that cytochrome C in $1.75 \times 10^{-5}M$ concentration was an active catalyst, but at higher concentrations ($3.5 \times 10^{-5}M$) it inhibited autoxidation. The effect was considered due to peroxide breakdown to inactive compounds. Lewis et al. (1963) have observed similar inhibitory effects with Hb, hemin and tissue homogenates.

The mechanism of the effect of NaCl in counteracting inhibitory amounts of Hb is not clear. There is some indication that the key to the pro-oxidant effect of NaCl could be in part due to its pH-lowering action. However, Banks et al. (1961) have observed that cytochrome C was particularly active at pH's above 6.0. Wills (1965) has shown that pH has little influence on heme catalysis, but a marked effect on metal catalysis.

Uncured meat, stored in the freezer, oxidizes very slowly although abundant quantities of heme pigments are present. The addition of NaCl causes a comparatively rapid rate of autoxidation in freezer-stored meat. The effect of NaCl on unfrozen meat has not been determined because of masking deteriorative changes due principally to microoragnisms. The influence of NaCl on meat tissue is probably manifold; however, a direct effect of NaCl on potential pro-oxidant catalysts operates. Lewis et al. (1963) observed that homogenates of liver, heart and spleen were catalytic of linoleic acid oxidation in dilute suspensions and inhibitory in high concentrations.

These results permit the postulation that the pro-oxidant effect of NaCl on meat may be due in some degree to a modification of the inhibitory action of relatively high concentrations of heme pigments and other components.

Freezer storage of hydrated gels

The hydrated gel remained physically stable when frozen and appeared applicable for use as a model system in the study of autoxidation factors under conditions of freezer storage. A comparison was made of the rate of peroxidation in the hydrated gel with and without 2.27% NaCl at 25°F. Autoxidation was extremely slow and reminiscent of freezer-stored meat in its pace. Appreciable PV's did not appear until after several months of storage. At the termination of six months, the NaCl-treated sample had a PV of 45 as compared to 5 for the control.

Thus, freezing converted an oxidation-inhibiting influence of NaCl to a pro-oxidative function, and this would seem to represent a direct action by the NaCl. Such experimental conditions should prove valuable in further study of factors influencing autoxidation in freezer-stored meats.

Storage of freeze-dried gels

Freeze drying the gels produced a spongy solid which could be ground into fluffy, fine particles. This preparation oxidized readily. However, induction periods were frequently longer than those of the hydrated gels.

Sodium chloride accelerated the autoxidation of lard in the freeze-dried solid. A sample containing 25% NaCl (2.3% NaCl as hydrated) reached a PV of 30 in 4 days as compared to a control's time of 9 days. This was a direct effect unaided by any agent unless it were trace metals. However, the possible influence of trace metals appears to be ruled out by the use of EDTA. Use of 2.59% EDTA (0.093% EDTA as hydrated) on the control increased the time to reach PV 30 from 9 to 14 days. Employment of 2.59% EDTA and 24.5% NaCl reversed the time from 14 to 10 days. This demonstrates that the chelator did not eliminate the pro-oxidant effect of NaCl.

Thus, inhibition by NaCl existed in the hydrated form and acceleration under dehydrated conditions (including frozen). Influence of pH would not be a factor here. These results agree with the research findings of Chang et al. (1950).

In other experiments results were essentially similar to those reported for the hydrated gels. Hb and Mb accelerated the autoxidation and were sensitized to a greater acceleration by NaCl. These results seem to definitely indicate that pH is not the only factor involved in the action of NaCl on heme pigments and meat.

An interesting and significant result was obtained when pork lean juice, pork lean aqueous extract and pork lean were incorporated with the lard gel. These experiments were set up only for the freeze-dried gel to avoid

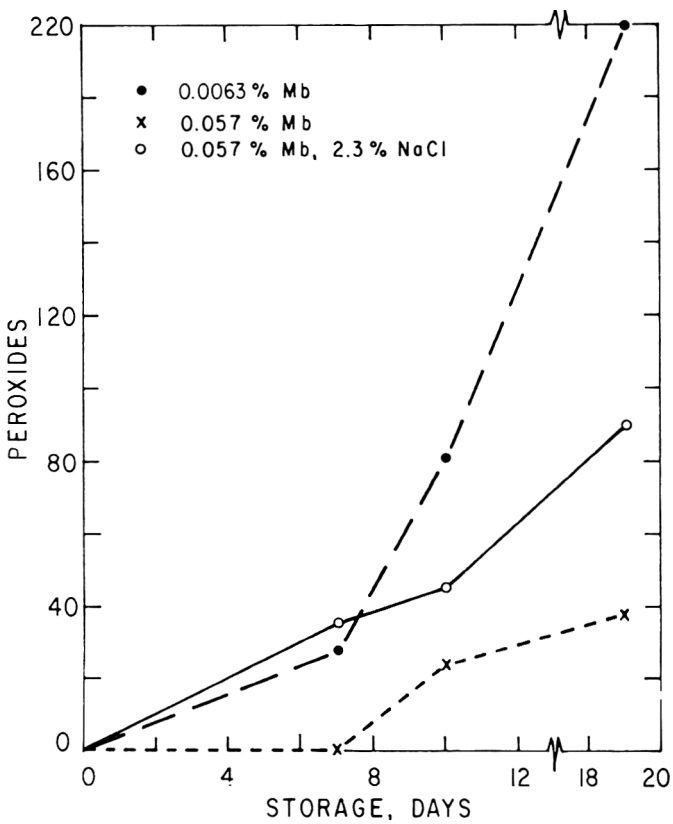

Fig. 3—Effect of NaCl on oxidation inhibiting amounts of Hb in a hydrated gel.

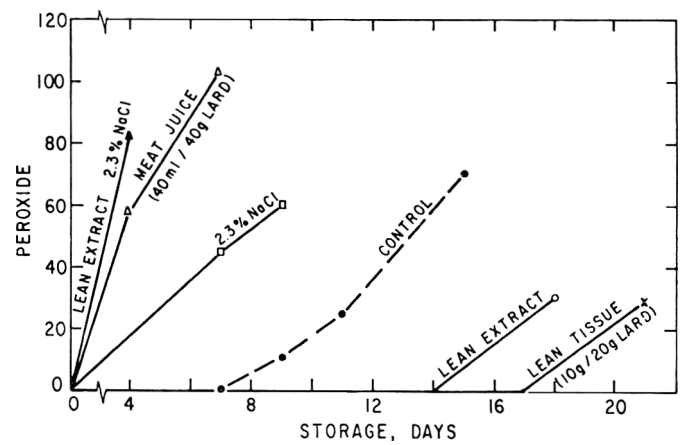

Fig. 4—Effect of meat fractions and NaCl on the autoxidation of freeze-dried gels.

microbiological spoilage. Meat juice was obtained by freezing and thawing pork lean. The aqueous extract was prepared by blending 10 g of lean pork for 2 min with 400 ml of water and filtering.

The results of those exploratory experiments are shown in Figure 4. The aqueous extract of the lean and the ground lean inhibited autoxidation. Addition of 2.3% NaCl to the aqueous extract produced an acceleration of oxidation. The pork juice accelerated oxidation. Experiments of this type should prove useful in further studies. The results indicate the possible presence of inhibitors and activators other than the heme compounds.

Autoxidation characteristics and monocarbonyl patterns

Monocarbonyl compounds generated were determined in some of the experiments. In the hydrated gels, in which NaCl inhibited autoxidation, there was little effect on the monocarbonyl/PV ratios and the individual monocarbonyl patterns. Mb, Hb and cytochrome C, which strongly accelerated autoxidation, had decreased monocarbonyl/PV ratios. This was surprising, since Ellis et al. (1968b) noted that, beginning with adipose tissue and increasing quantities of pork lean tissue, there was a progressive increase in monocarbonyl/PV ratios. Since the mechanism of heme catalysis involves hydroperoxide breakdown, it is apparent that much of the decomposition must be to nonmonocarbonylic secondary products.

The experimental data are shown in Table 2. When NaCl was used to accelerate the heme catalysis, the monocarbonyl/PV ratios increased but still were much lower than the controls. Differences in the individual monocarbonyl patterns were small, and more data would be necessary to determine their significance. The use of EDTA with Mb and NaCl-activated Mb produced a dramatic change in the major linoleate enal from C_8 to C_9 as shown in Table 2. This might indicate an influence of trace metals on hydroperoxide decomposition.

The monocarbonyl patterns of the oxidized freeze-dried gels differed from those of the hydrated samples. As shown in Table 3, the enal class was in greater proportion, and the C_9 enal was the major compound in that class. The C_9 enal was particularly dominant in the heme pigment catalyzed oxidations. In these cases, the quantity of C_9 enal was nearly as great as the C_6 alkanal. Both of these originate from linoleate hydroperoxide (Gaddis et al., 1961; Ellis et al., 1968).

Ordinarily in the oxidation of linoleate, C_6 alkanal is formed in very high percentages with very small quantities of C_7, C_8 and C_9 enals and C_{10} dienals. A mechanism by which such large amounts of C_9 enal might be formed is difficult to explain. This study indicates that the presence of water during autoxidation and hydroperoxide breakdown may have considerable influence on the monocarbonyls generated. Determination is needed of the effects of polarity, moisture and physical state since these conditions may obscure the influence of pro- and antioxidant additives.

The monocarbonyl data in Table 3 on the pork lean, pork lean juice and pork lean aqueous extract were of considerable interest and significance. Muscle juice had low monocarbonyl/PV ratios, and aqueous lean extract and lean had high ratios. Determination of the full significance of these results needs further investigation. However, the effect of the muscle juice resembles that of heme pigments. As referred to in Figure 4, the gels containing extract of lean were much more stable. The high monocarbonyl/PV ratios of these gels resemble those found by Ellis et al. (1968b) with pork backfat and lean mixtures.

Sodium chloride greatly decreased the stability of the lean extract gel, but the monocarbonyl/PV ratio remained high. The data are insufficient to determine whether this effect of lean and lean extracts is due to high proportion of heme compounds, protective factors or the presence of other prooxidant entities.

This investigation has indicated the value of lard CMC gels for the study of factors influencing the autoxidation of triglycerides. Such a model system resembled meat in some respects and appeared particularly adaptable for freezer storage investigations. Not all of the objectives of this study have been achieved, but progress has been made toward a

Table 2—The effect of additives on oxidation characteristics of hydrated lard gel.

Lard O. S. backfat, hydrated (20 g)	Heme	Additive (g)	NaCl (%)	PV	μm Monocarbonyl	Monocarbonyl/PV	Enal μm C_7	Enal μm C_8	Enal μm C_9
Experiment 45									
16 days	—	—	—	51	11.11	0.22	0.38	1.21	0.43
	81 mg Hb	—	—	57	4.92	0.09	0.11	0.56	0.16
	"	—	2.27	60	9.79	0.16	0.35	0.80	0.24
Experiment 47									
17 days	81 mg Hb	0.2 EDTA	—	31	1.45	0.05	—	tr[1]	0.23
12 days	"	"	2.27	28	2.37	0.08	0.09	0.15	0.24
	"	"	4.54	36	2.80	0.08	0.16	0.20	0.26
	"	"	6.81	46	3.71	0.08	0.15	0.20	0.31
Experiment 52									
9 days	—	—	—	41	4.26	0.10	0.19	0.46	0.20
	cytochrome C	—	—	50	2.68	0.05	0.09	0.16	0.14
	"	—	2.27	52	2.83	0.06	0.07	0.14	0.12
21 days	—	—	—	186	23.26	0.13	tr	2.62	1.08
	cytochrome C	—	—	201	11.16	0.06	tr	1.02	0.54
	"	—	2.27	215	12.16	0.06	—	—	—

[1] tr—trace.

Table 3—The effect of muscle and its fractions on autoxidation characterisitcs of freeze-dried gel.

Lard freeze-dried (40 g)	Heme	Additive	NaCl (%)	PV	μm Mono-carbonyl	Mono carbonyl/ PV	μm Alkanal C_6	Enal μm C_7	Enal μm C_8	Enal μm C_9
Experiment 40										
6 days	—	—	—	34	1.27	0.04	0.67	—	0.09	0.24
8 days	—	—	—	80	3.31	0.04	3.94	—	0.16	0.51
2 days	20 mg Mb	—	2.27	73	3.44	0.05	1.26	tr	tr	1.19
3 days	"	—	2.27	136	4.39	0.03	1.81	—	tr	1.42
Experiment 42										
6 days	—	—	2.27	48	2.27	0.05	1.07	—	0.11	0.31
Experiment 34										
4 days	—	Muscle juice	—	58	3.28	0.06	1.28	tr	tr	1.13
7 days	—	"	—	103	6.55	0.06	2.60	tr	tr	2.16
21 days	—	Lean muscle	—	29	4.27	0.15	1.87	tr	tr	1.08
Experiment 55										
18 days	—	Muscle extract	—	30	7.58	0.25	3.57	tr	tr	2.11
4 days	—	"	2.27	82	8.53	0.10	3.64	tr	tr	2.83

better understanding of the pro-oxidant activity of NaCl.

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Studies in Meat Tenderness. 8. Ultra-structural Changes in Meat During Aging

SUMMARY—During the aging of beef, the external loading required to stretch sternomandibularis muscle to its fullest extent declines 5–10-fold from the maximum of 2 Kg/cm² muscle attained at rigor onset. This loss of tensile strength is due to a weakening of the myofibrillar structures at the junction of the I filaments with the Z discs of the sarcomeres. The Z discs undergo progressive changes and lose ground substance as aging proceeds. There is no obvious structural change in the I filaments during aging although a weakening in the association of the constituent proteins of these structures apparently occurs.

INTRODUCTION

THE TENDERIZING that occurs in meat during storage above freezing temperature—so-called meat aging—has been the subject of two comprehensive reviews (Bate-Smith, 1948; Whitaker, 1959) and a number of research papers (Weinberg et al., 1960; Kahn et al., 1964; Fujimaki et al., 1965; Aberle et al., 1966; Davey et al., 1968a). Although a great variety of chemical and physical changes have been shown to accompany aging, a clear view of changes specifically associated with the phenomenon has only become possible now that more precise knowledge of muscle ultrastructure is available.

There are essentially three parallel components associated with muscle fiber—the myofibrils, the enveloping sarcolemma and the loosely-fitting endomysial sheath (Walls, 1960). These components are called upon to withstand considerable tensions in the living muscle of at least 4 Kg/cm² muscle (Weber et al., 1952) and for this reason alone are likely, in the cooked state, to offer considerable resistance to transverse shearing forces. Underlying changes of meat aging undoubtedly occur in one or more of these components.

Previous chemical and microscopic studies have shown that a number of distinct changes probably related to aging occur within the myofibrillar structures (Davey et al., 1967b, 1968a, 1968b, 1969a; Fukazawa et al., 1967). The first is a loss of adhesion of myofibrils normally held together through attachments of their Z discs to shared elements of the sarcoplasmic reticulum. The second is the loss of association of actin and tropomyosin within the I filaments of the sarcomeres. The third is a loss of material from the Z discs leading in some cases to a complete dissolution of these structures.

An electron-microscopic study of these changes within the myofibrillar components has now been made. The significance of the various changes in determining the increased tenderness of aged meat is discussed.

EXPERIMENTAL

Meat sampling

Bovine sternomandibularis muscles from animals 2–4 years old were prepared for experimental treatment within 90 min of slaughter under aseptic conditions (Davey et al., 1966). The initial length (L_o) of muscle strips (at least 15 cm long × 5 cm × 5 cm) was measured according to Davey et al. (1969b). The horizontally-supported strips were brought either to a cold-shortened state (Davey et al., 1969b) or to a slightly stretched state. Stretching was achieved by holding each strip between two

clamps, one of which was fixed while the other was attached through a cord and pulley to a vertically-hanging weight (10 g.).

With the onset of rigor mortis the meat samples became set at the length (L) so that the term $S = 1 - L/L_o$ defined the degree of shortening (+ve) or stretching (−ve).

Samples were taken for further study from pre-rigor muscle, 1.5 hr post mortem (PM), and from muscle stored at 15°C for 30 hr PM. These latter were considered to be fully in rigor mortis, (through attainment of the ultimate pH value) but with as little aging as possible. Samples were also taken from muscle stored at 15°C for 90 hr PM and thus approached the maximum limit of aging (Davey et al., 1967a).

Since samples stored for 90 hr PM were prone to microbiological spoilage and to dehydration, they were sprayed 24 hr PM with aureomycin (100 ppm) and chloramphenicol (100 ppm) and stored at 15°C and 80–85% relative humidity in a controlled-climate chamber. This procedure ensured that bacterial numbers, measured according to Davey et al. (1966) were contained at the low level of $< 10^2$ organisms/g muscle stored for 100 hr at 15°C.

Extension versus load determinations

Muscle strips of uniform cross section (at least 10 cm long x 1.5 cm x 1.5 cm) with fibers parallel to the long axis were cut from the muscles at 1.5, 30 and 90 hr PM. Each strip was hung vertically with a pan clamped to the bottom end for the addition of weights. A sample length for a given load was measured between two horizontal strips of gold leaf placed on the muscle surface one above the other and 2–3 cm apart. For consistency all extensions were measured 1 min after the application of each load.

Fiber-piece preparation

Muscle set in rigor, 30 hr PM, was homogenized under aseptic conditions in a Waring blendor for 1 min with 5 vol ice-cold 0.1*M* KCl containing 0.04*M* sodium phosphate buffer, pH 6.40. Microscopical examination showed that except for the presence of various cell components and debris, the preparations contained a small proportion of discrete myofibrillar units and a larger proportion of fiber pieces consisting of laterally-aligned myofibrils up to 40 in number. These fiber-piece suspensions were stored at 2°C in sealed tubes containing chloramphenicol (50 ppm) and aureomycin (50 ppm) for periods of up to 55 days at 2°C.

Preparation of samples for microscopic examination

Fixing and embedding. Fiber-piece suspensions (10 ml) taken at intervals during 50 days of storage were centrifuged (10 min, 600 × *g*) and the supernatant fluid discarded. The sediment was resuspended in a 2% solution of osmium tetroxide in 0.05*M* cacodylate buffer (pH 7.4) for 60 min at 20°C with gentle agitation to prevent clumping. Dehydration was carried out over a period of 60 min at 20°C in an ethanol series to 100% ethanol.

After brief centrifugation (5 min, 600 × *g*) the ethanol was discarded and replaced with acetone. Two mixtures of acetone and araldite (1:1 and 1:9 by volume) were then used in sequence to infiltrate the fiber pieces which were finally embedded in 100% Araldite as a shallow layer. The thin blocks of

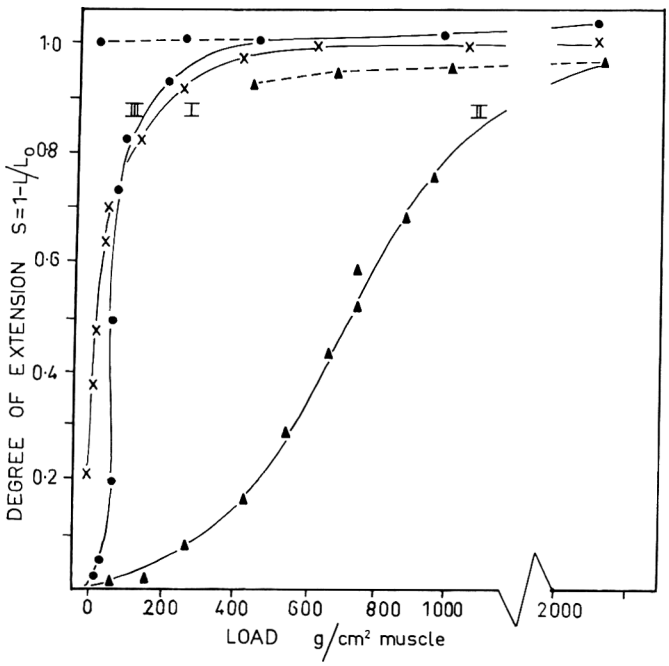


Fig. 1—Extension versus load curves for strips of bovine sternomandibularis muscle at different times post mortem. Curve I: Pre-rigor muscle, 1.5 hr PM; Curve II: Unaged meat, 30 hr PM; Curve III: Aged meat, 90 hr PM; (Broken lines, return curves with reducing loads.)

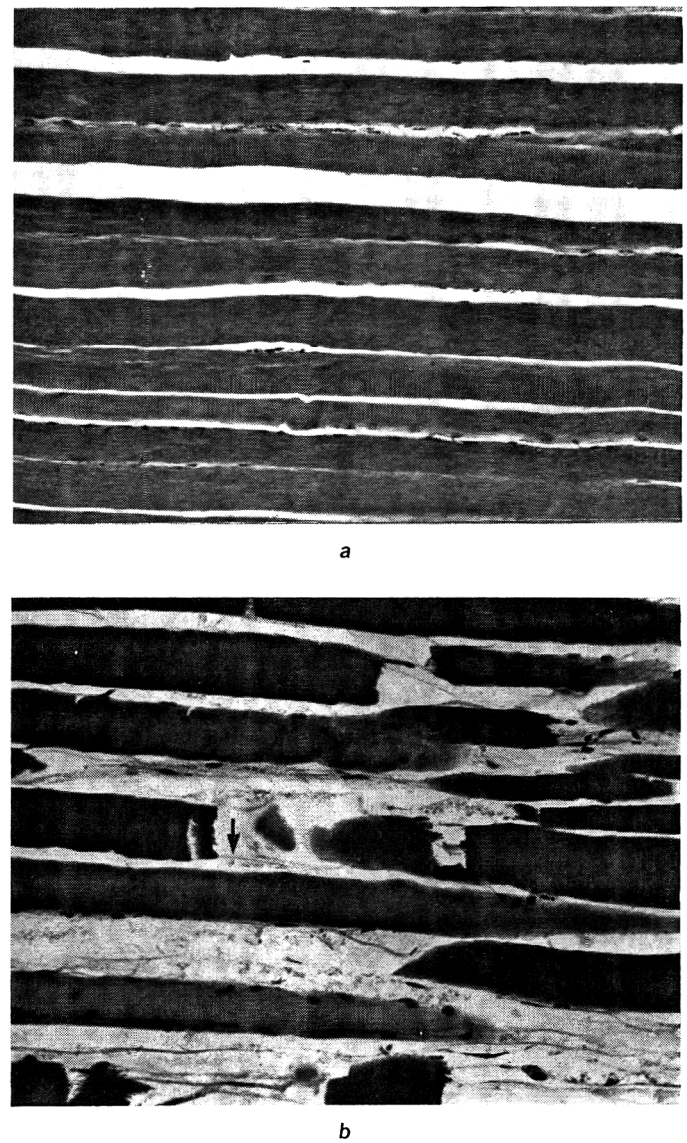


Fig. 2—Light photomicrographs of longitudinal sections from aged meat, 90 hr PM, close to resting length, S = 0.13. (a) Not subjected to extension. (b) After extending to approximately S = −2.0. Although some oblique sectioning occurs in the rather disorganized, extended samples, clear breaks are obvious in the myofibrillar material of the fibers, which are still enveloped in a thin sheath (arrow). (Magnification ×350).

embedded material (0.2 cm) were inspected with a phase-contrast microscope and typical fibers selected and trimmed for sectioning.

Whole muscle samples (1.5, 30 and 90 hr PM) were fixed as thin strips of parallel fibers (at least 6 cm x 0.2 cm x 0.2 cm) cut from the body of the meat. Samples subjected to a forcible extension were maintained at approximately 2 L_o by tying them under tension to glass rods. Fixation was carried out in two changes of 5% glutaraldehyde over a 4 hr period.

All samples were cut into small pieces, fixed for 2 hr more in fresh glutaraldehyde and finally washed in 0.1*M* phosphate buffer (pH 7.4) for 12 hr. A further fixation for 20 min was carried out in 2% osmium tetroxide followed by a post-fixative treatment (20 min) in 2% aqueous uranyl acetate.

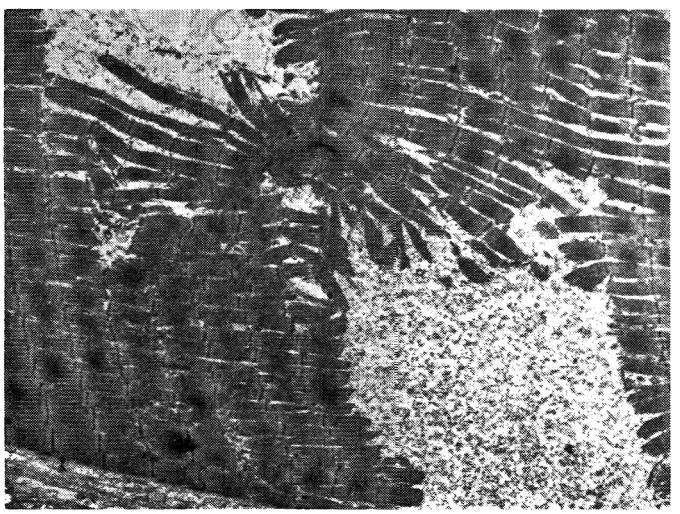
Sectioning, staining and microscopy. Samples for electron microscopy were cut with diamond knives on an LKB ultratome. After expansion with a trichloroethylene vapour, the sections were mounted on carbon-collodion films supported by 200 mesh grids, or without a supporting film, on 483 grids. Thicker longitudinal sections for light microscopy were cut from both unstretched and stretched, embedded samples and after mounting were treated with Van Giesen's stain. A Philips EM 200 with HT voltage of 60 Kv was used for the electron-microscopic study. Light micrographs were obtained with a Zeiss photo microscope.

Sarcomere-length determinations

Clearly-resolved striations were observed in the light microscope at a magnification of (×150) allowing sarcomere lengths to be measured on the micrographic negatives with a Nikon Comparator. Mean values of sarcomere lengths, together with standard deviation values were determined from at least 800 measurements covering micrographs of eight randomly-chosen fields from sections of each muscle sample.

RESULTS

THE LOAD-EXTENSION characteristics of muscle along its fiber direction are highly variable, changing with the physiological states of the material. Figure 1, typical of numerous such experiments, records the effect of external load on the degree or extension in muscle strips 1.5, 30 and 90 hr PM. In pre-rigor muscle, 1.5 hr PM (Curve I) the expected resting length-tension relationship was obtained. The stretching was freely reversible with little hysteresis. Since the muscle was held vertically, it stretched to an approximate



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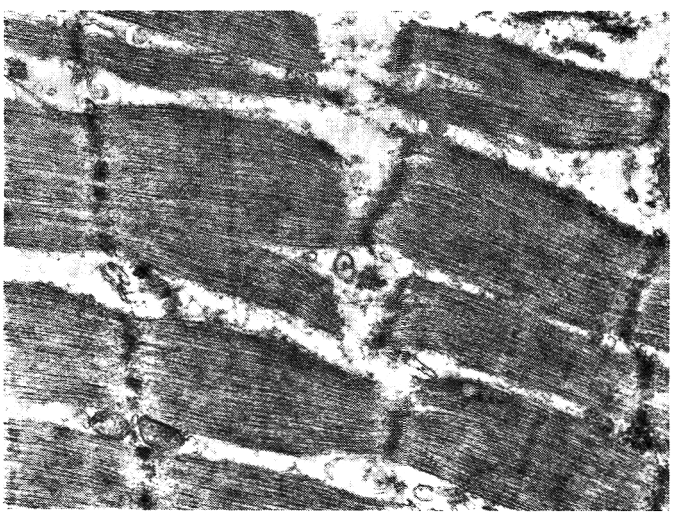

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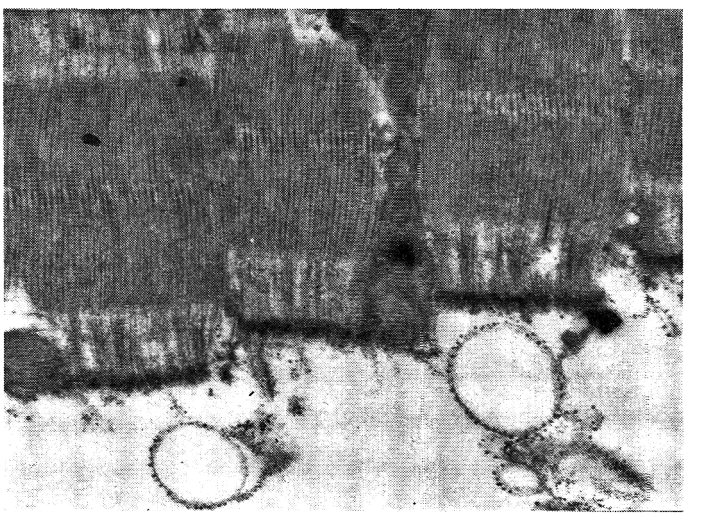
Fig. 3—Longitudinal section of aged meat, 90 hr PM, (S = 0.16) extended to approximately S = −2.0. Extension has not produced an increase in sarcomere length, but breakages have occurred beside the Z discs. (Magnification (a) ×3,000; (b) ×20,000).



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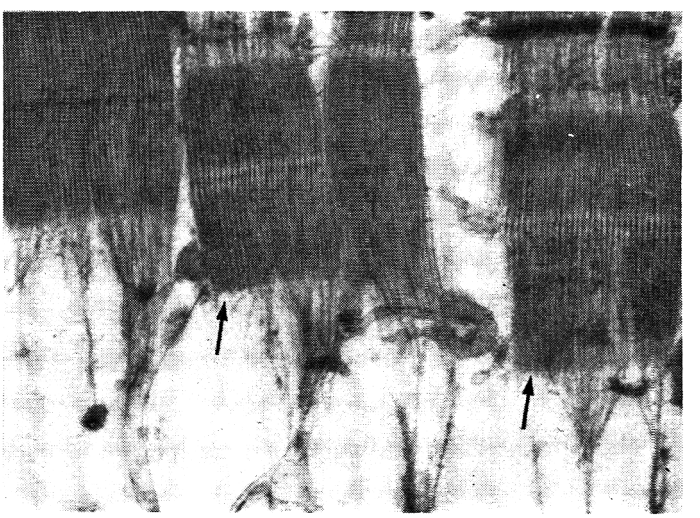


b

Fig. 4—Longitudinal section of aged meat, 90 hr PM, (S = −0.05) extended to approximately S = −2.0. (a) A preponderance of breakages has appeared at the junction of the Z discs and I filaments. (b) Occasional partial breakages (arrows) are encountered at the A band-I zone junction. On extending, the I filaments did not withdraw from between the myosin rods of the A bands. (Magnification ×20,000).

S valve of −0.2 under its own weight.

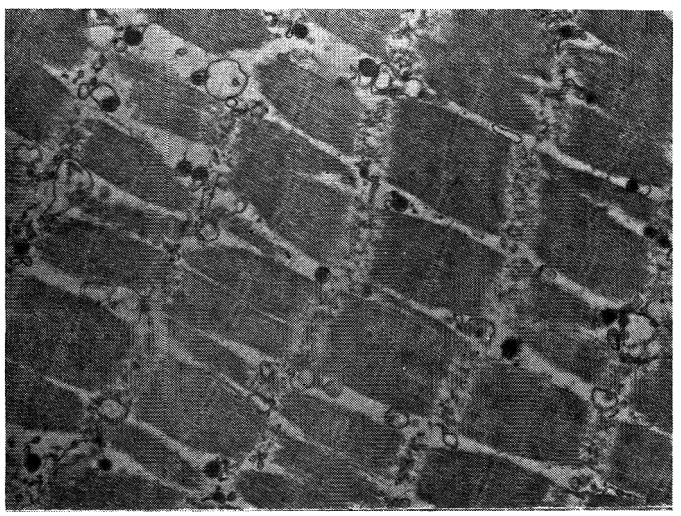
By comparison, meat in rigor 30 hr PM, was refractory and approached full extension (S = −1.0) only at loadings of more than 2 Kg/cm² (Curve II). Figure 1 shows that an entirely different pattern of extension is obtained with aged meat, 90 hr PM. Although such meat did not stretch under its own weight, it extended irreversibly (Curve III) at very small external loads (20–50 g/cm²). In all cases the limit of extension under load was virtually the same (S = −1.0 approximately) indicating that the connective tissue components had become fully stretched.

The ease with which aged meat samples extended when lightly loaded presumably resulted from weaknesses that appeared in the myofibrillar structures on storage. This is verified in Figure 2 which shows light micrographs of aged meat, 90 hr PM, before and after stretching. In the unstretched sample (Fig. 2a) the muscle fibers were aligned in parallel and unbroken order and had a mean sarcomere length of 2.30 μ (SD ± 0.18).

On the other hand, in the stretched sample (Fig. 2b) many breakages had occurred in the myofibrillar material along the muscle fibers which, however, remained surrounded by a stretched sarcolemma. The sarcomeres did not lengthen with stretching but remained at 2.30 μ (SD ± 0.22) characteristic of the unstretched meat.

The sites of fracture produced within the sarcomere on stretching are determined to some extent by the degree of muscle shortening during rigor onset. In the electron micrographs (Fig. 3) of a shortened sample (S = 0.16) breakages occurred solely in the shortened I zones. If muscle enters rigor in a slightly stretched state, (S = −0.05) the precise location of fracture on further forcible stretching in the I zone is more readily determined and occurs largely beside the Z discs (Fig. 4a) and also to some small degree at the A band-I zone junction (Fig. 4b).

Thus the much-reduced tensile strength of the myofibrils of aged meat, 90 hr PM, is associated with a weakening of sarcomere linkages largely between I filaments and Z discs. This weakening may be due either to a change in the Z discs or to a change in the I filaments themsleves. In the Z discs at least it has been shown by phase-contrast microscopy that



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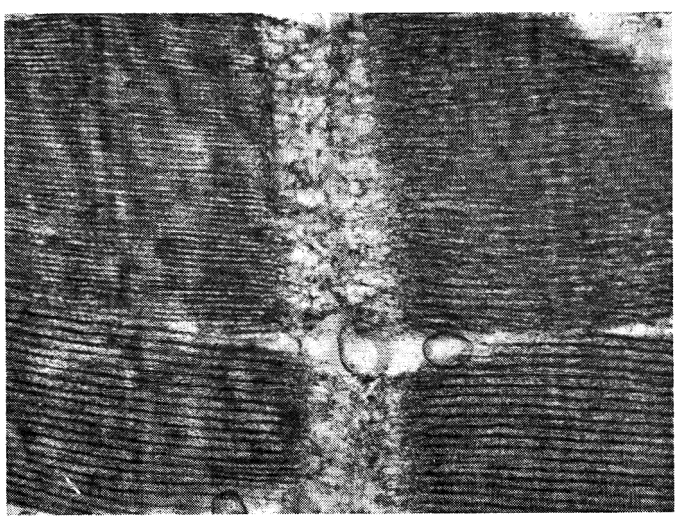
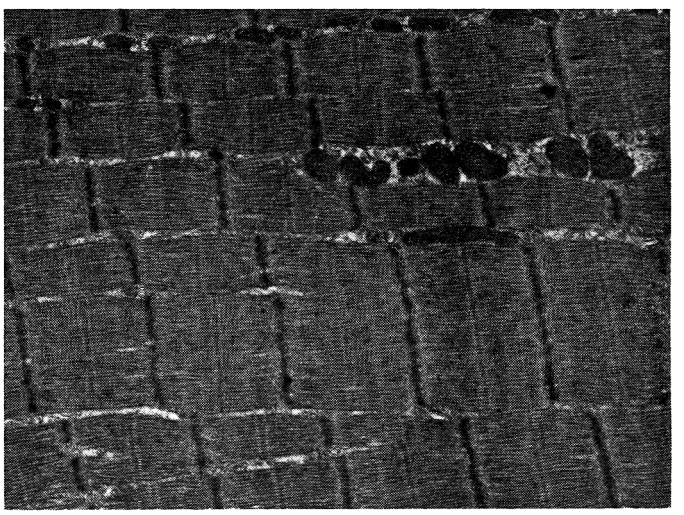


b

Fig. 5—Longitudinal section of a fiber piece from a suspension aged 40 days, 2°C. A general leaching out of ground material from the Z disc has occurred. The I filaments have lost their precise parallel alignment. (Magnification (a) ×10,000 (b) ×39,000).



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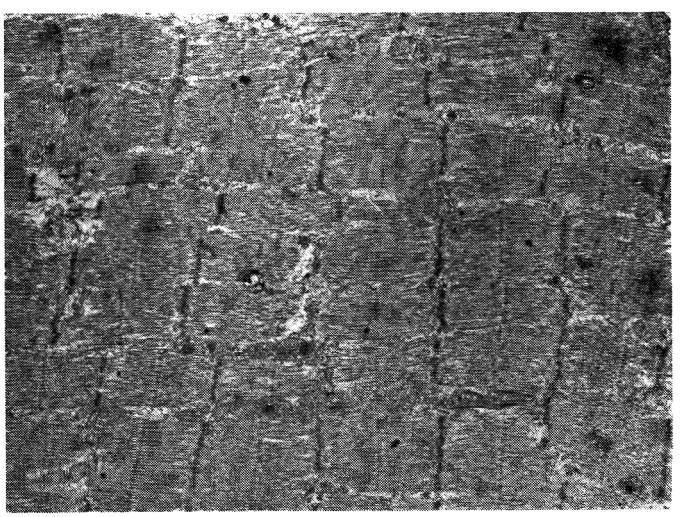

b

Fig. 6—Longitudinal section of whole meat, (S = 0.23) (a) Unaged, 30 hr PM. (b) Aged, 90 hr PM. Through aging Z discs have become less distinct and more wavy in appearance. (Magnification ×10,000).

there is a progressive disintegration of material during aging (Davey et al., 1969a).

This is confirmed in the electron micrograph of fiber-piece preparation aged for 55 days at 2°C (Fig. 5). Z discs are shown to have virtually disappeared through a loss of opaque ground substance while fibrous material in some disarray remained attached across the I zone.

Loss of Z discs in whole meat, 90 hr PM, is usually not so complete. However, it is shown in Figure 6 that discs remaining were not only much reduced in intensity through aging, but also had become more diffusive and wavy in appearance.

No discernible alteration in I filament ultrastructure has been encountered during storage for 90 hr PM (Figs. 5 and 6), although the increased disorder and loss of parallel alignment of the I filaments may well reflect some change.

DISCUSSION

CONSIDERABLE evidence of both a chemical and microscopic nature has now accumulated to suggest that during aging two distinct changes occur in the myofibrillar components of meat. The first is a weakening of lateral attachments which maintain myofibrils in precise register within the muscle fibers, probably at the level of the Z discs and involving elements of the sarcoplasmic reticulum (Davey et al., 1969a, Walker et al., 1968). The second is a weakening of the myofibrils themselves, resulting in a breaking at the I filament-Z disc junction and occasionally at the edge of the A band. How can these changes be of significance in describing the increased tenderness of aged meat?

We envisage that with the loss of the lateral inter-myofibrillar linkages, there will be a more limited dissipation of an applied shearing force throughout the body of the meat. With the resultant, more intense build-up of the stresses, aged meat will cleave at a lower shearing force than unaged meat. Expressed in terms of the breakages occurring on longitudinal stretching, it is envisaged that in the absence of lateral linkages tension will not build up uniformly but progressively among the myofibrils. For this reason each myofibril, to be released from its external stress, will break, thus allowing tension to be passed to the myofibril next in the progression.

If aging is due entirely to a loss of these lateral linkages, the sites within the sarcomere that yield on stretching will be the same for both unaged and aged meat. However, it has been clearly shown (Davey et al., unpublished data) that unaged meat, 30 hr PM, yields on forcible stretching (loadings 1–3 Kg/cm^2) largely through a withdrawal of I filaments from between the myosin rods of the A bands. On the other hand in aged meat a separation of I filaments from Z discs occurs under very low tensions indeed (50 g/cm^2 muscle), since the Z disc I-filament linkage is now the weakest link in the myofibrils.

From this evidence we conclude that meat aging is due largely to a loss of tensile strength within the meat fiber, through a weakening of the Z disc-I filament linkages, with the loss of lateral linkages among myofibrils playing a lesser role.

It remains to consider whether the Z disc-I filament weakening is due to changes in the properties of the Z-disc material or to changes in the I filaments. The present results show that the Z discs undoubtedly undergo progressive changes during aging. Fibrous components of the Z disc appear to be held together in rigid order by ground substance which disintegrates during prolonged storage. Uncharacterized proteins that may be derived from such material have been shown to leach from myofibrils coincident with the disappearance of the Z-disc structures (Davey et al., 1968b).

However, it must be kept in mind that the increased ease with which I filaments pull away from Z discs on stretching aged meat may actually be due to a change in the properties of the I filaments. Indeed, the close association between actin, tropomyosin and troponin within the I filaments is lost during prolonged aging (Davey et al., 1968b; Valin 1968).

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Infection Routes of Bacteria into Chicken Eggs

SUMMARY—*The area most prone to infection and consequent spoilage of eggs was identified. Different areas of the egg were coated with paraffin wax. The waxed eggs were exposed to infection by* Pseudomonas aeruginosa *and spoilage studied by ultraviolet light candling. The blunt end was found to be most vulnerable followed by the equitorial region and the small end.*

INTRODUCTION

IN RECENT YEARS, increased attention has been given to the mode of infection of eggs by bacteria. The organisms used most often for inoculation have been various species of *Pseudomonas*. These organisms are primary invaders and are relatively easy to detect.

Attempts have been made by several workers to correlate egg shell porosity with bacterial penetration (Walden et al., 1956, Fromm et al., 1960, Reinke et al., 1966). Romanoff (1943) states that the blunt end of eggs are more porous than the sharp end, however, Hartung et al. (1963) reported no difference between extreme porosity and indicated that capacity to resist penetration was not a function of porosity.

Tyler (1961a) determined the egg shell thickness by several methods and reported that the broad and narrow end of the egg shell had a thickness greater than most intermediate latitudes. The variation in the shell membrane thickness over various parts of the shell were reported to be considerable by Tyler (1961b), however, there was no relationship between membrane thickness and shell thickness. Reinke et al. (1966), using carbon dioxide penetration data, showed a low negative correlation between carbon dioxide permeability and bacterial penetration for top, bottom and equitorial shell sections of chicken eggs.

This laboratory found that eggs artificially exposed to *Pseudomonas aeruginosa* often reveal a definite greenish area of bacterial growth at the blunt (air cell) end. This study was undertaken to determine if there is any preferential area on an egg for bacterial penetration and growth resulting in spoilage.

MATERIALS & METHODS

Eggs

All eggs used in this experiment were from one strain of White Leghorn hens from the Cornell University farm. The eggs were of the same size and were one day old when used.

Washing of eggs

The eggs were washed in an immersion type washer using a sanitizer-detergent for 3 min at 45°C. The eggs were then rinsed in water (40°C) to remove residual sanitizer-detergent. They were dried at room temperature.

Dipping of eggs in molten wax

The washed, air dried eggs were dipped in molten paraffin wax that the unwaxed area consisted of the blunt end, small end, both ends and equitorial region. The detailed schematic for dipping is shown in Figure 1. Extreme care was taken to expose a similar amount of shell surface to paraffin wax within each treatment. The eggs were stored in a 35°C incubator for $1^1/_2$–2 hr before exposure to infection.

Exposure to infection

The waxed eggs were at about 35°C when removed from the incubator. They were dipped in water, infected with approximately 1 million cells of *P. aeruginosa* per ml. The temperature of the dip water was 15°C and the time of exposure to infection was 5 min. After exposure to infection, the eggs were removed and stored at room temperature (21–23°C).

Spoilage study

The infected eggs were stored at room temperature and the spoilage followed by candling every day using ultraviolet light. Eggs showing greenish florescence were considered positive for spoilage.

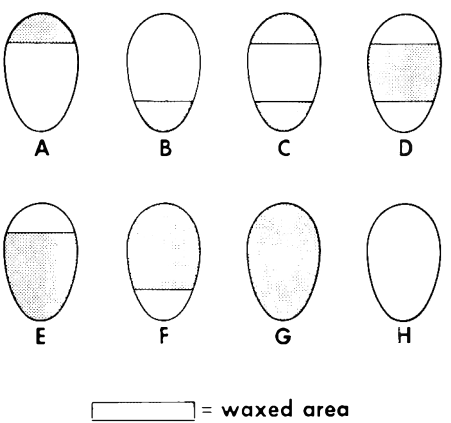

Fig. 1—Diagram of eggs showing different areas covered with paraffin wax.

RESULTS & DISCUSSION

TABLE 1 gives the results of bacteriological examination of the eggs that were waxed in different areas and were exposed artificially to infection by *P. aeruginosa*. The eggs were incubated at room temperature (21–23°C) for a maximum period of 30 days. Spoilage was considerably lower when the eggs were coated with wax at the blunt end or when all the shell was covered with wax except the small end. Spoilage was also minimal when the eggs were entirely covered with wax.

There was some spoilage even when the shell was completely covered with wax. This could be due to some minute areas of the shell which were not covered with wax or due to some native bacteria which were on the shell before waxing. Spoilage was very high when the small end was waxed. Similar results were obtained when both ends were left unwaxed or the blunt end was not waxed. However, spoilage was in between the two extremes when both ends were covered with wax.

Results show the air cell end is the area most vulnerable to infection. The de-

Table 1—Effect of various treatments as shown in Figure 1 on the spoilage of egg by Pseudomonas aeruginosa.

Treatment	Days of incubation				
	10	15	20	25	30
	Number showing spoilage[1]				
A	—	—	7	14	17
	—	—	4	7	11
B	—	1	9	21	32
	—	3	11	24	39
C	—	1	2	4	7
	2	4	9	12	18
D	—	6	13	19	40
	—	2	15	21	31
E	—	1	11	24	35
	1	3	13	26	39
F	1	2	4	5	7
	—	3	3	7	9
G	—	—	1	4	6
	—	—	2	5	9
H	—	3	12	24	35
	—	5	16	28	38

[1] Two trials, 40 eggs in each trial.
— No spoilage.

gree of spoilage decreases considerably if the blunt end is covered but it is not absolute. The small end appears to be the most resistant to penetration even when this is the only exposed area. The three regions rated according to their susceptibility are as follows: (a) blunt end, (b) equitorial region and (c) small end.

Kraft et al. (1958) and Reinke et al. (1966) reported a poor correlation between porosity and spoilage. Reinke et al. (1966) removed the membranes and were primarily studying the effect of shell porosity. However, in this study, the effect of all the exterior barriers including cuticle, shell and the two shell membranes were involved. Hartung et al. (1963) showed that membranes from eggs of varied shell porosities did not differ in their capacity to resist penetration by *Pseudomonas fluorescence*. Walden et al. (1956), indicated that shell at the blunt end contain a greater number of pores and was more permeable than the narrow end. It would appear that the mechanism of bacterial penetration may not involve permeability effect.

The data presented here indicate that eggs spoil faster if infection takes place at the blunt end as compared to other regions on the egg. Thickness of the shell and the membranes are not critical factors in penetration. Tyler (1961b) reported that shell thickness was greatest at the broad end, however, results indicate that this area is most vulnerable to infection. It is more probable that the air cell causes a vacuum or suction effect, thereby facilitating the passage of the organisms. The blunt end may provide better environmental conditions for growth and multiplication of the organisms once penetration has taken place.

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Chemical Destruction of Aspergillus niger Conidiospores

SUMMARY—Destruction of A. niger conidiospores at 20°C (68°F) by 20 ppm NaClO and 20 ppm iodine as iodophor yielded D values of 0.61 min and 0.86, respectively at pH 3.0 and 1.31 and 2.04 min, respectively at pH 7.0. On the basis of molar concentrations, iodine was slightly more effective than chlorine. A D value of 0.026 min was obtained with 4% NaOH at 60°C (140°F) indicating 4% NaOH at 60°C to be far more germicidal than 20 ppm of either halogen compound at 20°C. One per cent NaOH at 30°C resulted in an immediate and rapid release of amino acids presumably from the spore wall during the first 2 min of contact and a slower rate of release of RNA, with DNA released at the slowest rate.

INTRODUCTION

IN THE ASEPTIC processing of single strength orange juice two principal methods are presently in use for rendering glass containers free of spoilage microorganisms. One method uses a 4% NaOH wash followed by a chlorinated water rinse (Lund et al., 1966); the second method uses a rinse with 15 ppm iodophor (Neeb et al., 1967). The purpose of this investigation was to determine the relative germicidal effectiveness of these two methods for rendering glass containers free of viable mold spores and to assess the extent of marginal asepsis and potential spoilage engendered by both methods.

MATERIALS & METHODS

Preparation of conidiospores

Frozen orange concentrate was reconstituted with distilled water and clarified by vacuum filtration through a bed of Hyflo-Super-Cel. Fifty ml of the clarified orange juice were autoclaved for 10 min at 121°C in 250 ml Erlenmeyer flasks. They were then inoculated with a stock culture of *A. niger* and incubated statically for one week at 30°C for development of mature conidiospores. The spent juice was carefully poured off leaving the mat of growth intact. Distilled water (100 ml) was slowly added below the mat and then decanted to remove remaining juice and solids.

Sodium lauryl sulfate (100 ml of 0.1%) was added and the flask vigorously agitated to separate conidiospores from the mat. The spore suspension was then poured into a 250 ml Erlenmeyer flask containing glass beads and the flask was agitated at room temperature for 15 min on a rotary shaker to disperse clumps. The concentration of the spore suspension was then determined with a Petroff Hausser bacteria counting chamber and adjusted to 3.3×10^9 spores per milliliter.

Determination of destruction rates

Conidiospores of *A. niger* were subjected to 1, 2, 3 and 4% NaOH at six different temperatures (20, 30, 40, 50, 60 and 70°C). The destruction of *A. niger* conidiospores by halogen compounds at concentrations of 1, 3, 6, 10, 15 and 20 ppm was studied at 20°C. A 500 ml three neck flask (Levine et al., 1927) was used for determining destruction rates. One neck of the flask was equipped with a thermometer, one with a stirring rod and blade and the third with a stopper to permit aseptic sampling of the flask contents.

The flask was autoclaved, 299 ml of sterilizing solution added and placed in a Fisher Unitized Water Bath previously adjusted to the desired temperature, then 1 ml of a spore suspension containing 3.3×10^8

spores was added. One ml samples were removed periodically and transferred to 9 ml of appropriate chilled chemical inactivator (0.1 *M* $Na_2S_2O_3$ for halogens and 0.1 *M* to 0.4 *M* KH_2PO_4 for NaOH solutions).

When destruction rates were too rapid to allow sufficient time for germicidal inactivation with the flask method tubes were used for destruction studies. A series of 22 × 175 mm presterilized tubes were placed in the water bath, 9.9 ml of germicidal agent added, the temperature of the tubes allowed to become constant, then 0.1 ml of a spore suspension containing 1 × 10^6 spores per milliliter was added and shaken vigorously to give a uniform suspension.

After predetermined time periods, an equal volume of chilled KH_2PO_4 or $Na_2S_2O_3$ solution was rapidly added to inactivate the germicidal solution. Spore suspensions were further diluted using 9 ml dilution blanks of clarified orange juice at pH 4.0. One ml of the 10^1, 10^2 and 10^3 dilutions was plated in duplicate using orange serum agar (Difco) to determine survivors. Plates were incubated at 30°C for two days.

Preparation of germicidal solutions

The concentration of NaOH solutions in distilled water was determined by potassium acid phthalate titration (Horwitz et al., 1965). Solutions of a commercial iodophor (15% Butoxy polypropoxy ethanol-iodine complex, providing 1.75% available iodine, 9% phosphoric acid and 75.5% inactive ingredients) and sodium hypochlorite were prepared in buffer solutions. Mixtures of 0.1 *M* H_3PO_4, 0.1 *M* KH_2PO_4 and 0.1 *M* K_2HPO_4 were used as buffers to maintain the pH values (3, 5 and 7) of the halogen solutions constant. Iodometric titration was used to determine the concentrations of free chlorine (American Public Health Association, 1965). Free iodine concentrations were determined by starch-thiosulfate titration (Hays et al., 1967).

Assays for cell leakage

The method of Lowry et al. (1951) was used for the assay of ninhydrin positive amino acids using bovine serum albumin for a standard curve. Ribonucleic acid was determined by the orcinol method of Mejbaum (1939). Deoxyribonucleic acid was determined with the diphenylamine method of Burton (1956).

Leakage studies

Conidiospores were grown and harvested as previously described, washed three times with 100 ml of 0.1% sodium lauryl sulfate, once with 100 ml distilled water, and then adjusted to 1 × 10^9 spores/ml with distilled water. A series of 22 x 175 mm tubes was placed in a water bath, 5 ml of 1% NaOH added, the temperature allowed to rise to constant value (30°C), then 1 ml of the pretempered spore suspension was added and shaken vigorously to give a uniform cell suspension.

After predetermined time periods, an equimolar amount of chilled HCl was rapidly added to inactivate the NaOH solution and filtered immediately through a 0.45 micron porosity millipore filter. The filtrates were refrigerated overnight before leakage assays were performed. Zero time assays were performed by adding 1 ml of the spore suspension to chilled and previously neutralized 1 *N* NaOH prior to filtration. The use of phosphate or Tris buffer to neutralize the 1.0% NaOH in which the spores were suspended resulted in the release of considerable dark brown pigment from the spores which necessitated the direct addition of chilled HCl without a buffer for neutralization.

RESULTS & DISCUSSION

Destruction by sodium hydroxide

Destruction rates for *A. niger* conidiospores showed no lag and were exponential in 1, 2, 3 and 4% NaOH at all temperatures used (Figs. 1–4). With 1.0% NaOH the *D* value (time in min to destroy 90% of spores) at 20°C was 81.09 min while at 60°C the *D* value was 0.26 min. With 4% NaOH the *D* value at 20°C was 0.34 min while at 60°C the *D* value extrapolates to 0.026 mm (Table 1 and Fig. 5). The rates of destruction with 2 and 3% NaOH at 70°C and with 4% NaOH at 60 and 70°C were too rapid to determine experimentally with the methods used.

Table 1—Summary of D values for A. niger in sodium hydroxide.

Temperature, °C	D values in min			
	1% NaOH	2% NaOH	3% NaOH	4% NaOH
20	81.09	2.66	0.66	0.34
30	31.33	1.72	0.30	0.18
40	10.52	0.87	0.24	0.10
50	1.38	0.19	0.11	0.05
60	0.26	0.08	0.06	0.026[1]
70	0.12			

[1] Obtained by extrapolation from thermal destruction curve (Fig. 5).

The z values (number of degrees Fahrenheit required to alter the *D* values 10 fold) for the destruction of *A. niger* conidiospores in 1, 2, 3 and 4% NaOH were 29.5, 45.0, 69.0 and 67.0, respec-

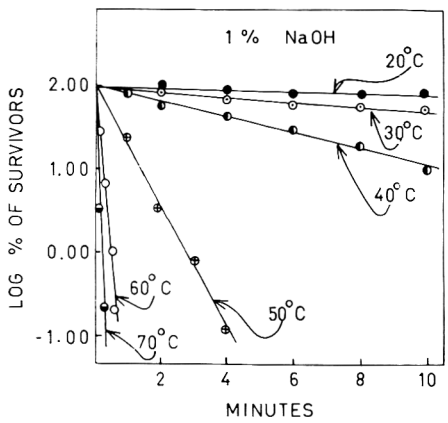

Fig. 1—Destruction of A. niger conidiospores at various temperatures by 1% NaOH.

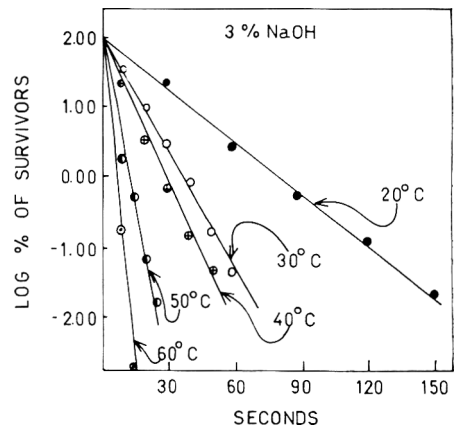

Fig. 3—Destruction of A. niger conidiospores at various temperatures by 3% NaOH.

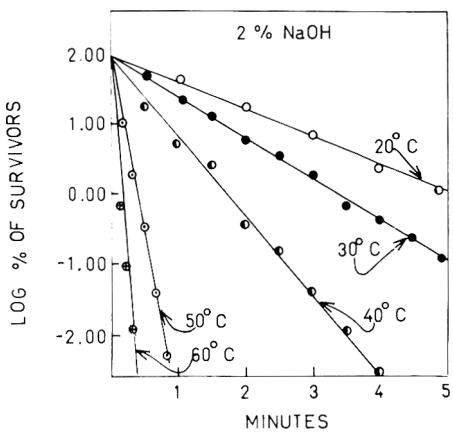

Fig. 2—Destruction of A. niger conidiospores at various temperatures by 2% NaOH.

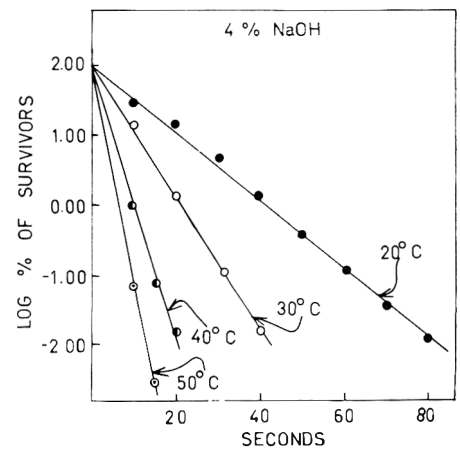

Fig. 4—Destruction of A. niger conidiospores at various temperatures by 4% NaOH.

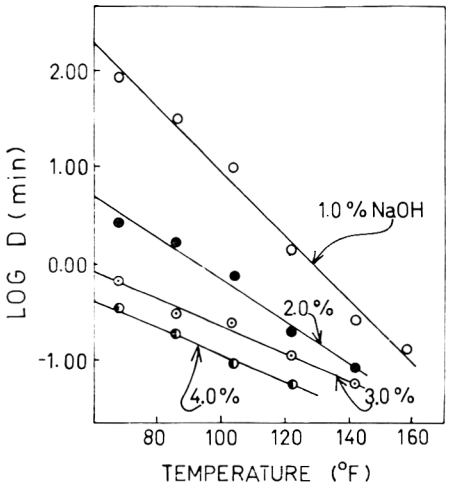

Fig. 5—Thermal destruction curves of A. niger conidiospores for various concentrations of NaOH.

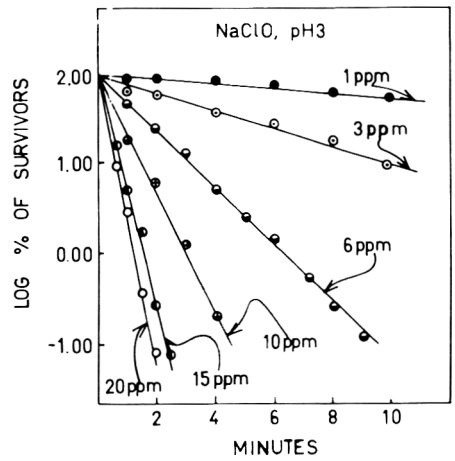

Fig. 8—Destruction of A. niger conidiospores by various concentrations of NaClO at pH 3.0.

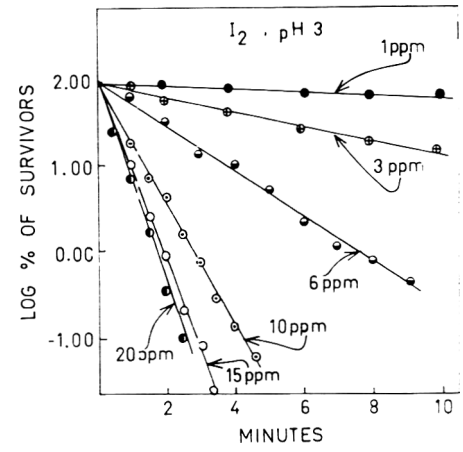

Fig. 11—Destruction of A. niger conidiospores by various concentrations of iodine at pH 3.0.

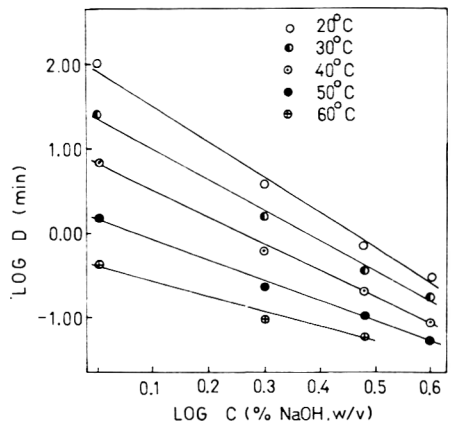

Fig. 6—Germicidal efficiency of NaOH against A. niger conidiospores.

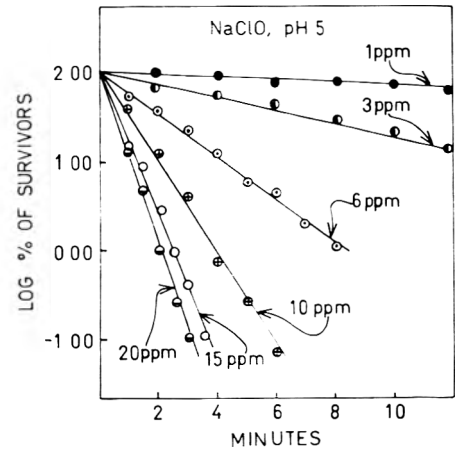

Fig. 9—Destruction of A. niger conidiospores by various concentrations of NaClO at pH 5.0.

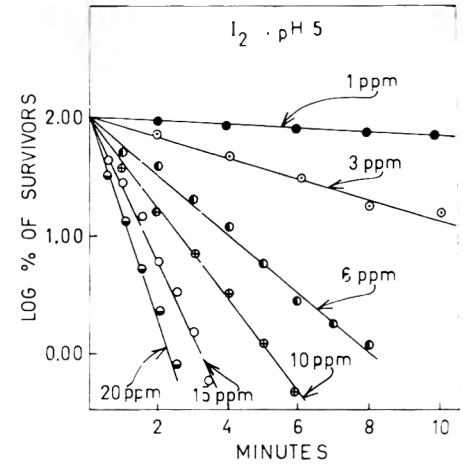

Fig. 12—Destruction of A. niger conidiospores by various concentrations of iodine at pH 5.0.

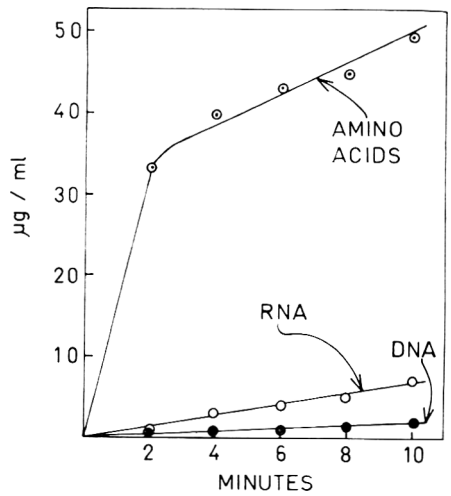

Fig. 7—Release of amino acids, RNA and DNA from A. niger conidiospores by 1.0% NaOH at 30°C.

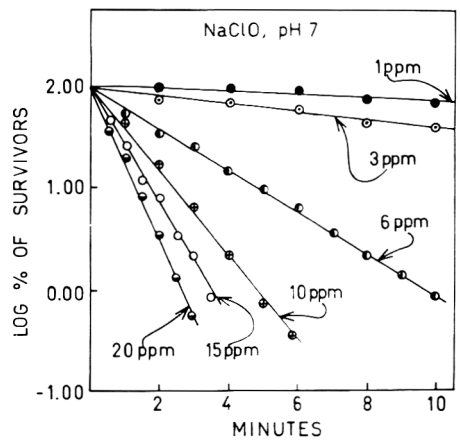

Fig. 10—Destruction of A. niger conidiospores by various concentrations of NaClO at pH 7.0.

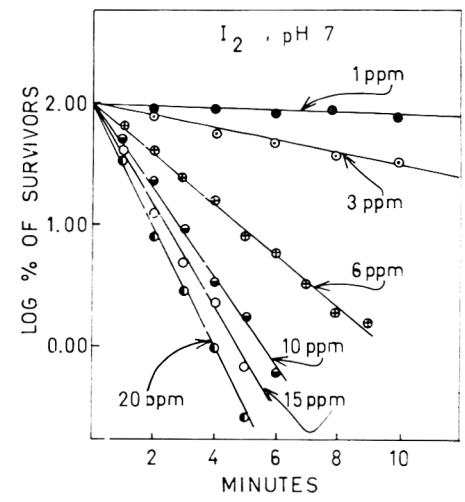

Fig. 13—Destruction of A. niger conidiospores by various concentrations of iodine at pH 7.0.

Table 2—Summary of D values of A. niger conidiospores in halogen solutions at 20°C.

Concentration of halogens, ppm	*D* values, min					
	pH 3		pH 5		pH 7	
	NaClO	$^1/_2$ I_2	NaClO	$^1/_2$ I_2	NaClO	$^1/_2$ I_2
1	55.56	40.82	69.40	56.82	100.00	90.90
3	10.10	10.37	13.50	10.89	22.72	19.23
6	3.20	3.76	4.17	4.00	4.85	4.77
10	1.49	1.41	2.00	2.56	2.38	2.81
15	0.78	0.94	1.21	1.61	1.71	2.32
20	0.61	0.86	1.04	1.15	1.31	2.04

tively (Fig. 5). Of interest are the nearly identical z values obtained with 3 and 4% NaOH which indicates a constant temperature characteristic for destruction at these two concentrations. This, however, should not be interpreted as indicating similar destruction rates with 3 and 4% NaOH at a given temperature. At 20°C the *D* value with 3% NaOH was 0.66 min whereas with 4% NaOH the *D* value was 0.34 min; applying an identical z value to these initial *D* values will not alter the approximately twofold difference in destruction rates (*D* values) maintained at any given temperature between the two concentrations.

The relationship between the logarithm of the concentration of NaOH and the logarithm of the *D* values was found to be linear (Fig. 6). This relationship was first expressed by Watson (1908) in the form $C^n \cdot t$ = a constant, where t is the time to destroy a specified number of organisms by concentration C (of disinfectant) and n is a constant. For purposes of calculation this expression may be written:

$$n \log C + \log t = \text{constant}$$

and indicates a linear relationship between log C and log t. Equations expressing these relationships for 90% destruction by solutions of NaOH were found to be:

at 20°C $\log t = 1.95 - 4.26 \log C$
at 30°C $\log t = 1.36 - 3.65 \log C$
at 40°C $\log t = 0.77 - 3.07 \log C$
at 50°C $\log t = 0.18 - 2.46 \log C$
at 60°C $\log t = 0.43 - 1.78 \log C$

Once established, these equations can be used to calculate the time required to destroy any number of the organism by any specific concentration of germicidal agent at the specified temperature.

Leakage studies

Studies on cell leakage were designed to determine the extent of damage to the spore membrane and cell wall by exposure of conidiospores to 1% NaOH at 30°C. This concentration and temperature were chosen from previous destruction studies so that approximately 50% spore destruction would result in 10 min. In this manner leakage of material would not arise from extensive damage of the entire cytoplasmic membrane, outer wall and coat but would reflect concurrent death of only half the cells by controlled chemical destruction.

A rapid increase in extracellular ninhydrin positive amino acids during the first 2 min of contact with the 1% NaOH occurred followed by a slower rate of increase (Fig. 7). Since there is no such change in the rate of spore destruction it is assumed that the release of protein or amino acids during the first 2 min is from the spore wall and external coat. This is consistent with the work of Horikoshi et al. (1964) who found the spore coat of *A. oryzae* to consist in part of protein. The rate of release of RNA and DNA from conidiospores in contact with 1% NaOH was much slower than the release of protein, with DNA released at the slowest rate.

Destruction of microorganisms by NaOH can readily be attributed to the lipoprotein nature of cytoplasmic membranes and the chemical affinity of NaOH for lipids. The structural aspects of lipids in biological membranes are discussed by van Deenen (1966) who observed the lysis of the cell membranes of *Neurospora crassa* by polyene antibiotics due to their affinity tor cholesterol in the membrane. The drastic degradation in the cell wall structure of microorganisms by NaOH is evidenced from the results of Northcote et al. (1958) who observed partial dissolution of the cell wall of *Chlorella pyrenoidosa* with 0.5–3.0% NaOH, resulting in an unmasking of the structural microfibrills of the wall.

Destruction by halogen solutions

Solutions of sodium hypochlorite and iodophor were found most effective at low pH values and high concentrations (Figs. 8–13, Table 2). On the basis of ppm free halogen, chlorine was more effectively sporicidal than iodine. The relationship between the logarithm of the molar concentrations of the halogens and the logarithm of the *D* values was found to be linear (Figs. 14–16). The equations of these lines were found to be:

for sodium hypochlorite (uM NaClO/l)
at pH 3.0
$\log t = 3.43 - 1.50 \log C$
at pH 5.0
$\log t = 3.44 - 1.43 \log C$
at pH 7.0
$\log t = 3.70 - 1.50 \log C$
for iodine as iodophor (uM $^1/_2$ I_2/l)
at pH 3.0
$\log t = 2.71 - 1.36 \log C$
at pH 5.0
$\log t = 2.73 - 1.28 \log C$
at pH 7.0
$\log t = 2.89 - 1.30 \log C$

At pH 3.0, 1 ppm NaClO and 1 ppm iodine yielded *D* values of 55.56 and 40.82 min, respectively; 20 ppm NaClO yielded a *D* value of 0.61 min and 20 ppm iodine yielded a *D* value of 0.86 min. At pH 7.0, 1 ppm NaClO and 1 ppm iodine yielded *D* values of 100.0 and 90.0 min, respectively, while 20 ppm yielded *D* values of 1.31 min with NaClO and 2.04 min with iodine (Table 2). These *D* values are all greater than those obtained with 4% NaOH at 20°C and above (Table 1).

Calculation shows:

1 ppm $^1/_2$ I_2 = 7.9 μM $^1/_2$ I_2
1 ppm NaClO = 13.4 μM $^1/_2$ Cl_2

Applying the ratio of 13.4/7.9 = 1.7 to the data in Table 2 indicates that on a molar basis iodine supplied as iodophor is equal to or slightly more effective than chlorine in destroying *A. niger* conidiospores.

Tsukahara et al. (1965) in an electron micrographic study of the structure of ultrathin sections of *A. niger* conidiospores demonstrated the presence of a thick-walled structure at the outer surface termed the outer hull. The outer hull was shown to vary in thickness from 0.1 to 0.5 microns. The authors suggest that the function of the outer hull is to protect the conidia from harmful physical and chemical agents. The spore coat or wall lying directly beneath the outer hull was observed by the authors to vary in thickness from about 0.05–0.10 microns depending on the stage of maturation. A broad intermediate space separating the spore coat from the spore membrane was observed to vary in thickness from 0.70 to 0.30 microns.

From the sum of the mean values of these measurements the spore membrane is found to lie at a mean distance of 1.2 microns from the outer surface of the outer hull. This represents a considerable distance for penetration of a germicidal agent and may account for the greater germicidal resistance of conidiospores compared to vegetative bacterial cells (Hays, 1967). Tanaka (1966) mentions the difficulties encountered in fixing and embedding mature conidiospores which

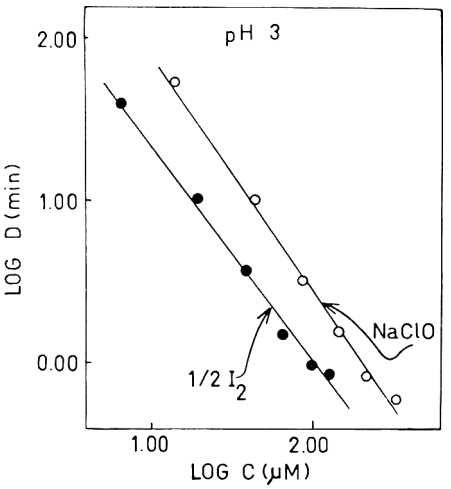

Fig. 14—Germicidal efficiency of NaClO and iodine against A. niger conidiospores at pH 3.0.

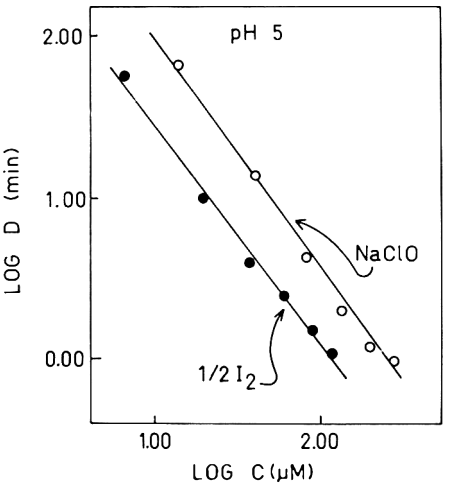

Fig. 15—Germicidal efficiency of NaClO and iodine against A. niger conidiospores at pH 5.0.

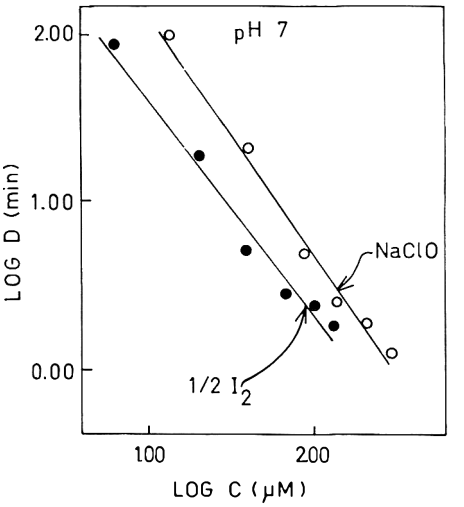

Fig. 16—Germicidal efficiency of NaClO and iodine against A. niger conidiospores at pH 7.0.

are not encountered with immature spores due possibly to the greater thickness and density of the walls of older spores.

The similarity in sensitivity between vegetative cells of *S. cerevisiae* and nonsporeforming bacteria (Hays et al., 1967) toward iodine and chlorine indicates failure of the thicker cell walls of *S. cerevisiae* to enhance germicidal resistance. Since the cell walls of yeasts approximate the thickness of conidiospore walls and from the ease vegetative yeast cells may be fixed and embedded (Bartholomew et al., 1955) vegetative yeast cell walls appear far more permeable to germicidal agents than conidiospore walls. Such a difference in permeability may also be reflected in the far greater resistance conidiospores exhibit toward ultrasonication than do yeast vegetative cells (unpublished data).

A. niger conidiospores were used throughout this study as representative of a common mold encountered in glass containers. Molds with less frequently occurring structures are known to be highly resistant to germicides. The marked resistance of *Neurospora crassa* ascospores to NaClO allows the application of this germicide at a concentration of 1.5% to ascospores as a means of selectively killing contaminating conidia in ascospore preparations (Emerson, 1948).

CONCLUSIONS

THE GERMICIDAL efficiency against mold spores of halogen solutions providing 20 ppm or less of NaClO or iodine at 20°C appears marginal. The application of 4% NaOH at 145°F followed by rinsing with chlorinated water appears to be the best method of choice for efficient destruction of *A. niger* conidiospores in glass containers.

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Factors Affecting the Distribution of Lactate Dehydrogenase Between Particulate and Soluble Phases of Homogenized Trout Skeletal Muscle

SUMMARY—Skeletal muscle of brown trout contained one electrophoretically distinguishable lactate dehydrogenase (LDH) isozyme. In homogenates of the muscle, release of the enzyme into the soluble phase was favored by high ionic strength and high pH. DPNH solubilized the enzyme and prevented binding of soluble enzyme to particulate matter at concentrations which contributed only negligibly to the ionic strength of the suspending medium. The other compounds involved in the LDH-catalyzed reaction, DPN^+, pyruvate, and lactate, were less effective. The effect of the latter two was due chiefly to their contribution to the ionic strength of the medium. Pyruvate, however, used with either DPNH or DPN^+ exhibited a synergistic activity. Effective solubilization showed remarkable specificity for DPNH and TPNH. Solubilization by DPNH was also dependent on the tissue concentration of the suspending medium. The lower the tissue concentration, the more readily LDH is solubilized by DPNH. In addition to certain metabolic implications, this information may be used to define assay conditions to allow the study of the kinetics of LDH in bound and soluble forms.

INTRODUCTION

AS TARR (1966) pointed out in a recent review, "the accumulation of lactic acid in fish muscles after death is of considerable importance technologically." Some important aspects include the effect of the drop in pH on bacterial growth, on struvite formation in canned fish, on water-holding capacity and subsequent drip, and production of a chalky condition in some fish. The sugars and sugar phosphates from which the lactic acid is derived will decrease concomitantly with the increase in lactic acid. This may cause a loss of sweetness in the flesh, and would also have an effect on the tendency of the fish to brown if it were to be canned or dehydrated.

Another aspect of the problem is that the accumulation of lactic acid is apparently a result of an attempt by the muscle cell to maintain a high level of ATP. When the muscle can no longer produce lactic acid, the ATP disappears and eventually hypoxanthine is produced with its corresponding bitter taste (Kassemsarn et al., 1963).

In post-mortem muscle, the principal system for maintaining a high ATP level (which at the same time produces the lactic acid) is glycolysis. Lactate dehydrogenase catalyzes the oxidation of reduced diphosphopyridine nucleotide (DPNH) to the oxidized form (DPN^+) utilizing pyruvate as substrate and producing lactate. As such, it is an important part of the glycolytic sequence. If DPNH were not oxidized, it would quickly accumulate to levels sufficient to inhibit 3-phosphoglyceraldehyde dehydrogenase and so stop glycolytic activity and the many concomitant changes that accompany glycolysis in muscle.

The concept of the glycolytic enzymes as soluble, cytoplasmic entities has been subjected to re-investigation within recent years. Green et al. (1965) postulated that all major metabolic pathways are actually membrane bound in vivo and it is the manipulative procedures leading to cell rupture which are responsible for the solubilization of the enzymes.

Paigen et al., (1962) found LDH in the soluble fraction of tissue homogenates. However, by reducing the ionic strength of the medium the cell particles could adsorb this enyme. The particulate nature of LDH in vitro appeared to be the result of a reversible adsorption with the point of equilibrium between the bound and soluble forms to be dependent on the salt concentrations of the immediate environment. The authors reasoned that the ionic strength prevailing in living cells would thus result in almost complete solubilization of the LDH. Amberson et al. (1965) have concluded that LDH is bound to the fibrous proteins of the myofibrils since they found 98% of the LDH activity associated with pressed muscle and 2% with the press juice.

Binding of other glycolytic enzymes has also been shown to have a pH and ionic strength dependency. Roodyn (1957) claimed that pH fluctuations in the extracting media were responsible for the distribution of aldolase between particulate and soluble fractions. By increasing the ionic strength of the media he noted a greater extraction of the enzyme. Hernandez et al. (1966) showed that the equilibrium between the soluble and particulate fractions of hexokinase was dependent on salt concentration and pH. These workers demonstrated that salts could induce a reversible solubilization of this enzyme.

Rose et al. (1967) found hexokinase to be solubilized by glucose-6-phosphate, ATP or by salts with differing pH dependencies. Magnesium caused rebinding of the enzyme independent of the conformational change imposed by glucose-6-phosphate.

In this laboratory the solubilization of LDH in homogenates from chicken breast muscle was shown to increase with increasing pH and increasing ionic strength (Hultin et al., 1966a). These basic factors can explain the difference in distribution of the enzyme between supernatant and particulate fractions prepared under different conditions such as concentration of tissue during homogenization, time of post-mortem aging, etc.

Hultin et al. (1967) ruled out the possibilities of isoelectric precipitation and entrapment as causative factors for the association of LDH with cell particulate matter. Retention and distribution of LDH obtained directly from a muscle homogenate were found to be similar to those caused by binding soluble LDH to a particulate muscle fraction previously freed of LDH (Hultin et al., 1966b). This led to the speculation that the soluble enzyme may have been adsorbed to the particulate fraction during homogenization. Thus, LDH may exist in the cells: (1) soluble in the cytoplasmic matrix, (2) bound to the subcellular particles, or (3) in equilibrium between soluble and non-soluble phases of the cell.

This study characterized the factors which affect the association of LDH with cell particulate matter in homogenates prepared from skeletal muscle of brown trout. In addition to pH and ionic strength, certain cell metabolites were observed to influence this association.

The purpose of this work was to (1) determine the similarity of the fish muscle to the avian (2) define conditions which would allow control of the partition of the enzyme between soluble and particulate phases of the homogenate (3) provide data to hopefully allow extrapolation from homogenates to conditions in situ and (4) provide basic information so that the kinetics of the activity of LDH could be studied with the enyme in soluble and bound forms. A preliminary report of some phases of this work has been made (Melnick et al., 1968).

EXPERIMENTAL

Materials

Brown trout (*Salmo trutta* Linnaeus) (9-in. length) were obtained from the Massachusetts State Hatchery in Sunderland, Massachusetts. Reduced diphosphopyridine nucleotide (DPNH), oxidized diphosphopyridine nucleotide (DPN^+) and adenosine triphosphate (ATP) were purchased from P-L Biochemicals Inc. Pyruvate and oxidized triphosphopyridine nucleotide (TPN^+) were products of Sigma Chemical Company. Reduced triphosphopyridine nucleotide (TPNH) and adenosine were obtained from Nutritional Biochemicals Corporation.

Methods

Centrifugation. All centrifugations were performed at 40,000 rpm for 30 min in a No. 40 rotor of the Beckman Model L or L-2 ultracentrifuge. This is equivalent to a maximal force of 144,000 G.

Preparation of bound and soluble LDH. Each day that a preparation was made, a fish was removed from an aquarium maintained at 4°C and sacrificed by stunning. The skeletal muscle was immediately cut out, weighed and placed in the proper homogenizing medium. All preparative operations were carried out at 0–4°C. The whole homogenate was obtained by blending the mixture of fish plus medium for 30 sec in a Waring blendor at full speed. The level of tissue in the homogenate was 5% (weight of tissue/volume of distilled water) unless otherwise stated. Samples of the whole homogenate were centrifuged.

The particulate or bound LDH fraction was defined as that portion of the enzyme which sedimented, while the soluble LDH fraction was that portion of the whole homogenate which failed to sediment under such conditions. The bound fraction was resuspended in media equal in volume to the decanted soluble portion.

LDH isozymic pattern

LDH isozymes were characterized by their electrophoretic pattern on cellulose acetate strips using phosphate-citrate buffer at pH 7.0 (0.067*M* in phosphate and 0.013*M* in citrate) (Preston et al., 1965).

Influence of pH and ionic strength on binding LDH

LDH-rich sediments from a 5% (weight of tissue/volume of homogenizing medium) homogenate were rehomogenized in solutions buffered with imidazole (15mM) at pH values ranging from 6.3 to 7.5. Particulate LDH-rich fractions were also rehomogenized in solutions of varying ionic strength (0.010–0.100), obtained with sodium chloride and calcium chloride as solutes, in 15 mM imidazole buffer, pH 6.7. The rehomogenized solutions were again centrifuged to obtain supernatant and sedimented fractions with LDH distributed between them as a function of pH or ionic strength.

Influence of cell metabolites on LDH binding

LDH-rich sediments were rehomogenized in 10 mM imidazole buffer, pH 6.7, with the following cell metabolites: DPNH, DPN^+, pyruvate, lactate, TPNH, TPN^+, ATP, ATP + $MgCl_2$, adenosine, nicotinamide, pyrophosphate and combinations of DPNH, DPN^+, pyruvate and lactate. The rehomogenized solutions were centrifuged, after which the supernatant and sedimented fractions were assayed to determine the effects of these agents on the solubilization of LDH from cell particulate matter.

LDH-rich sediments were freed of LDH by rehomogenizing them in 0.1*M* NaCl and centrifuging as above. The solubilized enzyme obtained after centrifugation was dialyzed overnight against distilled water. The dialyzed enzyme solution was rehomogenized with LDH-free sediments in the presence of DPN^+, DPNH, pyruvate and lactate. The rehomogenized solutions were centrifuged, after which the supernatant and sedimented fractions were assayed to determine the effect of these agents on the prevention of rebinding of LDH to particulate matter.

Assay for LDH

LDH activity was determined by the method of Wu et al. (1959) at pH 7.4. It was found that trout muscle LDH displays a constant activity over the pH range of 6.2–7.4 when saturating levels of pyruvate are used. The sum of the activities of the supernatant and sedimented fractions were compared to the activity of the homogenate to check on recoveries.

RESULTS & DISCUSSION

LDH isozymic pattern

From the electrophoretic studies it was concluded that skeletal muscle of brown trout contains only the muscle type of LDH isozyme. This conclusion was based on the observation of only one band for the whole homogenate, the soluble fraction and the particulate fraction, all traversing an equal distance and all less anodic than the LDH isozyme from trout heart.

A mixture of LDH obtained from muscle and heart tissue showed a definite separation in the electrophoretic test. Furthermore, electrophoretic determinations of heart- and muscle-derived LDH isozymes frozen and thawed together in neutral phosphate buffer containing molar NaCl failed to show the presence of the intermediate hybrids.

Table 1—Retention of LDH on particulate matter as a function of concentration of tissue in homogenate.

Concentration of fish muscle in whole homogenate[1] (%)[3]	LDH in sediment[2] (% of total LDH)[4]
2	37
5	26
10	20
20	13

[1] Whole homogenate was prepared in distilled-deionized water.

[2] Sediment fraction was obtained by centrifugation of whole homogenate at 40,000 rpm for 30 min.

[3] Expressed as weight per 100 parts of added water.

[4] This value was determined by dividing the LDH activity in the sediment fraction by the LDH activity in the supernatant and sediment fractions and multiplying × 100. The recovery of LDH in the separated fractions compared to the whole homogenate was 100 ± 10%.

This finding along with that of the single isozymic form of LDH is in agreement with the observation made by Fondy et al. (1965) that species which did not possess hybrids in vivo could not form hybrids in vitro from the heart and muscle isozymes of that same species. Furthermore, Market et al. (1965) reported that most trout species display only two or three isozymic bands in electrophoretic tests. However, Bouck et al. (1968) have reported 15 isozymic forms of LDH in rainbow, lake and brook trout and 27 in a brook-brown hybrid. They did not examine brown trout.

Distribution of LDH between fractions

Table 1 shows the relationship between the % of muscle in the whole homogenate (expressed as g/100 ml water) and the % of LDH retained on the particulate matter. The pH for whole homogenates varied between 7.05 and 7.25, and there were no consistent differences with tissue concentration. The greater the tissue concentration, the greater was the release (solubilization) of LDH from the bound fraction. For further studies, the 5% concentration was chosen since it provided a desirable retention of LDH in the sediment, with sufficient tissue and enzymic activity to minimize experimental error.

Influence of pH and ionic strength on solubilization

The two factors in the homogenizing media contributing most to the binding of LDH to the particulate matter of muscle homogenates are pH and ionic strength (Hultin et al., 1966a). Particulate fractions containing LDH were rehomogenized in media buffered from pH 6.3 to 7.5 with imidazole. The buffer concentration was maintained at 15 mM to keep the ionic strength of the media

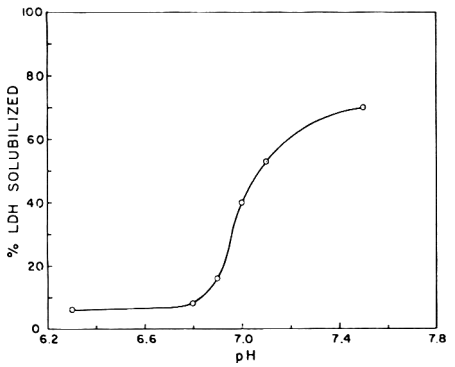

Fig. 1—Solubilization of LDH as a function of pH. Bound LDH fractions from a 5% whole homogenate were rehomogenized in 15 mM imidazole buffered at various pH values. The solutions were centrifuged for 30 min at 40,000 rpm. % LDH in the supernatant expresses the LDH activity in the supernatant fraction divided by the sum of the LDH activities in the supernatant and sedimented fractions.

low yet provide sufficient buffering capacity The pH of the media caused a redistribution of LDH between the soluble and particulate fractions. By assaying each of these fractions, a measure of the release of the bound LDH as a function of pH was determined.

In Figure 1 the % of the original particulate LDH released into the supernatant fraction was plotted against the pH of the suspending media. More LDH was released from particulate matter as the pH increased, and a break-point occurred at a pH of approximately 6.7. The ionic strength of these suspensions varied slightly due to the differing ionizing capacity of imidazole as a function of pH. The maximal value would be at the lowest pH; thus any adjustment in the curve to be made for this slight change in ionic strength would be to give less solubilization at the lower pH values and more at the higher. In other words, the effect of pH shown in Figure 1 was a minimal effect.

Particulate LDH-rich fractions were homogenized in solutions of varying ionic strengths. Figure 2 shows the % of the original particulate LDH solubilized into the supernatant fraction as a function of ionic strength. Sodium chloride and calcium chloride were used as the solutes. The pH in these experiments was maintained at 6.7. Similarity in results obtained with these two solutes indicated that it was the ionic strength of the two salts in solution which was responsible for the release of the enzyme. The contribution of the tissue was not taken into account in these experiments, but it was assumed to be small since the supernatant fraction from the original homogenate which probably contained most of the soluble salts of the tissue had been discarded and we worked only with the particulate LDH-rich fraction.

The contribution to ionic strength of the imidazole buffer was ignored in these experiments since the main interest was in seeing if there was an effect of ionic strength and in comparing a monovalent and a divalent cation. Recovery of enzymic activity was 80–90%.

The effect of the release of LDH by different molar concentrations of imidazole at pH 6.7 (the pH just at the break point of increased solubilization of LDH) was determined with the results shown in Figure 3. Although the higher concentrations afforded better buffering capacity, they also caused more enzyme release. From this figure a medium could be formulated to retain over 90% of the LDH in association with cell particulate matter. Such a medium would consist of imidazole buffer at a 10 mM concentration and a pH of 6.7. At pH 6.7, approximately 70% of the imidazole was in the protonated form, i.e., the ionic strength of the imidazole solution was 70% of the molarity.

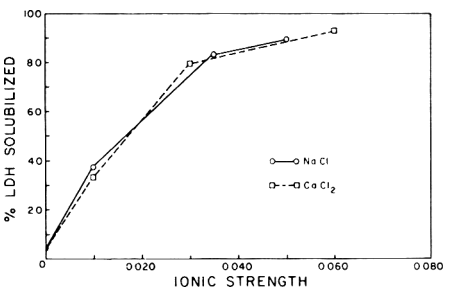

Fig. 2—Solubilization of LDH as a function of ionic strength. Bound LDH fractions from a 5% whole homogenate were rehomogenized in NaCl and $CaCl_2$ solutions. The solutions were centrifuged for 30 min at 40,000 rpm to obtain supernatant and sedimented fractions.

What appeared to be a lower solubilization in Figure 3 compared to the ionic strength obtained with NaCl or $CaCl_2$ as seen in Figure 2 was due to the fact that the contribution to the ionic strength by the buffer in the experiments of Figure 2 (approximately 0.01) was not taken into account.

Influence of cell metabolites on solubilization of LDH

The substrates and products of the LDH-catalyzed reaction were examined to see whether they could solubilize the enzyme (Table 2).

A concentration normally used for the assay of LDH activity ($1 \times 10^{-4}M$ DPNH) caused solubilization of about 70% of the enzyme. The contribution of this concentration of DPNH to the ionic strength of the medium is negligible. The effects by pyruvate and lactate were small, most likely due to their contribution to the ionic strength of the media. DPN^+ appeared capable of releasing somewhat less than half of the bound enzyme.

Table 2—Solubilization of LDH by DPNH, DPN^+, pyruvate, and lactate.

Reagent	Molar concentration	% in supernatant
DPNH	0.00	7.8
	2×10^{-5}	24.9
	5×10^{-5}	43.6
	1×10^{-4}	79.0
	1×10^{-3}	90.5
DPN^+	0.00	8.6
	1×10^{-5}	18.3
	1×10^{-4}	39.4
	2×10^{-4}	44.8
	3×10^{-4}	47.2
Pyruvate	0.00	7.5
	3×10^{-3}	22.8
Lactate	0.00	7.5
	3×10^{-3}	18.0
NaCl	0.00	4.5
	3×10^{-3}	14.4

LDH-rich sediments were rehomogenized in 10 mM imidazole buffer, pH 6.7, with the test reagents. The reagents were made up in 0.02*M* tris buffer, pH 7.4. The dilution of the reagents was such that there was no effect on the pH of the suspensions which remained at pH 6.7. The rehomogenized solutions were centrifuged at 40,000 rpm for 30 min to obtain supernatant and sedimented fractions. The percentage in the supernatant fraction is the LDH activity in the supernatant fraction divided by the sum of the LDH activity in the supernatant and sedimented fractions.

Experiments were conducted to see whether or not combinations of the substrates and products of the LDH-catalyzed reaction modified the respective effects of each on solubilization of the enzyme. In Table 3 the observed values for the combination of DPN^+ plus DPNH agree rather closely with the calculated

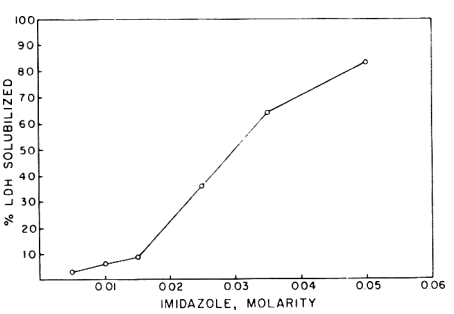

Fig. 3—Solubilization of LDH at different concentrations of imidazole buffer. Bound LDH fractions from a 5% whole homogenate were rehomogenized in solutions of imidazole buffer (pH 6.7) at several concentrations. The solutions were centrifuged for 30 min at 40,000 rpm to obtain supernatant and sedimented fractions.

Table 3—Solubilization of LDH by combinations of DPNH, DPN^+, pyruvate and lactate.[1]

Reagent and concentration	Solubilization (%) Observed	Expected[2]
$2 \times 10^{-5}M$ DPNH + $5 \times 10^{-5}M$ DPN^+	51.6	57.4
$2 \times 10^{-5}M$ DPNH + $1 \times 10^{-4}M$ DPN^+	68.0	72.7
$2 \times 10^{-5}M$ DPNH + $3 \times 10^{-3}M$ Pyruvate	80.7	54.5
$2 \times 10^{-5}M$ DPNH + $3 \times 10^{-3}M$ Lactate	61.1	53.5
$5 \times 10^{-5}M$ DPN^+ + $3 \times 10^{-3}M$ Pyruvate	79.8	58.7
$5 \times 10^{-5}M$ DPN^+ + $3 \times 10^{-3}M$ Lactate	63.1	56.5

[1] Same test conditions were used as described in Table 2.
[2] Calculated from solubilization values for each reagent measured individually at the time of the experiment.

Table 4—Solubilization of LDH by cell metabolites structurally related to DPNH.[1]

Metabolite[2]	% in supernatant
None	5.5
DPNH	87.7
TPNH	90.7
ATP	14.9
ATP + Mg^{++}	23.2
Adenosine	6.2
Nicotinamide	6.8
Pyrophosphate	22.8
TPN^+	32.2

[1] Same test conditions were used as described in Table 2.
[2] All concentrations were $4 \times 10^{-4}M$.

Table 5—Rebinding capacity of LDH in the presence of DPNH, DPN^+, pyruvate, and lactate.

Reagent	Molar concentration	% Rebound
DPNH	0.00	80.8
	2×10^{-5}	48.1
	5×10^{-5}	28.1
	1×10^{-4}	8.5
	1×10^{-3}	4.4
DPN^+	0.00	90.0
	1×10^{-5}	82.0
	5×10^{-5}	59.5
	1×10^{-4}	60.7
Pyruvate	0.00	84.5
	3×10^{-3}	77.7
Lactate	0.00	91.0
	3×10^{-3}	82.6

Enzyme solutions dialyzed against distilled water were rehomogenized with LDH-free sediments in the presence of the test reagents. The reagents were made up in 0.02*M* tris-HCl buffer, pH 7.4. The pH values for all of the rehomogenized solutions were approximately 6.7. These solutions were centrifuged for 30 min at 40,000 rpm to obtain supernatant and sedimented fractions. % rebound equals LDH activity in the sedimented fraction divided by the sum of the LDH activities in the supernatant and sedimented fractions.

percentages for release. The calculated values are the values obtained by adding the separate effects of each compound.

The combination of DPNH or DPN^+ plus pyruvate or lactate caused more solubilization than was expected. This effect was especially pronounced for pyruvate. Presumably the binding of DPNH (and DPN^+) to LDH results in conformational changes which affect the binding of the enzyme to the particulate structures. Binding of substrate then leads to further conformational changes in the enzyme which affect binding still further. Pyruvate and lactate can probably only affect binding in a significant way in the presence of cofactor since it is necessary for the pyridine nucleotide to bind before the pyruvate or lactate can (Bolotina et al., 1967). The greater affinity of the enzyme for pyruvate versus lactate probably explains why the synergistic effect on solubilization is greater with pyruvate.

The specificity of the solubilizing agents and the correlation between binding affinities of these compounds with LDH (Bolotina et al., 1967) and their efficiency as solubilizers indicate that the interaction of these compounds is with the LDH enzyme rather than with sites on the particulate structures.

To determine whether a portion or all of the cofactor molecule was involved in the solubilization, different cell metabolites with structural similarities to DPNH were tested for their effect on solubilization of LDH. From the values in Table 4 it was concluded that the phosphate groups of the DPNH molecule were more involved than nicotinamide or the nucleotide, adenosine. However, the phosphate group alone gave only 20–25% release. TPNH was as effective as DPNH in causing solubilization. Thus solubilization requires a structural configuration similar to DPNH or TPNH, but is not so specific as to differentiate the two.

The charge on the solubilizing agent is also important as seen by the different solubilization patterns of DPN^+ compared to DPNH (Table 2) and TPN^+ compared to TPNH. This difference may be due to the firmer association of the reduced nucleotides with LDH (Bolotina et al., 1967). The level of TPNH in skeletal muscle is low in comparison to DPNH, and it takes part in the LDH-catalyzed reaction only one hundredth as effectively as DPNH. However, the effectiveness of TPNH in causing solubilization together with its possible involvement in LDH catalysis (Navazio et al., 1957) may have implications in metabolic control of the pentose shunt.

After finding that the binding of LDH was dependent on the level of DPNH in the medium, further studies were conducted to relate the solubilization of LDH by DPNH at different tissue concentrations. Information at low protein concentrations would enable us to judge whether the enzyme remained bound during the assay, while results at high protein concentrations might aid in extrapolating results in vitro to actual conditions in situ.

Figure 4 shows that as the tissue concentration was lowered from 36 to 0.5%, LDH was more readily solubilized at equal concentrations of DPNH. These data strongly indicate that in the assay cuvette the enzyme is present solely in its soluble state even in the presence of particulate matter and at a pH and ionic strength which alone would not cause solubilization.

In the assay, the range of DPNH concentration was the same as was used for this solubilization study, but the enzyme-tissue concentration was much less, by as much as 500 to 1000 times relative to the 5% preparation. The enzyme in the assay was present at such low concentrations to insure that it would be saturated with substrate during the course of the reaction and give a reading which was not too fast to be followed. To retain greater than 90% of the enzyme on the particulate structures during the assay, the tissue concentration had to be kept at about 20% and the DPNH concentration below 0.01 mM.

The effect of tissue concentration on solubilization by DPNH was directly opposed to observations on the effect of tissue concentration in the original whole homogenate (Table 1). It must be emphasized, however, that the conditions were different in the two sets of experiments. The molar salt concentration in the experiments of Figure 4 was held constant, while in the experiments of Table 1, it varied directly with tissue concentration. This would indicate that solubilization by salt was a greater factor than solubilization by any DPNH (or TPNH) in the tissue in the latter case. Further, one would expect that most of the DPN in the homogenized muscle tissue would be present in the oxidized form (Long, 1961). This would make it far less effective as a solubilizing agent.

We had previously shown that solubilized LDH from chicken muscle could bind to the particulate fraction washed free of the enzyme (Hultin et al., 1966b). We studied the effectiveness of the metabolites involved in the LDH-catalyzed reaction in inhibiting this binding. Table 5 compares the rebinding capacity of solubilized LDH in the presence of DPNH, DPN^+, pyruvate and lactate. DPNH inhibited binding at concentrations which would cause only a negligible contribution to the ionic strength of the medium. The latter three agents were not

as effective as DPNH in preventing rebinding. Again the effects given by pyruvate and lactate were small enough to be the result of their contribution to the ionic strength of the media.

Heat lability of bound and soluble LDH

Heat inactivation characteristics of various forms of LDH were studied to determine whether binding imparted increased stability to the enzyme and thus offered a reason for LDH binding in situ. Figure 5 shows the heat lability of four forms of LDH with time at 46°C.

The "soluble" fraction, as opposed to the "bound" fraction, is that not bound to particles during the original homogenization and centrifugation. The other two fractions have been solubilized from the "bound" fraction by DPNH or NaCl. The LDH fraction solubilized by DPNH is apparently the most stable while the soluble and bound fractions are the least. With respect to heat stability, DPNH seems to make muscle-type LDH resemble the heart-type (Fondy et al., 1965). Thus, DPNH simultaneously increases the heat stability and decreases the binding ability of muscle-type LDH. Presumably these effects are brought about by conformational changes induced by the DPNH. We have not been able to demonstrate binding of heart-type LDH to cellular particulate structures.

An electrophoretic analysis demonstrated that brown trout skeletal muscle contained only one LDH isozyme, i.e., the muscle type. Only 25% of this enzyme was found to bind to cell particulate matter in a 5% whole homogenate prepared in distilled water. This binding value could be changed by varying the tissue concentration in the whole homogenate. The greater the tissue concentration, the greater was the solubilization of LDH from the particulate matter.

The limiting factor in the binding noted above (25%) was apparently not the number of binding sites on the particulate matter since if a more concentrated enzyme solution (that obtained from a 20% homogenate) is rebound to LDH-free particles, greater LDH activity is associated with the particulate fraction.

Homogenized chicken breast muscle at a 5% concentration retains 98–99% of its LDH with the particulate fraction (Hultin et al., 1966b) but has a lower pH than that of the trout muscle homogenate which is about 7.0. This lower pH is possibly caused by higher glycogen levels in the chicken muscle since the fish ordinarily were fasted before they were sacrificed. However, the pH of the whole homogenate was ruled out as the cause for the restricted binding in the trout muscle homogenate since a whole homogenate of fish muscle buffered at pH 6.4, the pH of a typical chicken homogenate, did not retain a higher percentage of LDH on the particulate matter.

Since certain metabolites (DPNH, TPNH, DPN+, etc.) can cause solubilization of LDH, it is possible that their presence in the homogenate, together with the pH and ionic strength, may render a considerable amount of the enzyme soluble. It appears that the LDH from trout muscle is more easily solubilized than that from chicken.

To study the kinetic behavior of LDH when it is bound to particles, the tissue level would have to be kept at about 20% and the DPNH concentration below 0.01 mM. However, under these conditions the standard spectrophotometric assay procedures cannot be employed. At such a high ratio of enzyme to substrate, the DPNH would be oxidized too rapidly to permit accurate rate determinations and the level of insoluble protein would be too high to allow use of a standard spectrophotometer. The activity of bound LDH will therefore have to be followed by a method which will allow measurement under these conditions.

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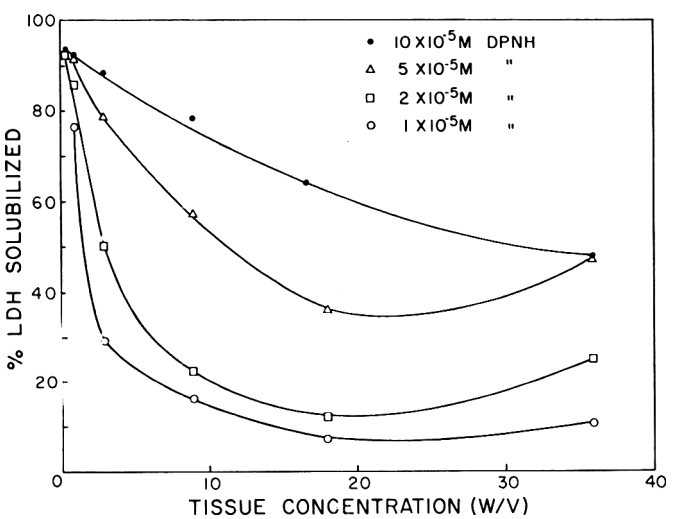

Fig. 4—Solubilization of LDH by DPNH as a function of tissue concentration. LDH-rich particulate fractions prepared from tissue homogenized at concentrations from 0.5 to 36% were rehomogenized in solutions buffered with imidazole (10 mM, pH 6.7) with increasing levels of DPNH. The solutions were centrifuged for 30 min at 40,000 rpm to obtain supernatant and bound fractions.

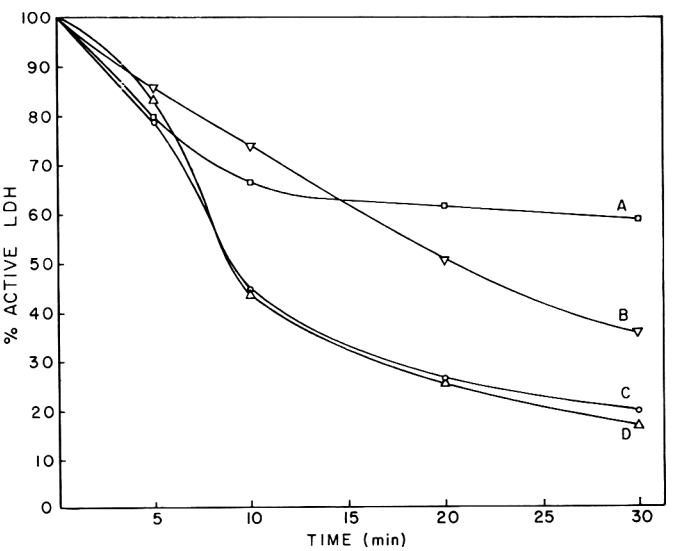

Fig. 5—Heat stability of LDH fractions at 46°C. A. Supernatant fraction obtained after centrifugation of LDH-rich sediments homogenized in a solution of DPNH, 1×10^{-3}M. Free DPNH was then removed by dialysis. B. Supernatant fraction obtained after centrifugation of a suspension of LDH-rich sediments homogenized in NaCl, 0.1M. C. Supernatant fraction obtained after centrifugation of 5% whole homogenate prepared in distilled water. D. Bound fraction of a 5% whole homogenate prepared in distilled water. Bound fraction was rehomogenized in 10 mM imidazole buffer, pH 6.7.

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Physicochemical Nature of Banana Pseudostem Starch

SUMMARY—Starch was isolated from banana pseudostem and its properties were compared with those of potato, corn and tapioca. The starch granules are irregular in shape and are bigger in size than those of the fruit starch. At 60°C, the granules start to swell, gradually increase in size, attain their maximum size at 75°C and do not rupture even after heating to 100°C. The intrinsic viscosity of the starch (2.05) is similar to that of potato starch (2.00). The amylose content of the starch compares well with that of banana fruit and potato (tuber) starch (21%). In general, banana pseudostem starch resembles potato starch.

INTRODUCTION

THE BANANA pseudostem contains 2–3% starch of good quality and it can be readily extracted (Subrahmanyan et al., 1957). In view of the importance of meeting the cereal food shortage in this and other countries, any new source of starch is welcome. The banana pseudostem, which is otherwise a waste material after the fruit is harvested, is a useful source of additional starch. It was, therefore, of interest to compare this starch with other well-known cereal and tuber starches so that it could be profitably utilized in various ways. The data collected during this investigation are presented in this paper.

MATERIAL & METHODS

STARCH was extracted from the pseudostem of the Maduranga variety by the method of Subrahmanyan et al. (1957). The material was disintegrated in a Waring Blendor and passed through a 100 mesh sieve. The slurry was channeled, and the starch collected was dried at 70°C. The starch was defatted with ether and taken for analysis.

Analytical methods

Ash, moisture and starch were determined by standard AOAC (1965) methods. Protein was estimated by "Kjeldahl's method" (Ma et al. 1942).

Swelling property. This was determined as follows: 0.5 g of starch was suspended in 30 ml water, heated to different temperatures (Fig. 1) and maintained at the desired temperature for 15 min. It was then cooled to room temperature (25–28°C), and the volume made up to 50 ml. The suspension was then centrifuged in a graduated tube and the volume of starch in the tube noted.

$$\text{Volume of granules \%} = \frac{\text{Volume of centrifuged granules} \times 100}{\text{Total volume of suspension}}$$

Intrinsic viscosity. This was determined by the method of Lansky et al. as mentioned by Kerr (1950). An Ostwald pipette No. 1, maintained at 35 ± 1°C in a thermostat, was employed for determining the specific viscosity.

Alkali number. This was determined by following the procedure given in Kerr, 1950.

Action of Takadiastase. To measure the change in the blue color of starch-iodine reaction during the enzymic breakdown of starch by Takadiastase, 100 ml of 1% starch suspension were prepared in a 250-ml flask and its pH adjusted to 4.5 with acetate buffer. The suspension was heated to 55°C, 0.1 g of Takadiastase added and the reaction mixture maintained at 55°C in a thermostat. At intervals of 0, 5, 10, 15, 20, 25 and 30 min, 5 ml aliquots of the reaction mixture were removed, the enzyme destroyed by boiling and the volume made up to 50 ml with water. To 10 ml of this, 2 ml of N/500 iodine solution were added, the volume made up to 25 ml and its light transmission measured at 650 μ in a Lumetron colorimeter.

Fractionation of starch. 20 g of defatted starch, suspended in 80 ml of water, were slowly added with constant stirring to a boiling mixture of butanol:water (1:10), heated on a steam bath. The paste was autoclaved for 3 hr at 18 lb pressure. To the suspension, a 1:1 mixture of butyl and amyl alcohol was added, and the suspension cooled slowly to 25°C, the beaker being wrapped with cotton for slow cooling. The supernatant was siphoned off carefully. The precipitated amylose was separated by centrifuging the left-over suspension. The precipitate was washed and centrifuged repeatedly with butanol-saturated water. It was recrystallized from boiling water in the presence of butanol. Amylopectin was precipitated from the supernatant by addition of an excess of methanol and dried by treating with fresh methanol.

A standard curve was drawn according to the method of Kerr et al. (1943), using different ratios of amylose and amylopectin of pseudostem starch. Composition of different starches was determined by making use of this curve.

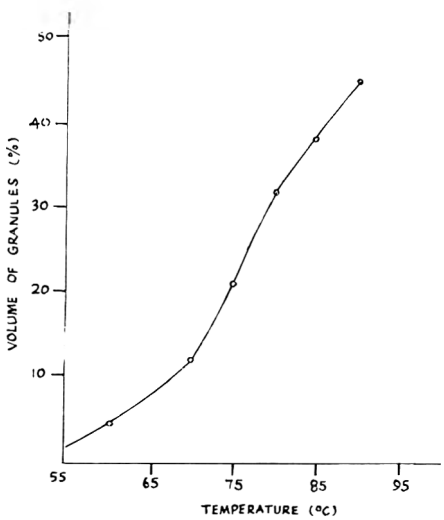

Fig. 1—Gelatinization of starch.

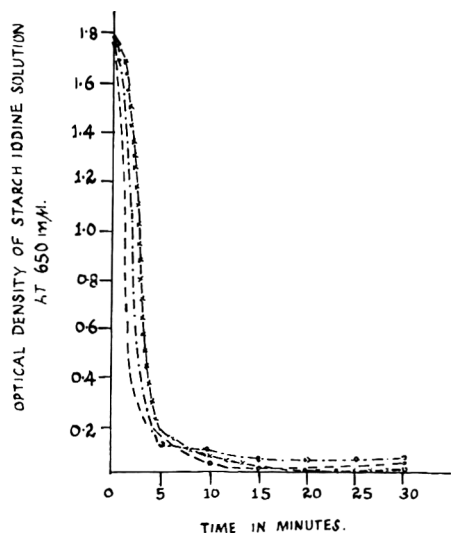

Fig. 2—Action of Takadiastase on starch. ○- - - - - -○ Banana starch (fruit); ○— • — • — • —○ Banana starch (pseudostem); ○— X — X — X — X —○ Potato starch.

RESULTS & DISCUSSION

Microscopic structure

The banana pseudostem starch granules (Fig. 3) resembled those of Curcuma starch, but were smaller in size (width 7 μ, length 53 μ), many of them being in the range of 30–50 μ. Their shape varied considerably, some of them having quadrangular, triangular, pear-like, curved and irregular shapes. Layers were not as distinct as in the case of fruit starch, whose granules were smaller (7–33 μ) and less irregular in shape.

Composition of isolated pseudostem starch

The composition of this starch was as follows: Moisture 11.30%, ash 0.30%, protein ($N \times 6.25$) 0.19 and starch value 87.8%.

Swelling property

The relative increase in volume with increase in temperature in the case of banana pseudostem starch is shown in Figure 1. The starch granules, which start swelling at 60°C, gradually increase in size and attain their maximum size at 75°C and above. They do not, however, rupture even when heated to 100°C

Intrinsic viscosity

Table 1 shows that the intrinsic viscosity of banana pseudostem starch (2.05) is lower than that of tapioca starch (2.55) and higher than that of corn starch (1.55), but nearly identical to that of potato starch (2.00).

Table 1—Intrinsic viscosity, alkali number and amylose content of banana pseudostem starch as compared with other starches.

Source of starch	Intrinsic viscosity	Alkali number	Per cent amylose
Banana pseudostem starch	2.05	4.75	21.0
Banana fruit starch	2.05	2.05	21.0
Tapioca	2.55	6.00	16.5
Potato	2.00	6.00	21.0
Corn	1.55	10.60	26.0

Alkali number

In its alkali number (Table 1), the pseudostem starch approaches tapioca and potato.

Color with iodine

The change in the optical density of the starch-iodine reaction color with the progress of enzymic breakdown of starch by Takadiastase is shown in Figure 2. The pseudostem starch behaves practically the same as potato starch and banana fruit starch in regard to enzyme hydrolysis.

Starch fractions

The butanol-precipitated amylose fraction of the pseudostem starch had a 6-lobed petal-like structure and resembled that of amylose in potato starch (Fig. 4). Table 1 shows that the pseudostem starch compares well in its amylose content with other well-known tuber starches. According to the data reported in the paper, banana pseudostem starch is similar to potato starch in many respects, but different from that of tapioca and corn.

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Fig. 3—Banana pseudostem starch granules.

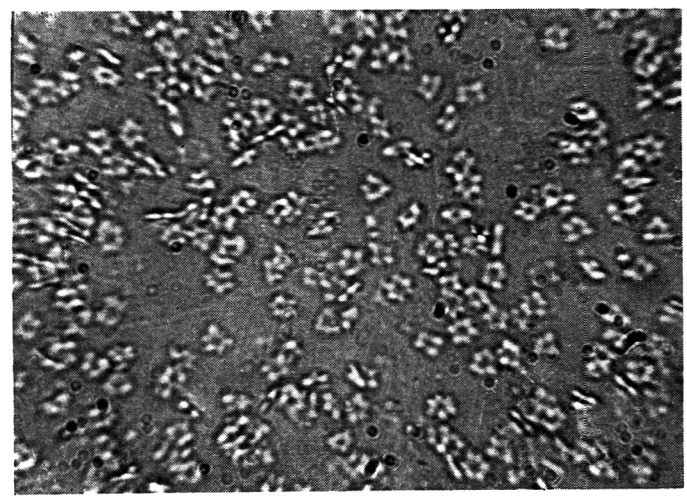

Fig. 4—Butanol-precipitated amylose fraction of banana pseudostem starch.

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Accumulation of Starch in Banana Pseudostem and Fruit

SUMMARY—The rhizome, pseudostem and the unripe fruit of the banana have a high starch content. Its concentration is higher in the middle fleshy leaf sheaths and increases gradually towards the rhizome downward along the length of the pseudostem. During the growth of the plant, starch accumulates in the pseudostem and reaches its maximum at the time of inflorescence and remains practically constant thereafter until the harvesting of the mature bunch. After the removal of the mature bunch, there is a gradual decrease in the accumulated starch, when the stem is allowed to stand in the field. If, however, it is felled and stored in the shade, the decrease in starch content is rapid and even after a period of two days, the starch content is reduced to such a great extent that its extraction becomes rather uneconomical. It is, therefore, necessary to utilize the pseudostem for processing soon after harvest of the bunch. This is a highly important consideration in the economic utilization of the pseudostem. Removal of the inflorescence, with a view to increase the starch content in the pseudostem by arresting its transfer to the fruit, is neither feasible nor economical.

INTRODUCTION

GREAT ECONOMIC importance is attached to the growing of bananas in many of the tropical countries of the Americas, Africa and Asia. Annual production in India varies between 1.7 and 2.0 million tons. World production is about 20 million tons. Once the plant has borne fruit, the bunch is cut and the trunk is generally left to decompose in the field. It has recently been shown that the trunk or pseudostem, which produces fibers of good quality, is also a source of high quality starch.

Production of starch from this source is, therefore, an economically important development. In view of the potentialities of banana pseudostem as a basic raw material for starch and paper pulp, a systematic investigation was undertaken to study the course of development and distribution of starch in different parts of the plant, to determine the time as well as pattern of its maximum accumulation and the changes that take place in the starch content after the bunch is harvested. The effect of removal of inflorescence at the very outset, on the accumulation of starch in the pseudostem was also studied. The results of the investigation are presented and discussed in this paper.

MATERIALS & METHODS

TWO VARIETIES of banana, *Maduranga* (*Musa paradisiaca* Linn) and *Rasabale* (*Musa sapientum* Linn), were selected from a 4-acre plantation in the local municipal farm. The plants were cultivated under normal commercial conditions of cultivation. The entire plant, including the rhizome and leaves, was removed at each stage and the different portions were sampled for analysis. These included leaf, leaf stalk, leaf sheath (which constitutes the pseudostem), central core, bract, flower, fruit and rhizome. The number of plants taken in each case is indicated in the tables.

The fresh weight of a plant ranged from 1 to 30 kg, depending upon the age of the plant and variety, as is shown in Tables 4 and 5. Portions of different parts of the plant were cut into small pieces and in each case random lots taken for further cutting into smaller pieces. From the latter, representative samples were taken at random on

Table 1—Distribution of starch in different parts of mature banana plant—(Var. Rasabale)—Age: 15 months.[1]

Sample	Fresh weight of different portions of the plant (kg)	Moisture (%)	Total starch present in different parts (g)	Starch on dry weight basis (%)
Leaf	12.00	68.7	147	3.90
Leaf base	2.00	91.5	17.5	10.30
Leaf sheath (Pseudo-stem)	24.00	86.50	832	25.50
Central core	0.12	94.80	1.18	19.00
Rhizome	7.00	90.40	212	31.60
Flower	0.25	88.40	3.36	11.20
Bract	0.60	91.20	10.8	20.00
Fruit	9.00	66.80	1773	59.60

[1] Data are the averages for 4 plants.

Table 2—Variation in starch content from the core to the outermost sheath (Var. Rasabale) —Age: 6 months. Dry weight basis.

Sheath number[1]	Plant No. 1 Fresh weight (g)	Plant No. 1 Total starch (g)	Plant No. 1 Starch content (%)	Plant No. 2 Fresh weight (g)	Plant No. 2 Total starch (g)	Plant No. 2 Starch content (%)
Central core	50	0.28	10.20	75	0.50	14.00
1	170	1.09	11.90	180	1.14	12.70
2	280	2.38	15.75	240	1.10	14.85
3	290	3.50	17.25	235	2.46	17.50
4	300	4.10	19.60	235	3.04	17.75
5	300	4.36	20.80	185	2.50	18.60
6	280	4.86	21.75	130	1.87	19.50
7	250	3.40	22.75	130	2.25	21.70
8	195	2.94	24.20	100	2.06	27.50

[1] Sheath numbers 1–8 represent the order of leaf sheath, sheath 1, being the inner most and sheath 8, outermost.

fresh weight basis.

Starch content of these was determined by the Takadiastase method (Allen, 1948) and sugars by Somogyi's method (Hodge et al. 1952). The analytical data are presented in tabular form. Table 1 shows the distribution of starch in different parts of a banana plant of the *Rasabale* variety (age 15 months) bearing a bunch of mature fruit. Data regarding the starch content of individual leaf sheaths in the case of two 6 months old *Rasabale* plants are given in Table 2. In Table 3, data are given for starch content in different portions of the pseudostem along its axis.

The starch content of the pseudostem at different stages of its growth is shown in Tables 4 and 5 for *Maduranga* and *Rasabale* varieties, respectively. The starch was extracted by the Blendor method of sieving and washing as described by Subrahmanyan et al. (1957). The data show the range as well as the mean values, based on the analysis of 2–6 plants.

In Tables 6 and 7, data are given for the distribution of starch in the leaf, pseudostem, rhizome and fruit at different stages of growth, in the case of *Rasabale* and *Maduranga* varieties, respectively. Table 8 gives data for the distribution of reducing and total sugars in the central core and sheath of the *Maduranga* variety.

To study the effect of removing the inflorescence on the starch content of the pseudostem, 12 plants of the *Maduranga* variety, which had just borne inflorescence, were selected. In six of the plants, the inflorescence was cut off and the remaining six, which served as control, were allowed to bear fruit. Two plants were removed in the case of each treatment, at start, after one month and after two months thereafter, for the determination of starch content. Inflorescence had just appeared on the plants 11 months after planting the suckers. The data are given in Table 9.

For the study of the post harvest variation in the starch content, 24 fully mature plants, from which the bunches had been harvested on the same day, were allowed to remain in the field for varying periods. Data are given in Table 10.

In the second series of experiments, the pseudostem was cut down soon after harvesting the bunch and stored in a cool place for varying periods. Data regarding variation in starch content in this case are shown in Table 11.

Table 3—Variation in starch content along the axis of the plant. Var: Rasabale,[1] Age: 8 months.

	Leaf	Stalk of the leaf	Middle portion of pseudo-stem	Base of the pseudo-stem
Fresh weight (g)	595	327	590	640
Moisture (%)	79.90	91.90	92.80	91.20
Total starch (g)	11.62	5.29	8.49	17.03
Starch (%) (dry wt basis)	9.83	20.70	19.90	30.10

[1] Data are the averages based on the analysis of six individual plants.

Table 4—Variation in the starch content of the pseudostem with the growth of the banana plant (Variety: Maduranga).

Stage of growth (mo)	Fresh weight of pseudostem (kg)		Moisture (%)		Starch calculated (%)		Starch extracted (%)		Yield of starch per stem (g)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
3 (Suckers)	1.10–1.37	1.19	93.6–95.0	94.3	8.6–9.8	9.4	0	0	0	0
6 (Suckers)	2.7–2.9	2.8	92.3–93.0	92.6	13.0–13.1	13.05	8.5–9.0	8.9	19.0–20.0	20
9	6.4–8.75	7.6	89.1–89.7	87.4	19.4–20.2	19.8	18.6–19.0	18.8	130–190	180
11 (Inflorescence stage)	15.4–25.4	19.8	87.1–90.5	88.9	28.7–39.8	32.95	27.7–37.1	29.8	480–730	653
13	20.5–25.4	22.3	89.3–91.7	90.5	24.1–31.50	27.9	19.00–21.2	20.06	410–430	424
14	17.0–20.5	18.5	89.3–91.5	90.4	21.5–30.7	25.43	15.2–20.3	17.7	250–320	314
15 (Harvesting stage)	18.0–21.1	19.8	90.1–91.4	90.7	21.8–25.5	23.7	17.0–20.8	18.8	340–350	345

Table 5—Variation in starch content in the pseudostem with the growth of the banana plant. (Var: Rasabale).

Stage of growth (mo)	Fresh weight of pseudostem (kg)		Moisture (%)		Starch calculated (%)		Starch extracted (%)		Total yield of starch (g)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
11	20.5–31.0	27.4	86.4–89.0	87.5	24.5–32.7	27.7	21.00–32.50	27.06	700–1230	927
12	22.0–28.5	25.2	85.4–86.4	85.9	28.0–28.6	28.3	27.5–27.6	27.5	830–1162	977
15	21.0–26.0	22.5	84.8–87.3	85.6	26.7–39.2	33.40	23.0–37.50	30.00	880–1130	972

Table 6—Distribution of starch in different tissues of the banana plant during different periods of its growth (Variety: Rasabale).[1]

Samples	Age of the plant (mo)																
	5			6			7			12				15			
	Leaf	Pseudo-stem	Rhi-zome	Leaf	Pseudo-stem	Rhi-zome	Leaf	Pseudo-stem	Rhi-zome	Leaf	Pseudo-stem	Rhi-zome	Green tender fingers (1-mo-old)	Leaf	Pseudo-stem	Rhi-zome	Green mature fruit (4-mo-old)
Fresh weight (kg)	0.59	1.46	0.67	0.49	2.19	1.15	0.81	2.90	1.18	11.00	24.00	5.00	4.00	12.00	24.00	7.00	9.00
Moisture (%)	79.50	92.00	80.00	76.10	93.80	84.35	77.20	92.00	83.50	65.20	85.40	74.70	86.80	68.70	89.00	90.40	66.80
Starch (%) (D.W.B.)[2]	5.80	8.90	14.20	5.70	8.30	15.50	3.50	7.30	26.10	2.22	28.50	34.40	14.20	3.90	29.09	31.60	59.60
Total starch present (g)	7.02	10.39	19.02	6.66	11.28	27.90	6.48	16.92	50.74	84.98	998	435	75	147	768	212	1773

[1] Data based on the mean for two plants.
[2] D.W.B. = dry weight basis.

Table 7—Distribution of starch in different tissues of the banana plant during different periods of its growth (Var: Maduranga).[1]

Age of the plant (mo)	4 Young suckers			12 Plant bearing small tender fingers				15 Plant with fully mature fruit ready for harvest			
Samples	Leaf	Pseudostem	Rhizome	Leaf	Pseudostem	Rhizome	Tender fingers (1-mo-old)	Leaf	Pseudostem	Rhizome	Raw fruit (4-mo-old)
Fresh weight (kg)	0.30	1.24	0.53	10.00	22.25	7.50	5.0	11.51	24.00	8.00	10.0
Moisture (%)	78.80	94.10	84.10	68.35	89.70	84.10	86.40	69.00	91.50	89.20	65.00
Starch (%) (D.W.B.)[2]	3.96	9.13	21.38	1.90	21.10	31.20	29.70	3.00	25.60	36.30	63.00
Total starch present (g)	2.50	6.66	18.02	60.00	482	372	195	107	520	312	2200

[1] Data based on the mean for two plants.
[2] D.W.B. = dry weight basis.

RESULTS & DISCUSSION

TABLE 1 shows that the moisture content of the different parts such as leaf, fruit, leaf sheath, rhizome, etc., is variable and so also their starch content expressed on dry weight basis. Regarding the distribution of starch, the mature fruit contains the highest amount (1773 g). Apart from the fruit, the pseudostem (832 g) is the richest source of starch in the plant, while the rhizome (212 g) comes next.

Variation of starch content

Table 2 shows that the central core of the pseudostem contains much less starch than the sheaths that enclose it. The percentage of starch increases from the central core to the outer sheath. The total amount of starch present in the middle sheaths is, however, considerably more than in the inner or outer sheaths. The percentage as well as the total amount of starch increases from the leaf stalk down to the base of pseudostem (Table 3).

Accumulation of starch in the pseudostem

As seen from Tables 4 and 5, the percentage of starch increases from the sucker stage onward, reaches its maximum when the plant bears inflorescence, then decreases slightly thereafter and remains practically constant until the time of harvesting the fruit bunch.

During the early period of growth (Tables 6 and 7), starch accumulates in the rhizome, while it remains practically constant in the pseudostem. After the initiation of the inflorescence, there is no further increase in the starch content of the rhizome or of the pseudostem, while there is an increase in that of the fruit. Leaves contain very little starch, the maximum amount found being only about 6% of their dry weight.

The starch, which accumulates in the rhizome until the onset of inflorescence, appears to be transferred, in the form of its enzymic hydrolysis products, namely sugars, for *de novo* synthesis in the developing fruit, with the result that there is no further increase in starch content in the rhizome.

The starch as well as sugar contents are maximal at the time of inflorescence. There appears to be subsequent transference of starch in the form of sugars for resynthesis in the developing fruit, through the central core of the pseudostem, as evidenced by the considerably higher sugar content in the core than in the outer sheath (Table 8). However, in the case of plants from which the inflorescence had been removed, soon af-

Table 8—Distribution of reducing and total sugars in the central core and sheath of banana plant (var: Maduranga).[1]

	Plant with inflorescence just set (11 mo) (Control)		30 days after the initiation of inflorescence (12 mo) (Control)		30 days after removing the inflorescence (12 mo)	
	Central core	Pseudo-stem	Central core	Pseudo-stem	Central core	Pseudo-stem
Moisture (%)	92.60	90.00	92.50	93.70	94.70	93.00
Reducing sugars (%) (D. W. B.)	25.40	11.30	23.70	25.20	18.00	18.30
Total sugars (%) (as reducing sugars) (D. W. B.)	39.00	15.50	25.00	31.70	19.60	25.40

[1] Data based on the mean for two plants. [2] D. W. B. = dry weight basis.

Table 9—Effect of removal of the inflorescence on the starch content of banana pseudostem (Var. Maduranga).

Stage of maturity	Fresh weight of stem (kg)		Per cent starch calculated (D.W.B.)[1]		Per cent starch extracted (D.W.B.)		Yield of starch per stem (kg)	
	Control	Inflorescence removed	Control	Inflorescence removed	Control	Inflorescence removed	Control	Inflorescence removed
1. Plant bearing the inflorescence (11 mo)	22.30 15.40	22.30 15.40	30.00 39.82	30.00 39.82	27.13 37.16	27.13 37.16	0.68 0.65	0.68 0.65
2. One month after the appearance of the inflorescence (12 mo)	25.40 21.00	26.00 21.00	24.10 28.00	30.80 32.30	24.10 28.10	28.42 32.30	0.41 0.42	0.89 0.88
3. Two months after the appearance of the inflorescence (13 mo)	20.50 17.00	12.00 11.5	21.50 21.50	15.00 13.00	17.60 15.10	9.40 7.00	0.30 0.27	0.17 0.13
4. Three months after the appearance of the inflorescence (14 mo)	21.10 20.30	14.00 9.00	25.50 24.10	4.30 1.40	18.60 17.00	Trace Trace	0.34 0.35	Trace Trace

[1] D.W.B. = dry weight basis.

Table 10—Post-harvest variation in starch content of banana pseudostem (Var: Maduranga).[1]

No. of days after harvesting of the bunch	Fresh weight of stem (kg)	Moisture (%)	Starch content (on dry basis) (%)		Yield of starch per stem (g)
			Calculated	Extracted	
0	13.25	92.50	30.70	24.80	246
4	10.70	87.38	25.20	20.20	273
6	9.25	90.80	24.50	20.00	170
10	10.00	90.70	18 00	8.05	75
12	10.00	92.30	16 10	8.00	62
16	9.00	92.30	16 20	7.30	49

[1] Data are the average values for 4 plants.

Table 11—Post-harvest changes in the starch content of pseudostem removed from the field and stcred in the shade at R.T. (25–28°C) (Var: Maduranga).[1]

No. of days after harvesting[2] of the bunch	Weight of stem (kg)	Moisture (%)	Starch content (on dry basis) (%)		Yield of starch per stem (g)
			Calculated	Extracted	
0	14.50	91.00	26.60	21 10	175
2	14.00	91.10	21.00	15.70	196
3	10.00	92.00	20.10	15.40	123
5	9.00	93.20	12.30	Trace	Trace
7	8.30	93.00	7.50	Trace	Trace

[1] Data are the average for four plants. [2] Harvested after 15 months.

ter its onset, neither the starch nor the sugar content increased (Tables 8 and 9). Further, the fresh weight of the pseudostem decreased, indicating disturbance in the development of the plant. The possibility of halting or arresting the transfer of starch from the pseudostem, by removing the inflorescence, does not, therefore, appear to be feasible or economical.

For the better utilization of the banana plant as a whole, the plant should be allowed to bear fruit, and after harvesting the mature fruit, the pseudostem removed for extraction of starch, in which case starch will be an additional factor of income to the grower.

It is of interest to note that *Rasabale*, which is a highly prized table variety, contains a higher amount of starch in its pseudostem than *Maduranga*, which is a cooking variety.

Post-harvest variation in starch content

After harvesting the mature fruit bunch, the banana plant is either cut down shortly after, or allowed to stand in the field for a few more days. It was, therefore, of interest to study the effect of these practices on the starch content of the pseudostem. As shown in Table 10 there is a decrease in the starch content of the pseudostem if it is allowed to remain in the field after the bunch is removed, perhaps due to the enzymatic degradation. Up to 4 days after the bunch is removed, the decrease in starch content is small. Beyond that period, this decrease is so high that utilization of the pseudostem for the extraction of starch would not be economical.

When the pseudostems are cut soon after harvesting the bunch and stored in a cool place to facilitate their handling for the extraction of starch, there is a rapid decrease in the starch content even within a short interval of 2 days (Table 1). It is, therefore, more economical to extract starch from them soon after harvesting the bunch.

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Water-Soluble Flavor and Odor Precursors of Meat. 3. Changes in Nucleotides, Total Nucleosides and Bases of Beef, Pork and Lamb During Heating

SUMMARY—Studies were made of the influence of heating on nucleotides and total purine nucleosides and bases of beef, pork and lamb muscle. Inosinic acid was the predominant nucleotide in all three species and it was degraded by heating. Adenylic acid increased during cooking in meat from all three species. Cytidylic, uridylic and guanylic acids were present in relatively low concentrations in meat from all three species and changed little during cooking. A rapid method for estimating total nucleotides resulted in greater variation than a specific method for measuring individual nucleotides.

INTRODUCTION

THE FIRST two papers of this series (Macy et al., 1964a, 1964b) outlined the importance of water-soluble constituents as precursors of cooked meat flavor. It was assumed that the constituent molecules or their heat-degraded products acted directly as flavorants of this food.

During the last few years, however, considerable interest has developed in compounds which enhance these flavors. Monosodium glutamate has gained widespread acceptance as a flavor intensifying agent for red meats and chicken. More recently, sodium salts of certain mononucleotides which have a hydroxyl group at position 6 on the purine ring structure have been shown to exhibit a similar effect. The flavor enhancing properties of these mononucleotides (inosinic acid, guanylic acid and xanthylic acids) have been investigated (Wagner et al. 1963).

Kuninaka et al. (1964) reviewed the history and development of nucleotides as food flavoring substances in Japan and further described their properties (Kuninaka, 1966). A synergistic effect with monosodium glutamate was reported. Desirable flavors were enhanced by addition of disodium inosinate, regardless of the type of meat or cookery employed. Disodium inosinate consistently produced an impression of greater viscosity and increased flavor. The basic tastes (sweet, salty, sour and bitter) were not changed in any consistent manner. Suppression of sulfury, fatty, burnt, starchy and hydrolyzed vegetable protein flavors was apparent.

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Batzer et al. (1962) found that in addition to glucose and an unidentified glycoprotein, inosinic acid (IMP) was necessary for development of meaty flavor in beef. Nucleotides and nucleosides are also potential precursors of free ribose and ribose phosphate, which have been implicated in Maillard browning reactions.

The objective of these experiments was to determine the stability of certain so called flavor potentiators and related compounds in beef, lamb and pork during heating at various temperatures.

EXPERIMENTAL

Preparation and extraction of beef round roasts

Five U.S. Good grade top rounds from different animals were obtained from a commercial source, excess fat was removed and each divided into three approximately equal parts. Portions from the same relative areas were treated similarly. One portion from each top round was not cooked. Another portion was cooked to an internal temperature of 49°C in a 163°C oven. The remaining portion from each animal was cooked to an internal temperature of 77°C.

Each portion was wrapped in aluminum foil prior to cooking and the exudate collected. Following roasting, the portions were quickly chilled, sliced and ground twice at −3.3°C through a 1/8 in. plate. The juices collected in the aluminum foil during roasting were thoroughly mixed with the respective ground samples prior to the second grinding. The uncooked samples were ground in a similar manner.

Duplicate 30-g samples of each ground roast were homogenized with 50 ml 0.6*N* perchloric acid and filtered. The remaining solids were re-extracted with 50 ml 0.6*N* perchloric acid, filtered and the residues washed with two 10 ml portions of distilled water. The extracts were neutralized with 30% (w/w) potassium hydroxide, the precipitated potassium perchlorate removed by filtration and the filtrates diluted to 150 ml with distilled water. The samples were also analyzed for fat and moisture.

Preparation and extraction of pork loin roasts

Three pork loins were boned out, extraneous fat removed and divided into 3 nearly equal parts. One part of each loin was not cooked. The other 2 parts were wrapped in aluminum foil and roasted in an oven at 163°C. One roast from each loin was roasted to an internal temperature of 49°C while the remaining roast was cooked to an internal temperature of 71°C. Temperatures of the loin roasts were monitored with thermocouples. The tissue was ground and extracted with perchloric acid as described previously for beef.

Preparation and extraction of lamb muscle

Six hanging tenderloin muscles from freshly slaughtered lamb carcasses were divested of as much fat and connective tissue as possible and ground 5 times through a 1/8 in. plate. Eight 20-g samples were packed tightly into 20 × 180 mm test tubes and heated in a water bath at 60°C. Samples were removed at 0, 5, 10, 15, 20, 30, 45 and 60 min and cooled in an ice water bath before extraction. Two 40-ml portions of 0.6*N* perchloric acid were used to extract each samples as described for beef samples.

Ion-exchange chromatography of nucleotides

The method of Lento et al. (1964) as modified by Macy et al. (1966) was used to determine cytidylic, adenylic, uridylic, inosinic and guanylic acids. 30 ml of each extract from beef and pork and 40 ml of the extracts from lamb were analyzed for individual nucleotides

Quantitation of the results was accomplished by separation and analyses of mixtures of authentic samples of the 5′-isomers of the individual nucleotides. Mixtures containing 0.5, 1.0, 1.5, 2.0 and 2.5 mg of the nucleotides were chromatographed and fractions containing the material for each absorption peak were combined and diluted to 200 ml with distilled water. The absorbance for each compound was then determined at its maximum as follows: cytidylic acid at 280 nm, adenylic acid at 257.5 nm, uridylic acid at 260 nm, inosinic acid at 249.5 nm and guanylic acid at 257 nm.

Absorbance of each compound was a

straight line function of its concentration. Concentrations of the nucleotides in the meat extracts were determined by reference to standard curves following appropriate dilutions.

Absorbance maxima of the individual nucleotides were obtained with a Cary spectrophotometer with authentic samples of the nucleotides.

The absorbance peak for cytidylic acid of the meat extracts contained small amounts of other UV-absorbing materials which was thought to consist mainly of the 2'- and 3'-isomers, and traces of some unknown materials. All of these UV absorbing materials were reported as cytidylic acid.

Analyses of total nucleotides and nucleosides

The batchwise method of Jones et al. (1964) as modified below was used for analyses of total nucleotides and total purine nucleosides and bases.

1 ml of each beef roast extract was mixed with 1-g of damp Dowex 1 × 8 (200-400 mesh) resin (formate form) for at least 15 min with intermittent stirring. The extract and resin were washed quantitatively into a funnel plugged with a small amount of glass wool. The resin, held by the glass wool, was washed with distilled water until a volume of 25 ml was collected. The absorbance of the material washed from the resin was then read at 248 and 260 nm. Total purine nucleosides and bases were calculated from the absorbance at 260 nm of the materials washed from the resin using the molar absorbtivity for hypoxanthine.

Total nucleotides absorbed onto the resin were given by the difference between the absorbances at 248 nm of the resin-treated samples and similar non-treated samples. The molar absorbtivity of inosinic acid was used to calculate total nucleotides.

The method was further modified for the analyses of the pork and lamb samples. The materials washed with water from the ion-exchange columns used for determinations of individual nucleotides were diluted to 500 ml with distilled water and the absorbances determined at 248 and 260 nm. 30 ml of extracts from pork and 40 ml of those from lamb were diluted to 500 ml and the absorbances read at 248 and 260 nm for samples not treated with ion-exchange resin.

Analyses of fat and moisture

These analyses were carried out by the standard methods of the AOAC (1960, sections 22.034 and 22.003).

RESULTS & DISCUSSION

Effects of heating nucleotides of beef round roasts

Mean values and standard deviations of nucleotides of 5 beef round roasts cooked to 49° and 77°C internal temperatures are tabulated in Table 1. Cytidylic acid was not greatly influenced by heating, although the mean for samples roasted to 49°C was lower than that for either the raw samples or those roasted to 77°C. Heating at both temperatures resulted in appreciable increases in adenylic acid. This possibly was due to hydrolysis of adenosine di- and tri-phosphates and pentose nucleic acid.

Table 1—Nucleotide content of raw and roasted beef.[1]

Nucleotide	Internal temperature (°C)		
	Raw	49	77
	mg/100 g dry, fat-free tissue		
Cytidylic acid	13.1 ± 2.0	10.6 ± 2.2	13.0 ± 3.2
Adenylic acid	12.2 ± 3.5	18.1 ± 7.7	41.3 ± 4.1
Uridylic acid	7.1 ± 1.2	4.2 ± 2.9	5.3 ± 2.5
Inosinic acid	278.4 ± 24.6	208.4 ± 40.6	170.3 ± 38.0
Guanylic acid	2.2 ± 4.9	1.2 ± 2.8	3.1 ± 4.6

[1] Results are given as means ± S.D. of 5 top round roasts.

The concentration of uridylic acid was relatively low in all beef samples and it was decreased by heating at both 49° and 77°C. Heating decreased the quantity of inosinic acid in the beef round roast, but not below the quantities necessary for human perception as reported by Wagner et al. (1963) for disodium inosinate. Guanylic acid, another nucleotide in beef roasts with flavor enhancing properties, was present in trace amounts.

The sums of individual nucleotides, total nucleotides (batchwise method) and total purine nucleosides and bases (calculated as hypoxanthine) are presented in Table 2. The sums of the individual nucleotides decreased while the total purine nucleosides and bases increased progressively with increased heating.

Total nucleotide concentrations determined by the batchwise method were similar to the sums of the individual nucleotides in the raw samples, but the total nucleotides increased during heating. This indicated that heating beef produced some materials which absorbed UV light and was adsorbed by the ion-exchange resin, but was not accounted for among the individual nucleotides. The nature of this unknown material was not determined but it was unlikely that it could be di- or tri-phosphonucleotides, as these compounds are easily degraded by heating and heating caused the unknown material to increase.

Effects of cooking on pork loins

To study the changes of nucleotides and related compounds in pork loins during roasting, the data for each cooked sample was divided by the corresponding data for the raw sample from the same loin. These results are tabulated in Table 3.

The influence of roasting on nucleotides of pork was similar to that on beef roasts. All nucleotides decreased during roasting except adenylic acid, which increased in quantity during cooking to 71°C as did total purine nucleosides and bases. The sums of individual nucleotides decreased during heating. Total nucleotides measured by the batchwise method increased during cooking to 49°C then decreased during cooking to 71°C.

Effects of cooking on lamb muscle

Although certain trends were established by the above data for pork, changes during heating at specific temperatures were not ascertained. The heating procedures described above for lamb tissue afforded better control of the cooking temperatures. Table 4 contains data on the influence of heating at 60°C for various lengths of time on nucleotides of ground lamb muscle.

Guanylic and uridylic acids were destroyed after 5 and 15 min heating, respectively. Cytidylic acid increased gradually throughout the heating period

Table 2—Effects of roasting on nucleotides and nucleosides and bases of beef.[1]

Internal temperature (°C)	Sum of individual nucleotides	Total nucleotides[2]	Total nucleosides and bases[2]
	mg/100g dry, fat-free tissue		
Raw	314	315	101
49	243	313	122
77	233	335	159

Results are given as means of 5 top round roasts.

[2] Batchwise method of Jones et al., 1964.

Table 3—Ratios of sums of individual nucleotides, total nucleotides[1] and total purine nucleosides and bases of cooked relative to those of raw pork.

Constituent	Ratio of chemical constituents in cooked and raw pork[2]		
		Internal temp (°C)	
	Raw	49	71
Cytidylic acid	1.00	0.67	0.58
Adenylic acid	1.00	0.80	1.81
Uridylic acid	1.00	0.83	0.84
Inosinic acid	1.00	0.90	0.78
Guanylic acid	1.00	0.75	0.67
Sum of individual nucleotides	1.00	0.87	0.83
Total nucleotides[1]	1.00	1.81	1.29
Total purine nucleosides and bases[1]	1.00	1.07	1.16

[1] Method of Jones et al., 1964.

[2] Results calculated from averages of duplicate analyses of 3 pork loin roasts at each temperature.

and nearly doubled the initial value at the end of 60 min. Heating caused adenylic acid to double in quantity after 30 min, then to decrease in concentration after 45 and 60 min heating. Apparently, precursors of adenylic acid were present in the tissue up to 30 min heating time, then after their depletion, degradation of adenylic acid occurred. Inosinic acid decreased very rapidly during the first 30 min at 60°C, then increased slightly during the second 30 min heating time.

Data concerning changes in the sums of individual nucleotides, total nucleotides, (batchwise method) total purine nucleosides and bases (as hypoxanthine) and the inosinic acid data in Table 4 of lamb muscle are presented in Figure 1. Total nucleosides and bases increased sharply during heating for 30 min, then continued to increase at a much lower rate during the second 30 min heating. Data for sum of individual nucleosides paralleled that of inosinic acid which decreased during the first 30 min heating and remained unchanged thereafter.

Values for total nucleotides (batchwise method) also decreased during the first 20 min heating. After this period, total nucleotides (batchwise method) increased slightly. This confirms that the batchwise method measured UV-absorbing material other than nucleotides and that this unknown material was produced by heating. This unknown material in lamb was qualitatively similar to the unknown UV-absorbing substance found in beef and pork. The increase in total nucleosides and bases during heating was greater than could be accounted for by the decrease in total nucleotides analyzed. This further supports the idea concerning the production during heating of some nonnucleotide base which absorbs at 260 nm.

The data also indicate that heating at 60°C results in rupture of phospho-ester and possibly glycosidic bonds of nucleotides.

Table 4—Effects of heating at 60°C on nucleotides of lamb.[1]

Heating time (min)	Cytidylic acid	Adenylic acid	Uridylic acid	Inosinic acid	Guanylic acid
	(μMoles/100 g wet tissue)				
0	9.3	8.6	14.5	209.1	21.8
5	12.7	6.9	13.3	150.8	19.5
10	11.8	7.5	11.7	88.5	—
15	11.1	7.5	12.0	54.0	—
20	13.9	14.1	—	39.9	—
30	13.9	15.6	—	28.1	—
45	15.8	10.7	—	31.3	—
60	17.3	8.6	—	33.3	—

[1] Results are from heating 1 sample for each time period.

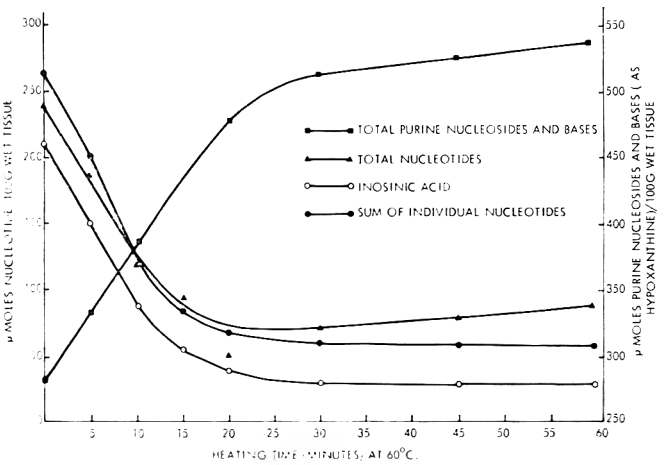

Fig. 1—Effect of heating 60°C on nucleotides and total purine nucleosides and bases of lamb muscle.

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Water-Soluble Flavor and Odor Precursors of Meat. 4. Influence of Cooking on Nucleosides and Bases of Beef Steaks and Roasts and Their Relationship to Flavor, Aroma and Juiciness

SUMMARY—Cooking resulted in significant increases in adenylic acid, total purine nucleosides and bases of 80 beef roasts of eight different cuts. It decreased the contents of inosinic acid, guanylic acid and sum of individual nucleotides (adenylic, cytidylic, uridylic, inosinic and guanylic acids) in these samples. Significant differences were also found between the various constituents of raw and cooked samples of the beef cuts.

INTRODUCTION

THE IMPORTANCE and interest in the nucleoside content of meat was outlined by Macy et al. (1970) and the subject has been discussed comprehensively (Kuninaka, 1967).

The work described in the present paper is a continuation of study of the effect of heating on the nucleosides, nucleotides and bases of beef roasts.

EXPERIMENTAL

Sample preparation and extraction

Ten cuts were studied in these experiments (Table 1).

These cuts were selected randomly from groups of 12 to 31 samples each of the eight different cuts. These roasts had been stored frozen approximately six months prior to their study.

Raw samples from each frozen roast were sawed in $^1/_2$ in. slices adjacent to the center cut portion which was subsequently cooked and analyzed subjectively. Lean portions of the cooked samples remaining after organoleptic evaluation were ground twice through a $^1/_8$ in. plate immediately after cooking. Raw samples were ground in a similar manner.

Perchloric acid extracts were made of these samples by a method similar to that described by Macy et al. (1970). The difference was in the dilution volume employed.

Ion-exchange chromatography of nucleotides. The method of Macy et al. (1966) was used.

Ion-exchange chromatography and analyses of total nucleosides, nucleotides and bases. The method described by Jones et al. (1964) was used for these analyses.

Analyses for fat and moisture. These analyses were carried out by the standard methods of the A.O.A.C. (1960).

Statistical analyses. Analysis of variance was used to analyze the chemical data and significant differences between means of various groups of samples determined by Duncan's multiple range test (Duncan, 1955).

RESULTS & DISCUSSION

Effect of cooking on different beef cuts

Results of analysis of variance indicated that no significant ($P < 0.05$) differences existed between means of cytidylic acid of the different cuts of raw or cooked beef or between raw and cooked samples of the individual cuts.

Cooking significantly increased mean adenylic acid values in all eight beef cuts studied (Table 2). This substantiates results from earlier experiments for beef, lamb and pork (Macy et al., 1970). The increase in adenylic acid of these samples was probably due to availability of nucleotides and also the inactivation of adenylic deaminase. There was possibly some phosphomonoesterase activity prior to heat inactivation of indigenous enzymes.

The uridylic acid of the raw samples was rather constant and did not change appreciably during cooking (Table 3). Cooking resulted in diminished content of this nucleotide in samples analyzed but this generally was not a significant decrease.

Mean concentrations of inosinic acid of raw and cooked beef roasts are presented in Table 4. Among raw samples, Choice rib steaks contained less inosinic acid than did other cuts but not significantly less than Good calf clod roasts.

Cooking decreased the inosinic acid of all samples and most of these changes

Table 1—Beef cuts studied.

Type of beef cut	U.S.D.A. grade	Type of cookery	Internal temperature, °C
Rib	Good	2 in. broiler steak	66
Rib	Choice	2 in. broiler steak	66
Calf rib	Good	2 in. broiler steak	66
Clod	Choice	Pot roast	77
Calf clod	Good	Pot roast	77
Round	Choice	Roast	66
Round	Good	Roast	66
Calf round	Good	Roast	66

Table 2—Means of adenylic acid for raw and cooked beef steaks and roasts.

U.S. grade and cut[1]	Means of adenylic acid[2] (mg/100 g dry, fat-free tissue) Raw	Cooked[3]
Choice rib steaks	17.8 FG	33.5 C
Good rib steaks	21.3 DEF	36.2 ABC
Good calf rib steaks	24.4 DE	35.2 BC
Choice clod roasts	18.7 EFG	37.4 ABC
Good calf clod roasts	13.8 G	37.5 ABC
Choice round roasts	21.9 DEF	42.3 A
Good round roasts	15.9 FG	41.6 AB
Good calf round roasts	26.1 D	39.7 ABC

[1] Ten different samples of each cut were analyzed.

[2] Means followed by the same letter are not significantly different ($P < 0.05$).

[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

Table 3—Means of uridylic acid for raw and cooked beef steaks and roasts.

U.S. grade and cut[1]	Means of uridylic acid[2] (mg/100 g dry, fat-free tissue) Raw	Cooked[3]
Choice rib steaks	14.8 ABC	13.9 ABC
Good rib steaks	17.4 A	15.6 AB
Good calf rib steaks	17.3 A	13.9 ABC
Choice clod roasts	11.2 BCDE	8.1 E
Good calf clod roasts	13.0 ABCD	10.2 CDE
Choice round roasts	15.2 AB	8.3 DE
Good round roasts	16.2 AB	8.5 DE
Good calf round roasts	12.9 ABCD	11.5 BCDE

[1] Ten different samples of each cut were analyzed.

[2] Means followed by the same letter are not significantly different ($P < 0.05$).

[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

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were significant. Inosinic acid of cooked samples varied significantly. The cooked rib steaks contained significantly more inosinic acid than did the other cuts and the clod roasts had less. The latter was undoubtedly due to the higher cookery end point.

Table 4—Means of inosinic acid for raw and cooked beef steaks and roasts.

U.S. grade and cut[1]	Means of inosinic acid[2] (mg/100 g dry, fat-free tissue) Raw	Cooked[3]
Choice rib steaks	269 DE	230 EF
Good rib steaks	322 BC	252 E
Good calf rib steaks	352 AB	250 E
Choice clod roasts	363 A	123 I
Good calf clod roasts	304 CD	122 I
Choice round roasts	339 ABC	131 I
Good round roasts	371 A	158 HI
Good calf round roasts	343 ABC	201 FG

[1] Ten different samples of each cut were analyzed.
[2] Means followed by the same letter are not significantly different ($P < 0.05$).
[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

Table 5—Mean values for ratios of adenylic acid to inosinic acid of raw and cooked beef roasts.

U.S. grade and cut[1]	Mean values for ratios[2] Raw	Cooked[3]
Choice rib steaks	0.066 E	0.148 CD
Good rib steaks	0.067 E	0.145 CD
Good calf rib steaks	0.071 E	0.144 CD
Choice clod roasts	0.052 E	0.332 A
Good calf clod roasts	0.047 E	0.327 A
Choice round roasts	0.067 E	0.340 A
Good round roasts	0.048 E	0.343 A
Good calf round roasts	0.080 DE	0.207 BC

[1] Ten different samples of each cut were analyzed.
[2] Means followed by the same letter are not significantly different ($P < 0.05$).
[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

Table 6—Means of guanylic acid for raw and cooked beef roasts.

U.S. grade and cut[1]	Means of guanylic acid[2] (mg/100 g dry, fat-free tissue) Raw	Cooked[3]
Choice rib steaks	16.5 CDE	10.3 EFGH
Good rib steaks	11.9 EFG	10.4 EFGH
Good calf rib steaks	27.1 A	22.2 ABC
Choice clod roasts	9.1 FGHI	4.4 HIJ
Good calf clod roasts	15.5 DE	8.0 GHIJ
Choice round roasts	19.6 BCD	5.4 HIJ
Good round roasts	3.3 IJ	2.1 J
Good calf round roasts	24.3 AB	14.8 DEF

[1] Ten different samples of each cut were analyzed.
[2] Means followed by the same letter are not significantly different ($P < 0.05$).
[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

The relationship between adenylic acid and inosinic acid is important because adenylic acid is the immediate precursor of inosinic acid, and the latter, the most abundant nucleotide in the beef tissue, is believed to be an important flavor enhancer (Kuninaka et al., 1964; Kurtzmann et al., 1964; Shimazono, 1964; Wagner et al., 1963). The mean ratios of adenylic acid to inosinic acid for the different cuts of raw and cooked beef are presented in Table 5. No significant differences were found between these values for the different raw samples.

The ratios of adenylic acid to inosinic acid for the different cooked cuts could be divided into three distinct groups. First, values for rib steaks from Choice, Good and calf did not differ significantly. Ratios for these cuts were significantly lower than those of the other cuts except Good calf round roasts which were not significantly different. Second, mean ratios for Choice and calf clod roasts, and Choice and Good round roasts were significantly higher than those of the other beef cuts. Third, ratios for Good calf round roasts were intermediate between values for the first two groups.

In all comparisons, ratios of adenylic acid to inosinic acid were significantly higher for cooked than for corresponding raw cuts. This is a reflection of the increase in adenylic acid and the decrease in inosinic acid during cooking at these temperatures.

Mean guanylic acid concentrations of the eight cuts of raw and cooked beef are presented in Table 6. Significant differences were observed in this constituent for the various raw and cooked samples. These differences were irregular except that, in general, the calf samples contained greater amounts of guanylic acid than more mature beef. Guanylic acid was low in concentration compared to inosinic acid. This finding confirms previously discussed results (Macy et al., 1970), where guanylic acid was found to be low in concentration in beef, pork and lamb as well as labile to heat. This is an important finding since this compound is thought to influence flavor desirability.

Means of the sums of individual nucleotides (adenylic, cytidylic, uridylic, inosinic and guanylic acids) of the eight raw and cooked cuts are outlined in Table 7. Since inosinic acid was the predominant nucleotide of beef and was destroyed by cooking, differences in total nucleotides were similar to those of inosinic acid. There was appreciable loss in nucleotides of all samples during cookery, particularly cooked to a higher point.

Total nucleotides of cooked and raw beef determined by the modified method of Jones et al. (1964) are presented in

Table 7—Mean values for sums of individual nucleotides for raw and cooked beef roasts.

U.S. grade and cut[1]	Means of sums of nucleotides[2] (mg/100 g dry, fat-free tissue) Raw	Cooked[3]
Choice rib steaks	332 DEF	302 FG
Good rib steaks	377 BCD	330 EF
Good calf rib steaks	437 A	335 DEF
Choice clod roasts	419 AB	186 J
Good calf clod roasts	365 CDE	192 J
Choice round roasts	409 ABC	200 J
Good round roasts	420 AB	223 IJ
Good round roasts	403 ABC	247 HI
Good calf round roasts	425 A	276 GH

[1] Ten different samples of each cut were analyzed.
[2] Means followed by the same letter are not significantly different ($P < 0.05$).
[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°, respectively.

Table 8—Mean values for total nucleotides of raw and cooked beef steaks and roasts.[1]

U.S. grade and cut[2]	Means of total nucleotides[3] (mg/100 g dry, fat-free tissue) Raw	Cooked[4]
Choice rib steaks	299 CDE	313 BCDE
Good rib steaks	487 A	302 BCDE
Good calf rib steaks	370 BC	300 CDE
Choice clod roasts	370 BC	206 FG
Good calf clod roasts	351 BCD	198 G
Choice round roasts	345 BCD	233 EFG
Good round roasts	341 BCD	244 EFG
Good calf round roasts	355 BCD	285 CDEF

[1] Batchwise method of Jones et al. (1964).
[2] Ten different samples of each cut were analyzed.
[3] Means followed by the same letter are not significantly different ($P < 0.05$).
[4] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

Table 9—Means for total purine nucleosides and bases of raw and cookec beef steaks and roasts.[1]

U.S. grade and cut[2]	Means for total purine nucleosides and bases[3] (mg/100 g dry, fat-free tissue) Raw	Cooked[4]
Choice rib steaks	252 GHI	269 FGH
Good rib steaks	231 I	273 EFGH
Good calf rib steaks	251 GHI	267 FGH
Choice clod roasts	289 DEF	289 DEF
Good calf clod roasts	302 CDE	296 CDEF
Choice round roasts	278 EFG	326 ABC
Good round roasts	247 HI	313 CD
Good calf round roasts	310 CD	351 A

[1] Batchwise method of Jones et al. (1964).
[2] Ten different samples of each cut were analyzed.
[3] Means followed by the same letter are not significantly different ($P < 0.05$).
[4] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°, respectively.

Table 8. Total nucleotides of raw beef samples were generally lower than the sums of individual nucleotides for the same samples. The exception to this observation was for Good rib roasts. Variation in total nucleotides of the cooked cuts was similar to that for sums of individual nucleotides analyzed.

There was very little difference between means for total nucleotides among the various groups of raw samples. Cooking significantly decreased total nucleotides in most samples analyzed. There were differences in total nucleotides among the cooked samples of the various cuts but many of these were insignificant.

Results of total purine nucleoside and base determinations of raw and cooked beef are in Table 9. The three cuts of raw rib roasts contained significantly less total purine nucleosides and bases than the raw clods, and Good calf rounds. Highest quantity of purine nucleosides and bases among the raw samples was in Good calf rounds. This was significantly higher than that found in other cuts of raw beef except clod roasts which were not significantly different. Other significant differences in total purine nucleosides and bases existed among the cooked samples; however, no particular trend was evident.

In general, cooking resulted in increased quantities of purine nucleosides and bases. Significant increases in total purine nucleosides and bases due to cooking were found in the Good rib steaks and in the round roasts. It has been thought that larger increases in total purine nucleosides and bases would be found in the different cuts of beef and that increases due to cooking would have been significant in all cases, since rather large amounts of inosinic acid were destroyed. Degradation of all nucleotides should contribute to increases in total nucleosides and bases.

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Water-Soluble Flavor and Odor Precursors of Meat. 5. Influence of Heating on Acid-Extractable Non-Nucleotide Chemical Constituents of Beef, Lamb and Pork

SUMMARY—Studies of the perchloric acid-extractable nitrogen, amino nitrogen, non-amino nitrogen, total carbohydrates, inorganic phosphate, free creatinine, creatine and total creatine plus creatinine of raw and heated beef, lamb and pork were made. Specific changes that occurred in the various constituents during heating are discussed. An extensive study of the influence of heating on certain chemical constituents of 80 beef samples of eight different cuts was also performed. Changes in chemical constituents during cooking of the various beef cuts were greatest in Choice clod and Good calf clod roasts, followed by round roasts and smallest changes occurred in rib steaks. Cooking caused significant increases in creatinine and decreases in amino and non-amino nitrogen, creatine and total carbohydrates. Creatine-creatinine was a better index of sensory quality than other chemical constituents studied.

INTRODUCTION

RESULTS of previous investigations suggest that numerous chemical compounds are involved in the production of desirable cooked meat flavor and aroma. It is also generally accepted that many precursors of cooked meat flavor and aroma are extractable from muscle tissue by cold water or dilute acid solutions (Macy et al., 1964). Certain compounds such as carbohydrates and amino acids react to produce meaty flavors and aromas. Certain other compounds, such as nucleotides, act to modify or intensify other flavors and aromas.

The purpose of this work was to determine the influence of cooking on certain non-nucleotide chemical constituents of beef, lamb and pork and to compare eight cuts of beef on the basis of their contents of these compounds.

EXPERIMENTAL

Sample preparation and extraction

These procedures were the same as previously described (Macy et al., 1970a and b).

Analysis of total amino nitrogen

The method of Rosen (1957) was used for this determination. Leucine standards (0.2*M*) were developed along with each group of suitably diluted extracts and all determinations were repeated at least once. Results were calculated as the average of the respective replicates.

Analysis of total extractable nitrogen

Total extractable nitrogen was determined

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Table 1—Effects of roasting on acid-extractable chemical constituents of beef.[1]

Chemical constituent	Cooking temperature (°C) Raw	49	77
	mg/100 g dry, fat-free tissue		
Total nitrogen	871	882	914
Amino nitrogen	126	136	148
Non-amino nitrogen	745	746	766
Total carbohydrates	1353	1628	1358
Reducing carbohydrates	635	829	750
Non-reducing carbohydrates	718	799	608
Inorganic phosphate	227	240	276
Free creatinine	53	95	232
Creatine	1006	918	815
Creatine plus creatinine	1059	1013	1046

[1] Results are given as means of 5 top round roasts.

Table 2—Effects of roasting to 170°F internal temperature on amino acids of beef.[1]

Amino acid	Raw	Cooked
	mg/100 g dry, fat-free tissue	
Taurine	75.3	125.4
Aspartic acid	6.4	7.8
Threonine	2.6	1.9
Serine	1.4	1.1
Glutamic acid	38.0	9.9
Glycine	10.5	15.3
Alanine	33.7	45.3
Valine	4.8	11.1
Methionine	2.6	3.6
Isoleucine	3.1	6.0
Leucine	3.7	8.6
Tyrosine	5.7	10.8
Phenylalanine	5.0	5.9
γ-Amino-*n*-butyric acid	0.3	2.5
Ammonia	36.3	42.5
Ornithine	9.4	10.7
Lysine	12.1	19.3
Anserine	116.5	207.5
Histidine	21.4	14.1
Carnosine	869.1	1191.3
Arginine	0.9	0.0
TOTAL	1258.8	1740.6

[1] Results are given as means of 5 top round roasts.

Table 3—Effects of roasting on chemical constituents of pork.[1]

Chemical constituent	Cooking temperature (°C) Raw	49	71
	mg/100 g dry, fat-free tissue		
Inorganic phosphate	99	127	105
Amino nitrogen	125	134	119
Total nitrogen	1566	1325	1496
Non-amino nitrogen	1441	1191	1337
Total carbohydrate	3682	3391	4059
Reducing sugars	2129	1718	1980
Non-reducing carbohydrate	1554	1673	2089
Percent reducing sugar of total carbohydrate	58	51	49
Free creatinine	68	77	96
Creatine	1215	872	932
Total creatine-creatinine	1283	939	1062
Percent free creatinine of total creatine-creatinine	5	9	9

[1] Results are given as averages of duplicate analyses of 3 pork loin roasts.

on 10 ml portions of the extracts by the standard Kjeldahl method of the A.O.A.C. (1960).

Analysis of total extractable carbohydrates

The anthrone method of Loewus (1952) was used for this determination. All analyses were repeated until replicate samples agreed within 0.05 Transmission units. Glucose standards were analyzed with each group of extract samples and the carbohydrate contents calculated as glucose.

Analysis of simple reducing sugars

The method of Folin et al. (1920) was used for this analysis in the preliminary experiments. Suitably diluted samples of the extracts were analyzed in duplicate along with glucose standards.

Analysis of total inorganic phosphate

The method of Fiske et al. (1925) was used. Suitably diluted samples of the extracts and standard phosphate samples were determined in duplicate.

Analysis of creatinine and creatine

The method described by Hawk et al. (1947) was used to determine individual amino acids of raw beef extracts and samples cooked to 77°C internal temperature in the preliminary experiment involving five beef top round roasts.

Sensory evaluation of flavor, aroma and juiciness

A trained panel consisting of six members scored cooked samples of the different types of beef cuts on an eight point desirability scale with eight as extremely desirable and one as extremely undesirable. The average score of each sample for flavor, aroma and juiciness was compared with chemical data.

Statistical analysis

Analysis of variance and correlation analysis was used to analyze data from the chemical analyses and sensory evaluations (Ostle, 1960). Duncan's multiple range test was used to examine significance of differences between means of the various groups of samples (Duncan, 1955).

RESULTS & DISCUSSION

Effects of cooking on beef round roasts

The effects of roasting on certain perchloric acid-extractables other than nucleotides of five beef round roasts are presented in Table 1. Total acid-extractable nitrogen slightly increased with heating, as did amino nitrogen and non-amino nitrogen.

Total carbohydrate increased considerably in the samples cooked to 49°C compared to the raw samples. Subsequent heating to 77° caused total carbohydrates to diminish to about the same value as that found in raw samples. These changes were paralleled by the reducing sugars and non-reducing carbohydrates. Heating to 77°C resulted in an increase in reducing sugars and a decrease in non-reducing carbohydrates.

Inorganic phosphate of the beef rounds increased progressively with temperature. This was anticipated since inosinic acid and adenosine di- and tri-phosphates are hydrolyzed during heating to inorganic phosphate and nucleotides. Free creatine decreased progressively during heating, and was apparently transformed to its anhydride, creatinine, during heating of the mildly acidic tissue. Total creatine plus creatinine (creatine-creatinine) changed little under these conditions.

Results of the analysis for individual amino acids of raw beef rounds and those cooked to 77°C are outlined in Table 2. All free amino acids of the beef roasts increased during cooking except threonine, serine, glutamic acid, histidine and arginine, which decreased. The over-all effect of cooking was an increase of 38.3% in the total amino acids. Total extractable amino nitrogen increased approximately 18% in the same samples during cooking. These increases were probably due to hydrolysis of protein and possibly involved cathepsins or other proteolytic enzymes in the tissue, since it has been shown that most free amino acids decreased during heating when isolated from the protein by dialysis (Macy, et al., 1964).

Taurine, anserine and carnosine increased to the greatest extent during cooking. These were also the major amino compounds present in both raw and cooked beef. Freeing of amino acids during cooking is important to development of meat flavor because they participate in browning reactions involving amino acids and reducing carbohydrate. In addition, some amino acids apparently contribute to the over-all flavor of meat (Macy, 1966).

Results previously reported (Macy et al., 1969a) concerning the effect of heating on nucleic acid derivatives of pork were in some cases paralleled by changes in other chemical constituents reported in this paper (Table 3). Inorganic phosphate increased in the intermediately cooked (49°C) samples and was less in the well-done (71°C) samples. Amino nitrogen also increased in the intermediately cooked samples, then decreased in the well-done samples to a level slightly below that of the raw samples. This probably represented hydrolyses of protein due to protease activity in the less severely heated samples. Heat-denaturation of proteolytic enzymes and browning reactions were

probably responsible for the lowered values for amino nitrogen in the well-done samples. Total acid extractable nitrogen decreased in the intermediate samples, and increased in the well-done samples. A large decrease in non-amino nitrogen was observed in the intermediate samples compared to either the raw or well-done samples.

Total acid extractable carbohydrate of intermediately cooked loins was less than that of the raw samples but carbohydrates of the well-done samples were higher than those of raw samples. Reducing sugar decreased in the intermediately cooked sample, and extractable non-reducing carbohydrates increased consistently during roasting. The percent reducing sugar of total carbohydrate decreased progressively with increased cooking.

Free creatinine increased with increased cooking. Creatine decreased considerably in the intermediately cooked samples then increased in the well-done samples. This pattern was also followed by total extractable creatine-creatinine. These values possibly reflect differences in the ability to remove creatine and creatinine from protein in the tissue rather than actual change of total creatine and creatinine due to heating (Hughes, 1960).

Effects of cooking on chemical constituents of lamb muscle

Results from the previous experiments indicated that heating conditions must be carefully controlled.

Table 4 is a summary of data obtained for changes in chemical constituents of lamb muscle during heating at 60°C. Inorganic phosphate increased during heating and this was probably due to dephosphorylation of nucleotides, especially inosinic acid, which decreased substantially during heating.

The data for the individual heating periods were somewhat erratic, although a trend toward increased amino nitrogen was apparent. Total extractable nitrogen decreased during heating for 15 min then increased steadily during the remainder of the experiment. The value for total nitrogen at 60 min heating was slightly less than that of the uncooked sample. There was a small increase in the percent amino nitrogen of total nitrogen and larger amounts of amino nitrogen-containing substances became extractable with perchloric acid as heating times progressed.

Total extractable carbohydrate decreased steadily during the first 20 min heating, then increased at 30, 45 and 60 min. This sequence was also followed by the reducing sugar content. The percent non-reducing sugar of total carbohydrates increased rapidly during heating. Non-reducing carbohydrate, probably glycogen, was rapidly degraded to reducing sugar.

Free creatinine of lamb muscle increased progressively during heating, but the increase was most rapid between 15 and 60 min heating. This was anticipated, due to the slightly acid conditions of meat which catalyze the removal of a molecule of water from creatine to form creatinine. In spite of the increased formation of creatinine, creatine increased slightly with heating. This could have been a result of extraction phenomena.

Total creatine-creatinine increased with heating time. This indicated that e ther a precursor of creatine was present in the tissue or the acid-extractability of these constituents was enhanced by heating. The former of these possibilities is more probable since phosphocreatine is present in muscle and this compound is quite labile to heating. Percent creatinine of total creatine-creatinine increased with heating. The rate of dehydration of creatine to creatinine may have been greater than the rate of formation of additional creatine from phosphocreatine.

Effects of cooking on eight cuts of beef

Data representing the acid-extractable nitrogen in the various cuts of raw and cooked beef are in Table 5. Raw Good calf clods contained less amino nitrogen than other raw samples but not significantly less than Choice rib roasts, Good calf rib roasts or Good round roasts.

Among the cooked cuts, Choice clod roasts had less amino nitrogen than other cuts but not significantly less than Good calf rib steaks or Good calf clod roasts. Good calf round roasts contained a significantly greater amount of amino nitrogen than other cooked beef except Choice round roasts and Choice rib steaks. Cooking at these temperatures resulted in significant decrease in amino nitrogen of all cuts except Good calf round roasts and Choice rib steaks.

The non-amino nitrogen data in Table 5 were more revealing than those for amino nitrogen. Raw Good rib steaks had less non-amino nitrogen than the other cuts but not significantly less than Choice rib steaks and Choice clod roasts. Among the cooked samples, the clod roasts had less non-amino nitrogen than the other cuts. Clod roasts contained significantly less non-amino nitrogen than the other cooked samples due to the higher cooking temperatures. All cooked samples contained significantly less non-amino nitrogen than their raw counterparts.

Table 4—Effects of heating at 60°C on certain constituents of ground lamb.[1]

Constituent	Heating time (min)							
	0	5	10	15	20	30	45	60
	Mg/100 g wet tissue							
Inorganic phosphate	18.8	19.3	20.5	20.3	19.9	22.5	22.7	23.3
Amino nitrogen	33.7	29.9	33.0	32.6	37.5	34.3	38.5	36.2
Total nitrogen	292.4	255.0	261.0	254.0	268.5	282.0	282.0	284.0
Percent amino nitrogen of total nitrogen	11.5	11.7	12.6	12.8	13.9	12.2	13.6	12.7
Total carbohydrate	574.5	550.5	450.0	300.0	237.0	319.5	307.5	274.5
Reducing sugars	114.7	131.2	130.5	126.7	107.2	147.7	111.7	159.0
Non-reducing carbohydrate	459.8	419.3	319.5	173.3	129.8	171.8	195.8	115.5
Percent reducing sugars of total carbohydrate	20.0	23.9	29.0	42.2	45.2	46.3	36.3	58.0
Free creatinine	11.6	14.0	13.5	12.2	17.8	17.8	16.4	23.8
Creatine	199.2	200.5	201.0	191.8	200.2	212.7	205.6	206.7
Total creatine-creatinine	210.8	214.5	214.5	204.0	218.0	230.5	221.0	230.5
Percent free creatinine of total creatine-creatinine	5.5	6.5	6.3	6.0	8.2	7.7	7.4	10.3

[1] Results are from duplicate analyses of samples from each time period.

Table 5—Means of amino nitrogen, non-amino nitrogen and total extractable nitrogen from raw and cooked steaks and roasts.[1]

U.S. grade and cut	Amino nitrogen[2]		Non-amino nitrogen[2]		Total nitrogen[2]	
	Raw	Cooked[3]	Raw	Cooked[3]	Raw	Cooked[3]
	mg/100 g dry, fat-free tissue					
Choice rib steaks	103.3 CDE	80.9 C	1140 C	962 E	1243 B	1043 C
Good rib steaks	108.3 BC	78.2 GH	1126 CD	1013 E	1247 B	1091 C
Good calf rib steaks	96.8 DEF	69.6 HI	1308 AB	970 E	1405 A	1040 C
Choice clod roasts	109.5 BC	62.9 I	1223 BC	731 GH	1332 AB	794 E
Good calf clod roasts	92.2 EF	69.2 HI	1250 AB	706 H	1342 AB	775 E
Choice round roasts	116.0 B	91.8 F	1276 AB	843 F	1392 A	934 D
Good round roasts	101.2 CDEF	75.4 GH	1258 AB	814 FG	1359 A	889 D
Good calf round roasts	105.7 BCD	101.6 CDEF	1329 A	1032 DE	1424 A	1133 C

[1] Ten different samples of each cut were analyzed.

[2] Means of the individual chemical constituents followed by the same letter are not significantly different ($P < 0.05$).

[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

Table 6—Means of creatine, creatinine and total extractable creatine-creatinine from raw and cooked steaks and roasts.

U.S. grade and cut[1]	Creatine[2]		Creatinine[2]		Creatine-creatinine[2]	
	Raw	Cooked[3]	Raw	Cooked[3]	Raw	Cooked[3]
	mg/100 g dry, fat-free tissue					
Choice rib steaks	1302 BC	902 E	37.9 J	124.5 E	1340 CD	1027 F
Good rib steaks	1363 AB	1029 D	51.8 HIJ	121.4 EF	1415 ABC	1150 E
Good calf rib steaks	1416 A	829 E	55.1 HIJ	132.9 E	1471 A	962 F
Choice clod roasts	1310 B	531 H	62.6 GHIJ	193.8 A	1372 BCD	725 I
Good calf clod roasts	1376 AB	614 G	42.9 IJ	162.8 ABCD	1419 ABC	777 H
Choice round roasts	1215 C	699 F	72.1 GHI	171.4 ABC	1287 D	870 G
Good round roasts	1297 BC	825 E	49.1 HIJ	181.7 AB	1346 CD	1007 F
Good calf round roasts	1338 AB	876 E	78.0 GH	140.8 CDE	1416 ABC	1017 F

[1] Ten different samples of each cut were analyzed.
[2] Means of the individual chemical constituents followed by the same letter are not significantly different ($P < 0.05$).
[3] Rib steaks were broiled to 66°C; round and clod roasts were roasted to 66° and 77°C, respectively.

Table 7—Means of total extractable carbohydrate for raw and cooked beef roasts.

U.S. grade[1] and cut[2]	Means of total carbohydrate	
	Raw	Cooked
	mg/100 g dry, fat-free tissue	
Choice rib steaks	2163 BC	1837 D
Good rib steaks	1849 CD	1542 DE
Good calf rib steaks	1437 EF	802 HI
Choice clods	1369 EF	823 HI
Good calf clods	1050 GH	630 I
Choice rounds	2329 B	1410 EF
Good rounds	2814 A	1623 DE
Good calf rounds	1622 DE	1152 FG

[1] Ten different samples of each cut were analyzed.
[2] Means followed by the same letter are not significantly different ($P < 0.05$).

Less total nitrogen was extractable from cooked samples than from corresponding raw samples (Table 5). Raw Choice and Good rib steaks contained significantly less total extractable nitrogen than other cuts except the clods which were not significantly different. The cooked beef roasts were divided into three significantly different groups according to total nitrogen. Cooked clod roasts contained significantly less total nitrogen than the other cuts. Good and Choice round roasts contained significantly less total nitrogen after cooking than the remaining cuts cooked to 77°C.

Free creatinine of the roasts increased significantly during cooking (Table 6). This observation agreed with the preliminary experiments for beef, lamb and pork and confirmed results of Bendall (1946). Creatinine of cooked rib steaks was significantly lower than that of Choice clod and round roasts and Good round roasts.

Cooked beef also contained significantly less creatine than uncooked beef (Table 6). This difference may be due to: (a) conversion of creatine to creatinine by heating under slightly acid conditions or (b) inefficient removal of these compounds from raw tissue with dilute perchloric acid.

Choice round roasts contained significantly less creatine than the other cuts except Choice rib steaks and Good round roasts which were not significantly different.

Cooked Good rib steaks contained significantly more creatine than other cooked beef. This was followed by cooked rib steaks from Choice and calf ribs and Good and calf rounds which had significantly higher creatine than the remaining cooked roasts. Cooked Choice clods had significantly less creatine than other cuts possibly due to their higher cooking temperature. In general, the cooked rib steak had higher amounts of creatine than rounds, and clod roasts had less creative than other cooked roasts.

The work of Hughes (1960) with herring indicated that cooking resulted in increased physical binding of creatine and/or creatinine to the tissue protein. Heat-processing of herring flesh at 115°C resulted in conversion of about one-quarter of the creatine to creatinine, but no overall loss of the two compounds was observed. Bendall (1946) reported, however, that high-temperature processing of beef resulted in destruction of creatine and/or creatinine, but did not indicate that this was due to lowered extractability of these compounds due to heat-processing.

Significant decreases in the amount of extractable creatine-creatinine were observed for cooked compared to raw beef. Losses of these compounds due to cooking reported by Bendall (1946) were relatively small and his cooking conditions were considerably more severe than those used in this study.

Data concerning total carbohydrate of raw and cooked beef roasts are presented in Table 7. Cooking resulted in significant decreases in total carbohydrate of samples in all cases except in Good rib steaks. The average decrease of more than 300 mg carbohydrate per 100 g dry, fat-free tissue from the Good rib steaks was not sufficient to attain statistical significance due to the extreme variation of carbohydrate within these samples.

Among the raw beef cuts, the Good round roasts contained significantly higher amounts of total carbohydrates than other samples. This was followed by rounds from Choice and Good beef roasts and Choice rib steaks. Raw Choice and calf clods contained significantly less total carbohydrate than other cuts of beef, followed by the raw rib cuts. Raw rounds contained the most carbohydrate. The exception was raw calf round, which had less total carbohydrate than raw rib roasts from Good grade beef. Calf roasts contained less carbohydrate than more mature beef regardless of grade.

Table 8—Correlation coefficients between chemical constituents of cooked roast and flavor, aroma and juiciness.

Chemical constituent	r		
	Flavor	Aroma	Juiciness
Total extractable nitrogen	−0.23*	−0.20	−0.08
Non-amino nitrogen	−0.24*	−0.20	−0.09
Amino nitrogen	−0.04	−0.07	−0.02
Total carbohydrate	0.08	0.15	−0.11
Creatine	−0.35***	−0.27**	−0.26*
Creatinine	0.31**	0.29**	0.18
Creatine-creatinine	−0.32**	−0.26*	−0.23*
Percent creatinine of creatine-creatinine	0.44***	0.26*	0.23

N = 80.
* ($P < 0.05$).
** ($P < 0.01$).
*** ($P < 0.001$).

Correlations for cooked beef

Correlation coefficients between sensory attributes and chemical content were low (Table 8). The best relationships between chemical content and flavor was the correlation between percent creatinine of total creatine-creatinine in cooked meat and flavor. The correlation was 0.44 ($P < 0.001$). The best relationship between aroma and chemical constituent was between creatinine and aroma (0.29; $P < 0.01$). The highest relationship between juiciness and chemical content was that found for creatine ($r = 0.44$; $P < 0.001$). All other correlations between results of sensory and chemical analyses were lower than those cited and most were insignificant despite the large number of samples analyzed.

Correlation coefficients between acceptability scores of cooked roast beef and ratios of N-containing compounds of

Table 9—Correlation coefficients between acceptability scores of cooked roast beef and ratios of N-containing compounds of cooked to raw beef roasts.

Chemical constituent	r		
	Flavor	Aroma	Juiciness
Total extractable nitrogen	−0.24*	−0.16	−0.30**
Non-amino nitrogen	−0.23*	−0.15	−0.30**
Amino nitrogen	−0.12	−0.17	−0.12
Creatine	−0.30**	−0.18	−0.34**
Creatine-creatinine	−0.25*	−0.14	−0.35***

N = 80.
* ($P < 0.05$).
** ($P < 0.01$).
*** ($P < 0.001$).

cooked to raw beef roasts are outlined in Table 9. All correlations between ratios of N-containing compounds of cooked beef roasts relative to those of raw beef roasts and flavor and juiciness except for amino nitrogen were significant. There were no significant relationships between aroma and chemical constituents.

Data presented in Tables 8 and 9 indicate that creatine and creatinine are better indices of flavor, aroma and juiciness of roast beef than other chemical constituents studied.

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A Reexamination of the Two-Stage Triangle Test for the Perception of Sensory Differences[a]

SUMMARY—A review of the theory and practice of the two-stage triangle test for the sensory perception of small differences leads to the conclusion that its disadvantages usually outweigh its advantages. When the test is used its information content is difficult to assess; and a "least objectional" method, based on a scoring scheme and statistical significance procedures, is here proposed.

INTRODUCTION

IN MANY CIRCUMSTANCES testing for marginal sensory differences is better carried out by way of simple paired comparison than by triangle test. This is well-recognized. But special appeal resides in the "modified" or "two-stage" triangle test. A comparative assessment of the flavor (or some other sensory characteristic) of a pair of products done by two-stage triangle test means that each individual trial consists of three coded items, two of one product and one of the other, with the double request to the panelist: "Which member of the triad, in terms of the relevant characteristic do you think is odd?" and, "Elaborate on the difference in [the way specified]."

At its simplest, the "way specified" will relate to the direction of the difference (e.g., a stronger or weaker flavor, or a smoother or coarser texture). Additionally, it may relate to degree of difference on an *ad hoc* scale. In another milieu it can relate to preference, either absolutely or by degree. This basic scheme, which, according to Bengtsson (1953), originated in the brewing industry in the 1920s, has the ostensible advantage of winnowing out those subjects who can't discriminate the two products. That is, the second-stage results of the correct oddity identifiers only are considered.

Nevertheless, in practice the two-stage triangle test often leads to interpretative difficulties, particularly associated with what may be called "the paradox of discriminatory nondiscriminators," some examples of which will now be considered.

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PARADOX OF DISCRIMINATORY NONDISCRIMINATORS

A PROBLEM to be faced is that "nondiscriminators" sometimes give non-random answers at Stage Two. It is natural to assume that a person who can't identify the oddity *ipso facto* can't discriminate, much less characterize, the difference between the two products. But results belying this assumption turn up (Amerine, et al. 1965). An early report of this nature is due to Byer et al. (1953). Table 1 shows three cases in which the comparative judgments of the panelists who misidentified the oddity were almost exactly the same, as regards distribution between the two items, as those of the rest of the panel. The three tests are disparate enough to invite individual comment.

Table 1—Results of some two-stage triangle tests.

Criterion and material	Number of selections of putative oddity that are Correct	Incorrect	Division of judgments for items A	B
Bitterness of quinine sulfate solutions[1]	67	—	54	13
	—	75	58	17
Tenderness of poultry meats[2]	92	—	56	36
	—	154	95	59
Preference between reconstituted skim-milk powders[3]	30	—	23.5	6.5
	—	30	21.5	8.5

[1] Byer et al. [1953 in Ref. List; the solutions were of known strengths, with A always stronger than B.

[2] Gridgeman [1964]; two different carcase treatments were at issue.

[3] Courtesy of Mrs. Elizabeth Larmond, Food Research Institute, Central Experimental Farm, Department of Agriculture, Ottawa. Some panelists were unable to express a preference, which accounts for the half-scores.

(1) The "bitterness" results are most surprising, because of the intimately related decisions involved in the two stages of the test. Yet more than half of the panelists misidentified the oddity and then went on to show themselves as good as the others at sensing the bitterer item. Clearly, the picking of the oddity was a psychosensorily confusing task. Bradley et al. (1964), following Ura (1960), have developed a probabilistic model, based on the formal variability of sensory response to a given stimulus, that can accommodate such results, but the amount of extra information so obtainable is small.

(2) The "tenderness" results could be explicable in terms of sample variation (poultry meat is far from homogeneous), and this was attempted in the report. The method of handling the data is, however, cumbersome and unrewarding. The high proportion of incorrect selections of the oddity in this experiment suggests that the judgments were made virtually at random.

(3) The "preference" results also indicate that the selection of the oddity was a bothersome task, and that the panelists behaved more rationally in a binomial than in a trinomial situation.

Of course, in some two-stage triangle tests those panelists who fail to identify the oddity behave with apparent consistency in that, as a group, they do not make significant distinctions in the second stage. Yet even here no advantage can be pointed to, because the panel might just as well have concentrated on paired comparisons instead.

Some side-by-side tests conducted by Mr. van den Berg of these laboratories are illuminating. Each member of a panel of seven made preference judgments by two-stage triangle test and, independently, by paired comparison (with ties allowed), the replications being so arranged that the same number of samples was assessed in each type of test. The results are shown in Table 2. The number of nondiscriminators (27) in the triangle tests was large, almost two-thirds of the total (which again implies that the judgments were made almost entirely at random).

Table 2—Results of parallel experiments, one by two-stage triangle test, the other by paired comparison, to check for preferential difference between the odors of chicken-leg meats from two treatments. (The same panel of 7 subjects was used. Each carried out 6 replicate two-stage triangle tests, and 9 replicate paired comparisons, all under code.)

	Number of selections of putative oddity that were Correct	Incorrect	Division of judgments for items A	B
Two-stage triangles	15	—	10	5
	—	27	14	13
	Preferred A	Preferred B	Undecided	
Paired comparisons	33	22	8	

Reference to statistical tables will confirm what is clear from inspection, namely, that the paired comparison results more sharply distinguish between the two treatments, A and B, than do the triangle tests. In fact only the paired-comparison difference (33 for A and 22 for B) is significant at the 5% probability level.

A "LEAST OBJECTIONABLE" SCORING SCHEME

IN VIEW of the tendency of some nondiscriminators to make meaningful second-stage judgments on the relation between the items, an inclination to take these judgments into good account cannot be avoided. In other words, should not all the results be pooled? To do so is not illogical or even unreasonable. However, it implies a rejection of the essence of the two-stage triangle test, which is thereby transformed to a set of ill-made paired comparisons.

And there is another consideration. Pooling necessarily means giving equal weight to the decisions of both discriminators and nondiscriminators. Maybe a realistic compromise would be to give less weight to the nondiscriminators' decisions? But how? This question expands to: What score scale should be attached to the four distinctive results that any one two-stage triangle trial can give rise to? No clearcut answer exists; however, a convenient one may be sought. More specifically, the problem is to choose a number to replace x in the end (score) column of Table 3 (which is in preference language, a though of course it applies to other criteria). The mean score is, clearly, $s_m = (x + 1)/2$, and the finding of the weights implicit in the scale must stem from the score deviations, negative for A and positive for B, from the mean, s_m. The weights given to the intermediate outcomes ("incorrect oddity chosen"), as a fraction of that given to the terminal outcomes ("correct oddity chosen"), is therefore $(s_m - s_{ia})/(s_m - s_{ca}) = (x - 1)/(x + 1)$. So a numerical value for this fraction must be decided on. First thoughts suggest $^1/_2$: that is, making the nondiscriminators' decisions half as valuable as those of the discriminators. This is achieved by the setting of $x = 3$, so that the appropriate score scale is 0, 1, 3, 4.

Table 3—Basic scoring scheme for the two-stage triangle test.

Possible outcomes of one trial	Number of forms of the outcome	Score
ca = correct oddity chosen; prefers A	1	$s_{ca} = 0$
ia = incorrect oddity chosen; prefers A	2	$s_{ia} = 1$
ib = incorrect oddity chosen; prefers B	2	$s_{ib} = x$[1]
cb = correct oddity chosen; prefers B	1	$s_{cb} = x + 1$
	6	

[1] The quantity "x" has to be decided on; see text.

Perhaps this is overly generous; so consider a smaller fraction, say $^2/_5$, obtained by setting $x = 7/3$, the corresponding score scale being 0, 1, $^7/_3$, $^{10}/_3$. Special mention is made of this because it was, in effect, chosen by Davis et al (1954) in a pioneer paper on weighing schemes for the two-stage triangle test. (Actually, their score scale consisted of what they term "I values" of 1, 4, 8, 11, which set is a linear function of, and therefore operationally equivalent to, the score vector 0, 1, $^7/_3$, $^{10}/_3$.)

Another possibility, here advocated, is to make the weight fraction 1/3; correspondingly, $x = 2$. The triadic nature of the test lends intuitive attraction to this figure. More importantly, it is statistically nice in that the resultant score scale is maximally simple, namely, 0, 1, 2, 3, and easy to handle in distribution contexts.

In all circumstances, it is desirable to carry out two-stage triangle tests in complementary pairs (one with A the oddity, the other with B) so as to cancel any "item bias" that may exist in the performance of single trials. To this end we shall deal only in even numbers of trials in replicated testing.

SIGNIFICANCE TESTING

THE DEVELOPMENT of tests of statistical significance is a little complicated. To exemplify the scoring of a set of trials, the data in Table 1 will be used. In the first test (bitterness of quinine sulfate solutions) there were $(67 + 75) = 142$ trials, so that the maximum possible score (if discrimination was perfect and every vote cast was for item B) is three times this, that is, 426. Now the observed score is $(54 \times 0) + (58 \times 1) + (17 \times 2) + (13 \times 3) = 131$; so the primary finding is that a fraction $131/426 = 31\%$ of the vote went to item B, and the rest, 69%, to item A. Likewise, $(738 - 321)/738 = 56.5\%$ of the test vote on poultry tenderness went to treatment A, and $(180 - 58)/180 = 67.8\%$ of the preference vote on reconstituted skimmilk powders went to product A.

In all three examples it is obvious that the vote splits were significantly different from the null hypothesis (of "no detectable difference between the items") of 50% each for A and B. However, suppose a statistical-significance test is asked for on, say, the skimmilk results. The procedure rests on two facts: (1) that the standard deviation of the mean (null hypothesis) score, $s_m (= 3N/2)$, in a set of trials is $\sqrt{11N/12}$, and (2) that when N is large the distribution of possible scores may be regarded as Normal. So here

$$S_m = 3 \times 60/2 = 90$$

and the standard deviation of this mean score is

$$SD(S_m) = \sqrt{11 \times 60/12} = \pm 7.4$$

Now the observed score is 58, which is $S_m - 12$, and as $32/(7.4) = 4.3$, it is apparent that the observed result is not fortuitous.

The statistical significances of the results of smaller numbers of trials (up to 30) are best ascertained from exact distribution data. This matter is discussed further in the Appendix.

It would of course be helpful if limits of error were assignable to any observed score. Unfortunately this is not practical, because any non-null score may involve up to three parameters (the discriminator: nondiscriminator split; and the A:B vote split within the discriminators and the nondiscriminators), which creates formidable difficulties. This is another limitation to the two-stage triangle test.

Table 4—Hypothetical details of results (as numbers of judgments), by two-stage directional triangle test, on poultry tenderness (with real differences).

Stage 1: selection of putative oddity	Stage 2: judgment of oddity's tenderness relative to other two items					
	Markedly less	Slightly less	Same	Slightly more	Markedly more	Sum
Correct	16	38	4	28	6	92
		56		36		
Incorrect	23	68	8	41	14	154
		95		59		

Table 5—Hypothetical details of results (as numbers of judgments), by two-stage directional triangle test, on poultry tenderness (the compared items being actually indistinguishable).

Stage 1: selection of putative oddity	Stage 2: judgment of oddity's tenderness relative to other two items:					
	Markedly less	Slightly less	Same	Slightly more	Markedly more	Sum
Correct	11	33	4	33	11	92
		46		46		
Incorrect	18	55	8	54	19	154
		77		77		

A SOURCE OF BIAS

THE TWO-STAGE triangle test is sometimes extended to embrace judgments on degrees of difference between the (putative) oddity and the residual pair. The kind of panel request involved is: "Do you consider the item you have picked as odd to be slightly, moderately, or markedly more (or less) sweet than the other two items?" Note that direction of difference (ranking) is demanded (in this particular case, "more" or "less" of the attribute).

Sometimes, however, degree without direction is asked for, the kind of question being: "Is the difference slight or marked?," or "Are you doubtful, reasonably sure, or confident of a difference?" This innocent-looking simplification introduces psychosensory bias. To be bias-free a sensory test must yield results whose long-run expectation, when the rival items A and B are in fact undistinguishable, is unambiguous, no matter how careless or prejudiced the panel may be. The null hypothesis must be statistically well-defined.

To illustrate the ambiguity introduced by nondirectional degrees of difference, a hypothetical example, based on an extension of the "poultry tenderness" results in Table 1, may be useful. In that investigation the second-stage question was: "Do you think that the putative oddity is more or less tender than the companion pair?" Now suppose that two degrees of tenderness difference (with direction, that is, "more" or "less") had been asked for.

A suitable phrasing of the question would have been: "Do you think that the putative oddity is slightly or markedly more or less tender than the companion pair?" It would be advisable to add: "If you feel that your selection of the 'oddity' was really arbitrary, so that you find no difference in tenderness, register this." (The experiment is of course assumed to be adequately balanced as regards codings and the two triangles, AAB and ABB.) Then the results might well have been as shown in Table 4—which, so to speak, collapse to those in the middle of Table 1 when the degrees ("slightly" or "markedly") are abandoned and when the neutrals ("same") are equally divided between the two directions ("less" and "more").

Table 6—"Folded" results of tables 4 or 5.

Stage 1: selection of putative oddity	Stage 2: judgments of oddity's degree of tenderness difference relative to other two items			
	none	slight	marked	Sum
Correct	4	66	22	92
Incorrect	8	109	37	154
	12			

The number of neutrals (12 in all) has deliberately been made small under the supposition that the panel is prejudiced in favor of the existence of a real tenderness difference. To show that this prejudice cannot bias the results, consider how they might most likely have appeared if in fact no real tenderness difference existed (Table 5). The inference from the experiment would have been unequivocal, namely, that the judgments pro and con

cancel out and that samples A and B are indistinguishable.

Some interest lies in a consideration of what results would most likely have been obtained (by the same prejudiced panel, testing samples that are in fact indistinguishable) if the second-stage question had been concerned with nondirectional degrees of difference, such as: "Do you think that the putative oddity you have selected is slightly or markedly different from the other pair? If you feel that your selection of the 'oddity' was really arbitrary, so that you find no difference, register this." The most likely results are given in Table 6, which is in effect a folding of Table 4 *or* Table 5 about the vertical centerline (the neutral frequencies), and which is therefore consonant with both. This is the ambiguity, rooted in psychosensory bias, that can mar the nondirectional test.

Sometimes it is hard to tell from the wording whether a two-stage triangle test was directional or not—the examples used by Bradley [1964], for instance, seem to be nondirectional and therefore open to bias. It is important to make abundantly clear, in reportage, exactly how sensory tests were conducted, with the instructions to the panel spelled out.

CONCLUSIONS

AN UNQUESTIONABLE advantage of the common triangle test is that it allows the panel to be instructed in the most general terms. A specific quality need not be mentioned in the briefing; the panel is merely asked if any one of the triad of items appears to be sensorily different. This property can be turned to further advantage with the ancillary question: "If you think that one item is different, try to characterize the difference." This question is preferably accompanied by a short list of relevant possibilities, such as texture, flavor, color, etc.

In practice, however, this sort of situation is less frequent than might be expected. Usually, the experimentalist has a specific quality firmly in mind from the start, and he wishes to restrict attention to this. Here, again unquestionably, paired comparison is the best design medium. It is structurally the simplest and psychosensorily the easiest.

Supplementation of the triangle test with a second-stage question to elicit information on the kind or direction or degree of difference between the nominal oddity and the remaining companion pair is an alluring notion. However, as has been shown, it entrains many difficulties, some stemming from the psychosensory situation itself, and some from statistical considerations. Furthermore, intersubject variability is likely to be greater in this milieu than in a simpler one.

The two-stage triangle test is normally something to be avoided. If it is used the simple scoring scheme, plus significance tests, described above, furnishes a minimally objectionable method of assessing the results.

APPENDIX

ONE TRIAL of a two-stage triangle test has four possible outcomes and, as explained in the text, the scores and the relative frequencies of these on the null hypothesis are as follows:

Table 7—Certain score probabilities (between 0.0000 and 0.3000) on the null hypothesis.

Score = ≤X or ≥(3N − X) where X is	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
0	0.0556	0.0015													
1	0.2767	0.0139	0.0006												
2		0.0633	0.0036	0.0002											
3		0.1929	0.0159	0.0010	0.0001										
4			0.0519	0.0042	0.0003										
5			0.1347	0.0142	0.0011	0.0001									
6			0.2890	0.0398	0.0039	0.0003									
7				0.0951	0.0116	0.0011	0.0001								
8				0.1967	0.0299	0.0034	0.0003								
9					0.0676	0.0092	0.0010	0.0001							
10					0.1367	0.0222	0.0028	0.0003							
11					0.2487	0.0485	0.0071	0.0008	0.0001						
12						0.0963	0.0164	0.0022	0.0002						
13						0.1749	0.0349	0.0054	0.0007	0.0001					
14						0.2925	0.0685	0.0121	0.0017	0.0002					
15							0.1242	0.0253	0.0041	0.0005	0.0001				
16							0.2094	0.0490	0.0090	0.0013	0.0002				
17								0.0888	0.0184	0.0031	0.0004				
18								0.1508	0.0353	0.0066	0.0010	0.0001			
19								0.2406	0.0639	0.0134	0.0023	0.0003			
20									0.1090	0.0256	0.0049	0.0008	0.0001		
21									0.1758	0.0462	0.0098	0.0017	0.0003		
22									0.2686	0.0791	0.0186	0.0035	0.0006	0.0001	
23										0.1287	0.0335	0.0072	0.0013	0.0002	
24										0.1992	0.0576	0.0135	0.0027	0.0005	0.0001
25										0.2940	0.0943	0.0244	0.0053	0.0010	0.0002
26											0.1476	0.0420	0.0099	0.0020	0.0003
27											0.2210	0.0693	0.0178	0.0039	0.0007
28												0.1094	0.0308	0.0072	0.0015
29												0.1658	0.0510	0.0130	0.0028
30												0.2413	0.0811	0.0226	0.0053
31													0.1242	0.0375	0.0096
32													0.1831	0.0602	0.0166
33													0.2603	0.0929	0.0277
34														0.1386	0.0446
35														0.1996	0.0695
36														0.2781	0.1047
37															0.1526
38															0.2154
39															0.2947

outcome:	ca	ia	ib	cb
score:	0	1	2	3
relative frequency:	1	2	2	1

Now two replicate trials, each so scored, will clearly have seven possible outcomes, with scores ranging from 0 to 6, with the following relative frequencies:

score:	0	1	2	3	4	5	6
relative frequency:	1	4	8	10	8	4	1

This set of relative frequencies, summing to 36 (the square of 6, the sum of the relative frequencies in a single trial) can easily be worked out from elementary considerations. More formally, it is reached by a squaring of the frequency vector (1,2,2,1).

Further replication, say N times, analogously calls for the Nth power of the vector, which will contain $3N + 1$ frequencies, with of course a score maximum of 3N. The set of frequencies, summing to 6^N, can then be expressed as cumulative probabilities. Thus, for duplicate trials, $N = 2$, and the probability, on the null hypothesis, of a score of zero *or* six is $(1 + 1)/36 = 0.0556$, while that of a score of unity or less *or* of five or more, is $(1 + 4 + 4 + 1)/36 = 0.2773$.

Observe that "two tail" probabilities must be used, because the chances of a high score are the same as those of the complementary low score. (The direction of the score scale is mathematically arbitrary in the sense that "wins" for item A can be recorded as "losses" for item B.)

The accompanying table (Table 7) gives exact probabilities for all scores in the statistically critical regions for even N up to and including 30. At $N = 30$ the score must be ≤ 31 (or ≥ 59) for statistical significance at a probability level of 0.01, and ≤ 34 (or ≥ 56), correspondingly, at $p = 0.05$. If the Normal approximation is used for the case of $N = 30$ (as described in the text) the critical scores will be found to be very close to these (≤ 31.4 instead of ≤ 31, and ≤ 34.7 instead of ≤ 34), so that the Normal approximation can be safely used for all N greater than those tabulated.

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Resistance of Clostridium perfringens to Varying Degrees of Acidity during Growth and Sporulation

SUMMARY—Viable cells of each of 6 strains of Clostridium perfringens *were exposed to 4 levels of acidity during phases of the growth cycle. The selected strains included 4 which had been recovered in association with food poisoning outbreaks and 2 strains not so associated. The growth media tested included Fluid Thioglycollate Medium, DS-sporulation medium and the CP-2V medium proposed by Hauschild.*

The level of acidity, length of exposure of the cells, the growth medium employed and the phase in the growth curve influenced the survival of C. perfringens. *Exposure of cells grown in DS-sporulation medium to buffers pH 6.0 had little effect on the survival over the 8-hr test period, with somewhat greater sensitivity of cells being demonstrated at pH 4.5. Exposure of cells, similarly produced, to buffer pH 1.0 or 2.0 was much more effective in reducing the percentage of survival, particularly during early log phase and at the onset of sporulation. Based on the 3 growth media utilized, calculated survival curves resulting from exposure of cells to pH 1.0 or 2.0 were erratic in shape, and percentage survival was almost universally less than 10%.*

Source of the strain, whether food poisoning or non-food poisoning associated, appeared to have no significant effect on the acid resistance of the cells. The comparatively regular increase in the percentage of survivors after the initiation of sporulation suggests that spores exhibit a greater resistance to acid stress than vegetative cells.

Incubation at temperatures of 37° or 0°C, during the time of treatment with the test buffers pH 1.0 or 2.0, produced no consistent change in the percentage of survivors when the cells were grown in FTG.

INTRODUCTION

THE POSSIBLE resistance of *Clostridium perfringens* to the stress of acidic environments, as found in the gastric juice and in foods, is important from the standpoint of possible food poisoning. Although the exact means by which *C. perfringens* causes gastroenteritis is not fully understood, several volunteer experiments have confirmed the pathogenicity of the oral ingestion of large numbers of organisms (Dische et al., 1957; Hauschild et al., 1967b; Hobbs et al., 1953). If the illness is due to the ingestion of cells, the cells must exhibit an unusual degree of resistance to the acidic environment of the stomach.

It seemed possible that strains of *C. perfringens* might vary in their ability to withstand acid stress. Such a difference might account for any predisposition of some strains to cause food poisoning, while others are less likely to do so. This study attempted to ascertain whether there is a strain difference based upon survival subsequent to exposure to varying degrees of acidity. Results permitted a comparison of the percent survival of 6 strains of *C. perfringens* exposed, during various phases of the growth cycle, to four levels of acidity for pre-determined lengths of time. Special consideration was given to the possible effect of the growth medium and the experimental design included production of cells in 3 different media.

Hauschild et al. (1967a) reported that vegetative cells of strains of *C. perfringens* isolated from materials associated with food poisoning outbreaks varied during the stages of the growth cycle in their resistance to synthetic gastric fluid. They observed also that the ability of cells of one strain to induce food poisoning symptoms in human beings was apparently related to the age of the cells when ingested.

Table 1—Percent survival of *Clostridium perfringens* cells of varying ages exposed to 4 levels of pH for 20 or 40 min[1,2,3]

Age (hr)	pH 6.0 Minutes exposed		pH 4.5 Minutes exposed		pH 2.0 Minutes exposed		pH 1.0 Minutes exposed
	20	40	20	40	20	40	20
0	131.6	117.8	61.4	65.8	1.8	0.3	<0.005
1/2	129.2	121.9	70.5	54.4	9.1	0.08	<0.005
1	103.5	106.7	42.2	21.0	0.3	0.02	<0.005
2	79.4	66.2	40.8	30.0	5.3	0.04	<0.005
3	107.6	94.9	33.3	34.3	2.1	0.01	<0.005
4	102.0	70.5	21.2	7.3	1.5	0.4	<0.005
5	52.6	45.9	19.8	12.8	0.03	0.005	<0.005
6	95.8	94.5	75.6	59.0	0.6	0.4	0.1
7	107.3	95.1	121.6	104.1	1.5	1.3	0.5
8	107.7	112.0	69.5	85.1	18.0	8.1	0.7

[1] Original inoculum—approximately 10^9 cells/ml.
[2] Cells grown in DS-sporulation medium.
[3] Values represent the average for six strains, except at pH 4.5 for which only two strains were tested (NCTC 8238 and ATCC 3624).

MATERIALS & METHODS

Strains

Six strains of *C. perfringens* were employed for the acid resistance studies. The four strains which had been recovered in association with food poisoning outbreaks included: National Collection of Type Cultures (NCTC) 8238 (Hobbs' Type 2), isolated from boiled salt beef; T-65, isolated in this laboratory from cooked turkey; 214a, isolated in the Health Department of Milwaukee, Wis., from beef and gravy; and IU-1168, recovered by Dr. L. S. McClung from the feces of a patient suffering from food poisoning symptoms. The two strains not known to have been implicated in food poisoning outbreaks were American Type Culture Collection (ATCC) 3624 (classical Type A) and 215b, isolated from beef liver at the University of Wisconsin.

Inoculum

Stock spore suspensions of each of the selected strains were prepared in the sporulation medium of Duncan et al. (1968) as previously described. The three growth media employed included Fluid Thioglycollate Medium (BBL), subsequently referred to as FTG; the CP-2V growth medium of Hauschild et al. (1967a), and the sporulation medium of Duncan et al. (1968). (The latter medium will be referred to in this paper as DS-sporulation medium.)

To obtain an active culture for inoculation into either DS-sporulation or FTG media, 0.1 ml of the stock spore suspension was inoculated into 10 ml of FTG, heat shocked 20 min at 75°C and incubated 16–20 hr at 37°C. The inoculum for the CP-2V medium was prepared by adding 0.1 ml of the spore suspension to 10 ml of the inoculum medium described by Hauschild et al. (1965) and heat shocking at 75°C for 20 min. After cooling to room temperature, this suspension was transferred to a larger volume of inoculum medium and incubated at 37°C for 16–18 hr. FTG and DS-sporulation media were each inoculated with a 0.1% by volume active culture, whereas a 7% by volume inoculum was used with CP-2V medium. In all cases, a magnetic stirrer was used to distribute the culture evenly in the growth medium and thus facilitated sampling from the flask through a serum-stoppered side spout. A water seal for gas evolution was provided.

Sampling

Two 5-ml samples of a bacterial suspension were removed immediately after inoculation of the respective growth media, and additional samples were drawn hourly for 8 hr. The control samples were diluted 1:2 with citrate-phosphate buffer pH 7.0 or 7.3 (Colowick et al., 1955), depending upon the pH of the suspension at a given time interval. The number of viable cells found to be present on plating the control samples at each of the time intervals provided the values used in constructing the growth curve. The test sample was diluted 1:2 with the buffer under investigation.

The pH values for the 4 buffers employed

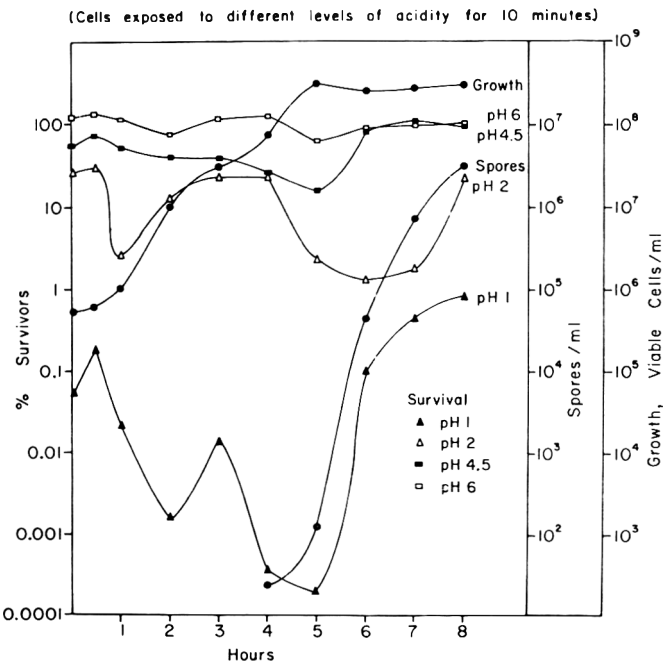

Fig. 1—Growth, sporulation and survival of Clostridium perfringens *grown in DS-sporulation medium. Values represent average for six strains, except for pH 4.5 at which acidity only two strains were tested.*

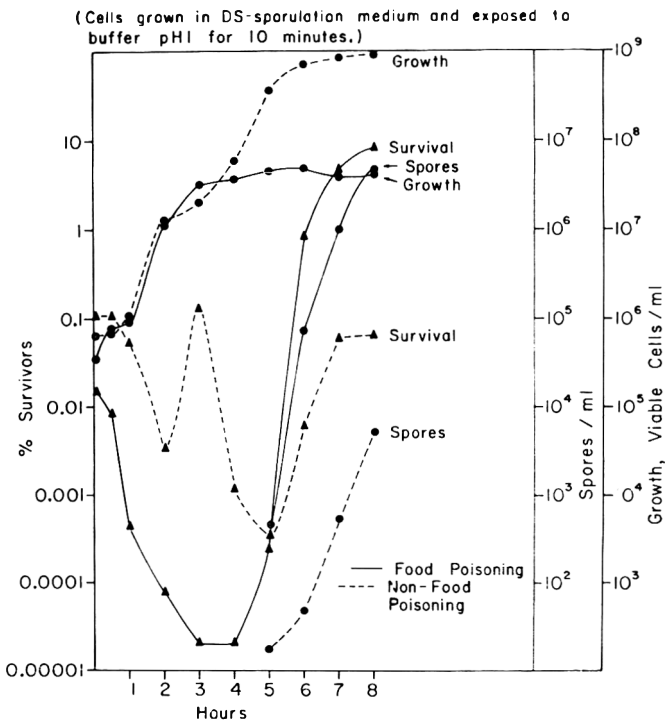

Fig. 2—Growth, sporulation and survival of food poisoning and non-food poisoning associated strains of Clostridium perfringens. *Values shown represent four food poisoning and two non-food poisoning associated strains.*

in testing the acid resistance of the selected strains were pH 6.0, 4.5, 2.0 and 1.0. Buffers of pH 6.0 and 4.5 were prepared from 0.1*M* citric acid and 0.2*M* $Na_2HPO_4 \cdot 7H_2O$, while buffers pH 2.0 and 1.0 contained 0.2*M* KCl and 0.2*M* HCl (Colowick et al., 1955).

The control and test samples of cell suspensions in DS-sporulation medium, and one series of those in FTG medium, were held at ice-water temperature during the period of exposure to the respective buffers. A second series of samples prepared in FTG medium, and the cell suspensions in CP-2V medium, were incubated at 37°C during the time of exposure to buffers of varying pH values. Predetermined time intervals for acid exposure were 10, 20 or 40 min. In all cases, at the end of the time of exposure to acid, a 1.0-ml specimen was removed immediately from the test sample and transferred to 4 ml of buffer pH 7.0 or 7.3. Appropriate dilutions of the neutralized test sample and the control sample were made in 0.1% peptone water, and each was plated in duplicate in SPS agar (BBL).

Plates were incubated at 37°C under a gas mixture of 90% nitrogen and 10% carbon dioxide. Viable counts were made after 48 hr. All experiments were replicated twice.

To obtain the number of spores produced in DS-sporulation medium, a 4-ml sample was removed beginning 4 hr after inoculation, a time interval at which previous experiments in this laboratory had indicated sporulation to begin. The samples were heat shocked 20 min at 75°C and cooled before plating in SPS agar as described above. This time-temperature relationship has been recommended for destroying vegetative cells of *C. perfringens* (Gibbs et al., 1956).

RESULTS & DISCUSSION

Survival of cells grown in DS-sporulation medium

The 6 strains of *C. perfringens* grown in DS-sporulation medium were each exposed to varying levels of acidity during which time they were held in ice water for three different exposure intervals.

The survival of *C. perfringens* during a 10 min exposure to environments of varying pH values was influenced both by the level of acidity and the phase of the growth cycle (Fig. 1). Increased time of exposure to acid stress decreased the percent survival in most cases (Table 1). The percentage survival of *C. perfringens* was only slightly affected when exposed to buffers of pH values 6.0 or 4.5. Survival of cells exposed to buffer pH 2.0 or pH 1.0 decreased during 1 or 2 hr of growth and then increased during the second or third hr of growth, respectively. With the onset of sporulation, the number of survivors again decreased and then increased quite regularly during the next 2 or 3 hr. This suggests that the presence of spores increased the ability of the bacterial population to withstand acid stress.

A comparison of the growth and survival of cells at pH 1.0 over an 8-hr period for 4 strains of *C. perfringens* originally associated with food poisoning, and 2 non-food poisoning associated strains, is shown in Figure 2. When exposed to buffer pH 1.0, the survival of the non-food poisoning associated strains was higher than the food poisoning strains during the first 5 hr of growth. During the last 3 hr of growth, the survival of the food poisoning-associated strains was higher than that of the non-food poisoning strains. A similar relationship was observed in 2 of 3 samplings when strains were exposed to buffer pH 2.0.

Any interpretation of the data presented in Figures 1 and 2 must include a consideration of the variation in performance of the individual strains when subjected to acid treatment, as well as the recognition of the low percentage of cell survival, particularly at pH 1. Figure 2 attests to the difference between strains. This variation is further emphasized in that, at the point of greatest divergence among the strains—hour 3, pH 1 (Fig. 1)—a 10,000-fold difference in number of survivors was demonstrated among the 6 strains. Similar points of wide variation included hour 8, pH 1, and hours 3 and 5, pH 2, when 1,000-fold differences occurred in each instance. Thus, the calculated survivor curves as presented represent, at some points, rather widely divergent individual values.

Based on the limited number of strains observed, it would appear that there is no difference in resistance to stress of acid environment between strains of *C. perfringens* associated with incidence of human food poisoning, and strains not so associated. For the two groups the percentage survival at the varying time intervals fell into no regular pattern, and apparently merely reflected the individual strain difference.

In no instance did a single strain, when observed during different phases in the growth cycle, respond to exposure to an acidic environment in such a manner as to produce a survival curve which would indicate a regular relationship between age of cells and ability to withstand acid treatment. This latter situation persisted for all strains at all times of acid exposure.

Survival of cells grown in FTG

Strains NCTC 8238 and ATCC 3624, each grown in FTG medium (an asporogenic medium) and exposed to two different acidity levels, were held in ice water or were incubated at 37°C for varying time intervals. The purpose of these experiments was to test the effect of acidity on only vegetative cells, and to compare the effect of two temperatures of incubation during the time the samples were exposed to acid treatment.

Survival of vegetative cells exposed to buffer pH 1.0 and held in ice water for 10 min is shown in Figure 3. The number of survivors decreased rapidly and was at a minimum after $^1/_2$ hr of growth and increased during the next $^1/_2$ hr. A decline in survival was observed after 4 hr of growth followed by an increase during the next 3 hr. Between the 7th and 8th hr, percent survival again decreased. A somewhat comparable relationship was observed when cells were exposed to buffer pH 2.0. The percent survival was at a minimum after 2 hr of growth and maximal after 7 hr of growth, with variable percentage survival at the intervening time periods. Increased exposure times to acid reduced the number of survivors in most cases.

Similar experiments were carried out utilizing FTG as a growth medium, but substituting an incubation temperature of 37°C during the buffer treatment, since it seemed possible that the incubation at ice water temperature might have placed a double stress on the organisms. Over the 8-hr growth period, however, there was no regular difference exhibited by cultures when incubated at the two temperatures. Erratic but non-parallel curves representing approximately similar magnitudes of survivors were obtained.

When percentages of surviving cells grown in DS-sporulation medium and in FTG medium and exposed to buffers pH 1.0 or 2.0 were compared, a higher number appeared to remain viable among those produced in the DS-sporulation medium. However, in all cases, the percentage remaining viable was small and the differences were of doubtful significance.

Survival of cells grown in the CP-2V medium of Hauschild

C. perfringen strains NCTC 8238 and ATCC 3624, grown in the CP-2V medium (Hauschild et al. 1967a), were exposed to buffers pH 1.0 or 2.0 and incubated in a 37°C water bath for 10, 20 or 40 min.

Comparison of the results for growth and survival of vegetative cells of *C. perfringens* produced in CP-2V medium, and exposed to simulated gastric juice without pepsin, as reported by Hauschild et al. (1967a), or exposed to buffer pH 1.0 is presented in Figure 4. The survival curves resulting from these two treatments are quite dissimilar in shape. When buffer pH 1.0 was employed, the maximum percent survival occurred during early log phase and survival was at a minimum at the end of the growth phase. This result is in direct contrast to that obtained when simulated gastric juice without pepsin was present.

The irregularity in the shape of the calculated survivor curve obtained in the present studies when the cells were exposed to acid buffers is further underscored by the fact that although 3 different growth media were utilized for producing the cells, survivor curves, espe-

cially following exposure to pH 1.0 and 2.0, were always erratic in shape, except subsequent to the initiation of sporulation when the curves became quite regular. The latter result suggests that the presence of spores increased the ability of the bacterial population to withstand stress.

The percentage of surviving cells as observed by Hauschild et al. (1967a) during the 8-hr growth period was always higher than that obtained in the present study, although in no case, in either series of somewhat comparable experiments, were resistant cells present in excess of 10%. Variation which did appear may have been due to the performance of individual strains when exposed to acid stress, since this study revealed that, indeed, strains did differ in this regard. A second factor to be considered is the possible effect of the composition of the solution in which the cells were suspended during the acidity tests. Meyers (1928) concluded that different buffer mixtures of approximately the same pH exerted very different germicidal effects.

Although irregularly-shaped calculated survival curves were produced in this study, the regularity of the control growth curves suggests that transient changes generally do not exist at various phases of the growth cycle. The results obtained do confirm that the survival of cells of *C. perfringens* exposed to conditions of low pH varies with the growth phase, but that the influence of specific age of the cells is difficult to predict. This is not an unexpected result since earlier workers noted a variation in sensitivity of cells at the time of rapid cell division to such factors as low temperature, heat, phenol NaCl and kind of acid. (Cohen et al., 1919; Sherman et al., 1923; Srivastava et al., 1965; Stark et al., 1929.)

The practicability of observation of the type reported here is open to question. The effectiveness of the acidity of the stomach as a bactericidal bar is mitigated by the simultaneous presence of protein, as shown by Hauschild et al. (1967a), although their results indicated a considerable range in values between proteins and between different samples of the same protein. Likewise, it is known that the pH of the stomach is considerably increased during the time food is present. Also, there is the question of how long the food remains in the stomach. The evidence accumulated here indicated that usually the longer the period of association between organism and buffer of lower pH value, the greater the bactericidal action. Hauschild et al. (1967a) did show a difference in occurrence of food poisoning symptoms in human beings when vegetative cells of *C. perfringens* representing different ages were consumed just previous to lunch.

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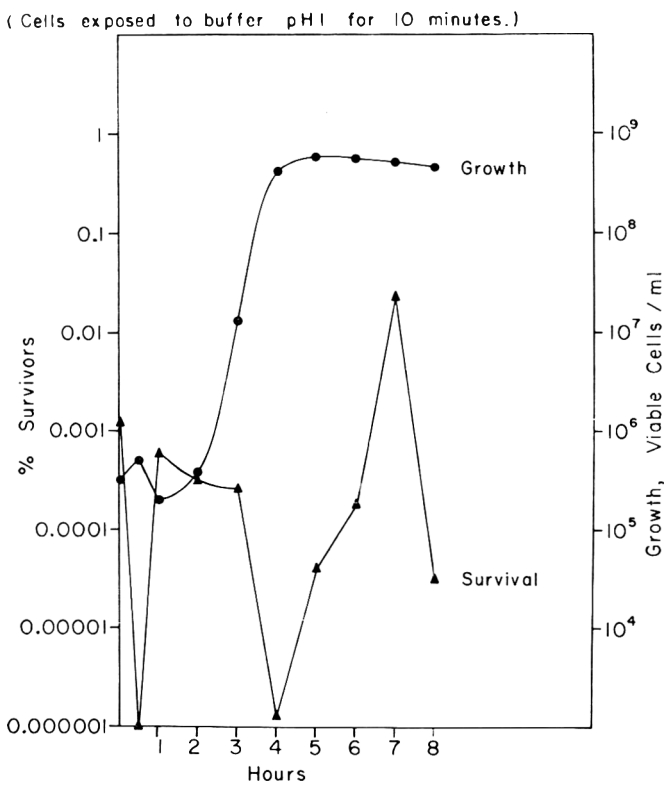

Fig. 3—Growth and survival of Clostridium perfringens *grown in fluid thioglycollate medium. Values represent average for two strains.*

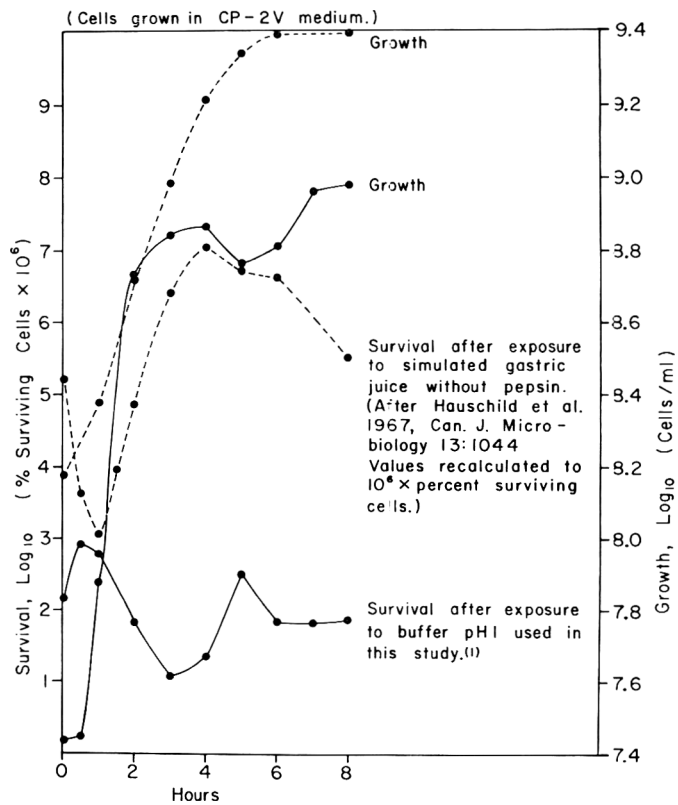

Fig. 4—Comparison of the growth and survival of Clostridium perfringens *cells exposed to simulated gastric juice without pepsin and to buffer pH 1 for 10 min. (The values for two strains are for our work only; Hauschild tested one strain.) Values represent average for two strains.*

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Two curves in Figure 4 reproduced by permission of the National Research Council of Canada from the Canadian Journal of Microbiology, **13,** p. 1044 (1967).

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Determining the Odor Thresholds for Some Compounds in Alcoholic Beverages

SUMMARY—Determinations have been made of the sensory odor threshold estimates of four alcohols, five esters, seven acids and diacetyl found in alcoholic beverages, by means of the triangular test. The threshold levels ranged among alcohols from 5 to 8 ppm except for isobutyl alcohol with the level of 75 ppm. Among esters there were three with the level of 0.2–0.6 ppm and two with the level of 14–17 ppm. Among acids three levels were found, one with the range of 4–9 ppm, one with 15–20 ppm and isovaleric acid with 0.7 ppm. Diacetyl had the lowest level—0.0025 ppm. The determination of thresholds was standardized by calculation of the percentage-above-chance-scores; by testing the usefulness of the amateur panel, and by specification of the threshold estimates in statistical terms. The results attained a fair goodness-of-fit of the model and the smell perception was found to vary with the logarithm of the stimulus. The distribution of scores follows the normal probability function.

INTRODUCTION

THE DEVELOPMENT of analytical methods has made it possible to isolate and identify many volatile compounds related to aroma. The mere identification of compounds does not reveal important qualitative features. Aroma—a characteristic, pleasant smell—is the response of the sense of smell to stimulating volatile compounds. Intensity of stimulation depends on a combination of the potency of the substance and its concentration in the inhaled air space (Harrison, 1967). Some knowledge must be gained of both these factors. In practice one must measure the minimal levels at which substances can be detected by a panel of judges. Berg et al. (1955) and Keith et al. (1968) have determined flavor thresholds: Patton et al. (1957) and Guadagni et al. (1963) odor thresholds when the solvent is liquid. With respect to alcoholic beverages, Harrison (1963, 1966, 1967) studied the sensory odor thresholds of compounds of beer which had been analyzed by gas chromatography and were chemically different, by application of the pair test and the use of a professional panel.

To make it possible later to correlate gas chromatographic data with sensory flavor, a beginning was made with relatively few compounds involving some of the most important peaks of chromatograms (Powers et al., 1968). The aim was to develop a method for the determination of sensory odor threshold of some common aroma compounds of alcoholic beverages, with concentration at first on the sense of smell alone. The purpose was to get information on sensory odor perception in a situation adequate to a natural use of alcoholic beverages. The work is preliminary in nature, with an amateur panel. An endeavor has been made to find the origin of variance and the nature of the standard deviation and if practicable, to appraise the sensation and propose a model for it.

EXPERIMENTAL

THE TRIANGULAR test was used in the selection of panel members, in determination of the threshold proper and in testing the reliability of the results obtained. The triangular test has proved to be statistically most advantageous (Hopkins et al., 1955); it has also proved advisable for panel selection (Bengtsson, 1953, Helm et al., 1946) and is suitable for threshold studies (Dawson et al., 1963). As the panel was an amateur one, moreover, the triangular test was selected to ensure that the part played by chance would remain moderate.

Those composing the panel were selected from among the laboratory staff, to provide members who possessed an average sense of smell. For the purposes of selection, use was made of obviously perceptible and suprathreshold concentrations of isoamyl alcohol and β-phenylethyl alcohol. In the first selection, with clearly perceptible concentrations, all those accepted could smell three out of six combinations (AAO AOA OAA OOA OAO AOO (A = sample with a studied compound added, O = blank i.e. alcohol/water solution), when pure chance would amount to 32%. In the other selection, with supra-thresholds, the rejection limit was 16/24, and chance remained below 10%. Personal thresholds were determined for 20 selected persons and, on the basis of these and the selection tests, the panel members were ranked in order of superiority so the groups of 10 persons making daily judgments were comparable, despite different compositions.

The following substances were chosen: diacetyl (Fluka AG), isobutyl alcohol (E. Merck), isoamyl alcohol (J. T. Baker), *n*-hexyl alcohol (Fluka AG), β-phenylethyl alcohol (B.D.H.), ethyl acetate (E. Merck), ethyl caprylate (Fluka AG), ethyl lactate (B.D.H.), isoamyl acetate (Fluka AG), β-phenylethyl acetate (Fluka AG), propionic acid (Fluka AG), butyric acid (Fluka AG), isobutyric acid (Eastman), isovaleric acid (Fluka AG), caproic acid (Fluka AG), caprylic acid (Fluka AG) and capric acid (Fluka AG). All the compounds were commercial preparations and of the highest degree of purity available; at least 97.5%.

Highly rectified grain spirit with an alcohol content of 94.4% (w/w) was used as the solvent. The compounds were diluted with strong spirits to stock solutions of 10,000 ppm and diluted further with water, purified with ion exchange columns and active charcoal, to solutions of 1000 ppm, and then to test solutions by addition to the solution of water and spirits 9.4% (w/w), i.e., strong spirits diluted with water in the ratio 1:10. On every test day, the fresh samples were presented to the panel and pure ones were substituted if any smell of impurity was identified. The smelling was conducted in the laboratory at a temperature of 20°C and a relative humidity of 50%. The test liquids were served in colorless aroma glasses of 150 ml covered with watch-glasses. Before the liquids were portioned out, the glasses were rinsed with the solution of water and spirits to ensure the same conditions on each occasion. The volume of the sample was 30 ml.

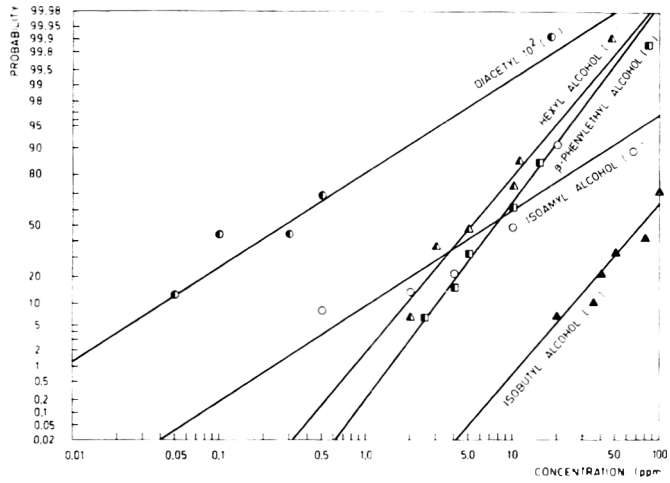

Fig. 1—Response reliability of smell as function of concentration; alcohols and diacetyl.

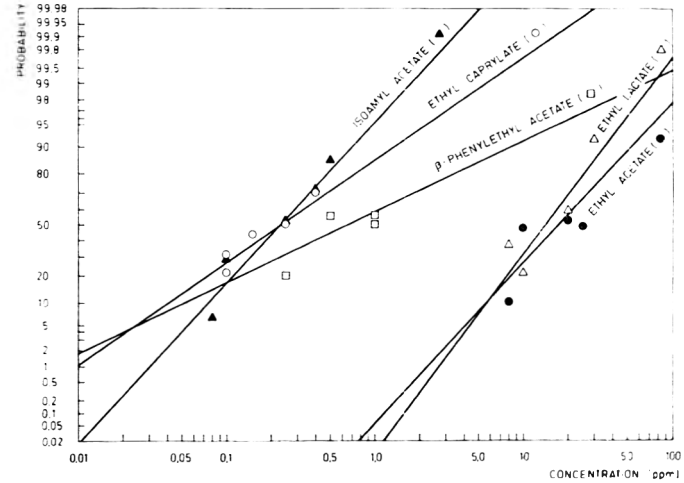

Fig. 2—Response reliability of smell as function of concentration; esters.

Five members of the panel were chosen to search for the concentrations which would be studied from a series of 5–8 dilution samples. The concentration which they could just identify was called the start concentration. In the determination proper of thresholds, the panel received this start concentration—and dilutions 2, 5 and 10 times stronger and lighter—until every substance had five tested concentrations. In every test situation, the test series of four triangular sets, drawn from six possible combinations, was passed to each judge. Every set contained one or two samples of a studied compound with the concentration in question in water/alcohol solution and one or two blank samples—pure water/alcohol solution—the relation of which was determined by randomizing of triads. Every judge was required to check odd samples with the knowledge that two samples were the same and one different. A judge was advised to guess if no difference could be found. The probability of chance equaled $^1/_3$.

To explain the standard deviation and personal variation, 5–8 dilutions of β-phenylethyl alcohol and ethyl acetate were given to the panel, and each judge had to indicate whether the odd sample he selected was pure alcohol or contained some added aroma compound. The probability of chance was then $^1/_6$. When the judge had selected correctly at least two consecutive concentrations, the lower one was regarded as his daily test threshold level. The panel had 10 replications and the judges 5 to 8.

In the triangular test the number of odd samples correctly identified can be regarded as the test score, i.e. the percentage of correct judgments can be a measure of the intensity of sensation and a measure of the difference in intensity of sensation between two samples (Lockhart et al., 1952, Berg et al., 1955). When no detectable sensory difference exists, it is probable that a chance percentage of correct identifications equal to 33.3 will be scored by a panel working with a triangular system. Consequently, it is more meaningful to calculate the percentage-above-chance-scores. In binomial designs, the following formula can be applied:

$$P_c = 3/2\ (P_o - 33.3)$$

where

P_o = the percentage of correct judgments observed in the test

P_c = the percentage of correct judgments over and above chance, expressing the score of the intensity of smell sensation, and used as a relative score for comparison of the threshold levels.

Attention has been given to the significance of differentiation, along with the percentage-above-chance-scores.

It is expected that the smell perception, according to Fechner's law (Amerine et al., 1965) varies with the logarithm of the stimulus value and it is assumed that the distribution of scores around the halfway point follows the normal probability function. When a graphical model in which the concentrations were plotted against percentage-above-chance-scores on log-probability paper (Harrison et al., 1950, Lockhart et al., 1952), was applied to these data, it seemed to fit a straight line. Thus the regression of the percentage-above-chance-scores on logarithmic concentration of the studied compounds was

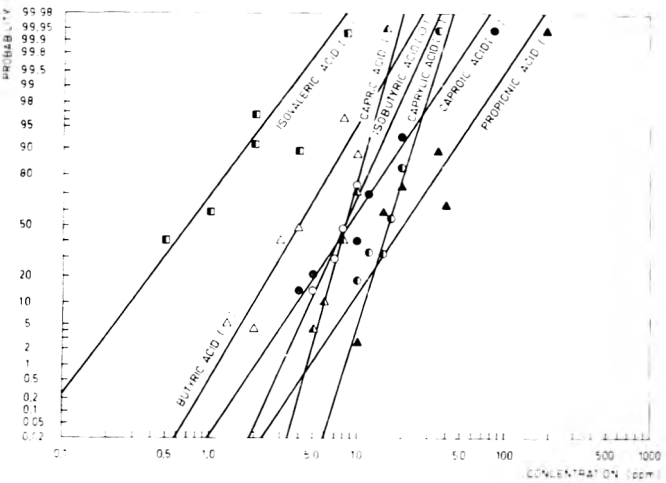

Fig. 3—Response reliability of smell as function of concentration; acids.

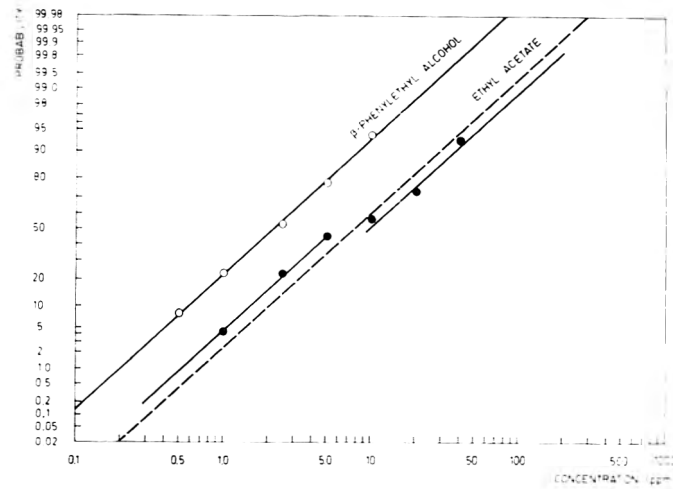

Fig. 4—Cumulative frequency functions of daily replications of the panel.

Table 1—Judgments in the triangle tests of different concentrations of aroma compounds calculated percentage-above-chance-scores, and the significance between correct and total judgments.

Compounds	Concentration (ppm)	Judgments		Percentage-above-chance-score
		Correct	Total	
Diacetyl	0.0005	15	36	12.6
	0.001	25	40	43.8***
	0.003	20	32	43.8***
	0.005	35	44	68.9***
Isobutyl alcohol	20.0	15	40	6.3
	40.0	19	40	21.3*
	50.0	20	36	33.5**
	80.0	27	44	41.4***
	100.0	29	36	70.8***
Isoamyl alcohol	0.5	17	44	7.8
	2.0	20	44	13.8
	4.0	19	40	21.3*
	10.0	29	44	48.9***
	20.0	30	32	90.8***
n-Hexyl alcohol	2.0	15	40	6.3
	3.0	23	40	36.3**
	5.0	29	44	48.1***
	10.0	33	40	73.8***
	11.0	36	40	85.1***
β-Phenylethyl alcohol	2.5	15	40	6.3
	4.0	19	44	14.9
	5.0	22	40	32.4**
	10.0	30	40	61.4***
	15.0	36	40	85.1***
Ethyl acetate	5.0	12	40	
	8.0	15	36	10.1
	10.0	26	40	47.6***
	20.0	30	40	62.6***
	25.0	26	36	58.9***
Ethyl caprylate	0.05	13	40	
	0.10	21	44	21.6*
	0.10	24	44	31.8**
	0.15	25	40	43.8**
	0.25	24	40	40.1**
	0.40	32	40	69.6***
Ethyl lactate	5.0	8	40	
	8.0	21	36	37.5**
	10.0	19	40	21.3*
	20.0	29	40	58.8***
	30.0	34	36	91.6***
Isoamyl acetate	0.05	12	40	
	0.08	15	40	6.3
	0.10	21	40	28.8*
	0.25	27	40	51.3***
	0.40	32	40	69.6***
	0.50	36	40	85.1***
β-Phenylethyl acetate	0.20	10	44	
	0.25	20	40	25.1*
	0.50	31	40	55.8***
	1.00	27	40	51.3***
	1.00	31	44	55.8***
Propionic acid	10.0	14	40	2.6
	15.0	29	40	58.8***
	20.0	33	40	73.8***
	35.0	37	40	88.8***
	40.0	30	40	62.6***
Butyric acid	2.0	12	36	4.2
	3.0	24	40	40.1***
	4.0	26	40	47.5***
	8.0	39	40	96.3***
	10.0	33	40	87.6***
Isobutyric acid	5.0	17	40	13.8
	7.0	19	36	29.3
	8.0	26	40	47.5***
	10.0	32	40	70.1***
Isovaleric acid	0.5	24	40	40.1***
	1.0	29	40	58.8***
	2.0	37	40	91.0***
	2.0	43	44	96.8***
	4.0	37	40	88.8***
Caproic acid	4.0	17	40	13.8
	5.0	19	40	21.3
	10.0	24	40	40.1***
	12.0	32	40	70.1***
	20.0	38	40	92.6***
Caprylic acid	10.0	18	40	17.6
	12.0	20	36	33.4**
	15.0	22	40	32.4**
	17.0	28	40	55.4***
	20.0	32	36	83.1***
Capric acid	5.0	13	36	4.2
	6.0	16	40	10.0
	8.0	24	40	40.1***
	10.0	31	40	70.1***
	20.0	32	32	100.0***

* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0.1% level.

Table 2—Correlation coefficient of the regression lines of the studied compounds, F-values and statistical significance.

Compound	Correlation coefficient	F-values	df	Significant
Diacetyl	0.87	6.13	1/2	$P < 0.20$
Isobutyl alcohol	0.97	48.81	1/2	$P < 0.01$
Isoamyl alcohol	0.91	15.30	1/3	$P < 0.05$
Hexyl alcohol	0.97	38.69	1/3	$P < 0.01$
β-Phenylethyl alcohol	0.99	179.81	1/3	$P < 0.001$
Ethyl acetate	0.82	4.05	1/2	$P < 0.20$
Ethyl caprylate	0.89	11.44	1/3	$P < 0.05$
Ethyl lactate	0.91	9.76	1/2	$P < 0.01$
Isoamyl acetate	0.97	41.27	1/3	$P < 0.01$
β-Phenylethyl acetate	0.84	4.77	1/2	$P < 0.20$
Propionic acid	0.79	4.92	1/3	$P < 0.20$
Butyric acid	0.96	31.94	1/3	$P < 0.05$
Isobutyric acid	0.99	77.25	1/2	$P < 0.05$
Isovaleric acid	0.82	4.62	1/3	$P < 0.20$
Caproic acid	0.96	39.05	1/3	$P < 0.01$
Caprylic acid	0.92	16.33	1/3	$P < 0.05$
Capric acid	0.995	300.54	1/3	$P < 0.001$

calculated. Furthermore, the regression lines were tested for measures of the goodness of fit of the line to the data with the analysis of variance. The direction of the lines was tested by comparing the slope of the lines. Since the standard deviation proved to be large, the homogeneity of variance was tested by the Bartlett test. To compare replications in testing the reliability of the results, t-values were also calculated. All the calculations were effected on a GE 415 computer owned by the Finnish State Alcohol Monopoly (Alko).

RESULTS & DISCUSSION

TABLE 1 INDICATES the judgments obtained for the concentrations of substances studied, along with the calculated percentage-above-chance-scores and the significance in the triangular test. In Figures 1–3, the concentrations have been plotted against the percentage-above-chance-scores on the log-probability paper. The regression lines formed appear to describe the response of smell perception as a function of logarithmic concentration. The correlation coefficients of the lines were as high as $r = 0.78 - 0.995$ (Table 2), but the judgments of some compounds (diacetyl, ethyl acetate, β-phenylethyl acetate, propionic acid and isovaleric acid) could not be explained as statistically significant lines. Nevertheless, the major proportion of judgments gives statistically significant goodness-of-fit of the line, and the figures relating to β-phenylethyl alcohol and caprylic acid are highly significant. Since the F-values (Table 2) of even unsignificant lines were relatively high ($0.1 < P < 0.20$), and since the explanation of the model will always be above 60% ($100r^2$), the percentage-above-chance-scores, representing the response of smell perception, may be regarded as a linear function of the logarithmic concentration.

The plots of the response of smell perception have been selected in an effort to obtain criteria similar to those commonly used in the determination of sensory threshold levels. Swets (1961) studied sensory thresholds and plotted the proportion of right responses to signals added to a background stimulus against the responses obtained to the noise alone. The plots of the psychometric function form a curve of one or more line segments, and the threshold can be calculated from these. Swets arrived at the result that a two-line curve fitted the yes-no data reasonably well and described best the relations between stimulus and sensation. In this work, so few estimates were determined for every substance only five concentrations—that it was impossible to test whether a two-line-model would give a better explanation of the results. The results in regard to some compounds (hexanol, ethyl acetate) suggested that the plot of smell response might be composed of more than one line segment.

For explanation of the origin of the variance, replications made with β-phenylethyl alcohol and ethyl acetate have been observed, first with individuals and second with test days as variables. The distribution of the replications indicates that the cumulative frequency values of the replications plotted on the log-probability paper form a linear function of logarithmic concentration (Fig. 4). As the cumulative sum curve significantly approaches a straight line, the logarithmic values of concentration can be considered to be normally distributed. Ethyl acetate proved to be interesting, because the curve of the sum function was formed by two line segments, so that the distribution has two peaks. This result could be expected, as the threshold levels of ethyl acetate determined by different persons were divided into two quite different groups.

The daily replications of the panel indicated that although the standard deviation was rather large ($100\delta/\bar{x}$ = ca 30%), different replications could be considered reliable, as no statistically significant difference was demonstrable between replications tested on different days (ethyl acetate $F = 1{,}06$ df. 9/96 $P < 0.20$, β-phenylethyl alcohol $F = 1.74$ df. 9/98 $0.05 < P < 0.10$). Moreover, the variance notwithstanding, its magnitude proved to be homogeneous on the application of Bartlett's test (ethyl acetate $\chi^2 = 3.73$ df. 9 $P > 0.90$, β-phenylethyl alcohol $\chi^2 = 4.01$ df. 9 $P > 0.90$). One replication of β-phenylethyl alcohol differed from the others, giving statistically significant t-values, but with ethyl acetate there were none. The reason for the difference with β-phenylethyl alcohol was found to be the participation on the same day of too many judges insensitive to this particular substance.

When the individuals were variables, with β-phenylethyl alcohol there were very significant differences between judges ($F = 2.79$ df. 1 6/91 $0.001 < P < 0.01$) and with ethyl acetate highly significant differences between judges ($F = 11.61$ df. 17/88 $P < 0.001$). Nevertheless, the variance was still homogeneous (β-phenylethyl alcohol $\chi^2 = 6.81$ df. 16 $P > 0.95$, ethyl acetate $\chi^2 = 16.81$ df. 17 $P < 0.30$). The order of superiority for the judges equals the results as far as β-phenylethyl alcohol is concerned, but inequalities were revealed with regard to ethyl acetate. One judge smelled β-phenylethyl alcohol "too well" if no attention is paid to his values, the significance between individuals diminishes to 95%.

The results with ethyl acetate divided the judges into two groups, but the ability of some persons to smell alcohol varied significantly from their ability to smell acetate. Consequently, it is possible to have panels on different days which are so unequal that the results can no longer stand comparison, although the selection of judges can noticeably restrict this hazard. However, there is evidence of so large a variation in ability to smell different substances that the order of superiority found for one substance does not apply to others.

Table 3—Sensory odor threshold levels of studied compounds, with percentage-above-chance-scores of 50% in grain spirit solutions of 9.4% (w/w).

Compound	Odor threshold (ppm)	(mM)
Diacetyl	0.0025	0.000035
Alcohols		
Hexyl alcohol	5.2	0.05
Isoamyl alcohol	7.0	0.08
β-Phenylethyl alcohol	7.5	0.06
Isobutyl alcohol	75.0	1.00
Esters		
Ethyl caprylate	0.25	0.0015
Isoamyl acetate	0.20	0.002
β-Phenylethyl acetate	0.65	0.005
Ethyl lactate	14.0	0.11
Ethyl acetate	17.0	0.20
Acids		
Isovaleric	0.7	0.007
Butyric	4.0	0.045
Isobutyric	8.1	0.09
Capric	8.2	0.05
Caproic	8.8	0.075
Caprylic	15.0	0.11
Propionic	20.0	0.27

Before it is possible to consider a threshold as a characteristic of a sensory system, the threshold value of a stimulus has to be specified in statistical terms, as abundant evidence exists of continuous physiological change in large numbers of receptive and nervous elements in various sensory systems (Swets, 1961). The concentration of a particular flavor or odor which, on the average, can be detected 50 percent of the time, is known as the threshold concentration (Guilford, 1936). Table 3 contains estimates, made on this basis, of the thresholds of the compounds studied, read from the intersection of the regression line at the 50 percentage-above-chance-score. On examination of the values, it can be said that the esters have the lowest threshold levels. Diacetyl has the lowest level. Isobutanol has the highest.

The order of thresholds does not follow any systematic order of acids or esters and the isomeric compounds, such as butyric and isobutyric acids, have different thresholds. According to the sensory odor thresholds determined in this work, and the quantities of these compounds found in alcoholic beverages, it seems that isoamyl alcohol and isobutanol can be considered as large components among the alcohols. The threshold level of isoamyl alcohol is about one tenth that of isobutanol. In turn, β-phenylethyl al-

cohol can be a small but very significant compound. The importance of hexanol would perhaps remain of no consequence.

In regard to the quantitative values and odor threshold values of the esters studied, it seems that ethyl acetate would also be a large component, with its importance dependent on its quantity. Ethyl caprylate, isoamyl acetate and β-phenylethyl acetate comprise a rather homogeneous group. The importance of ethyl lactate is doubtful.

Nykänen et al. (1968) have determined in some distilled beverages 90–600 mg/l total volatile acids calculated as acetic acid, when acetic is approximately 40–95% of the volatile acids. Comparison of the threshold levels of the acids studied, and their average relative amounts, indicates that by reason of the smell perception of humans the small component isovaleric acid may be more important than the large components capric and caprylic acids. Isovaleric acid seems to be a significant component for both Finnish beer (Arkima, 1968) and distilled beverages. Butyric, isobutyric and caproic acids may be less prominent.

The importance of propionic acid is probably significant as regards odor perception. When these odor threshold values were compared with the flavor values found by Harrison (1963, 1967) in Irish beer, these proved to be about one tenth of the flavor threshold values. But the interrelations of the compounds in nearly all the groups were apparently the same, even if the values were not quite commensurable.

It has been observed in sensory tests that 2–4 mg/l diacetyl in pure spirits can cause an impure flavor or odor (Wessel, 1955). On comparison of the threshold level found in the test (0.0025 ppm) with the values given above, it seems likely that the odor contamination might be more significant than flavor contamination, particularly in pure spirits. In 1963, Harrison reported a taste threshold of 0.005 ppm in Irish beer, which is close to the level obtained in this test; in later studies however, Harrison (1967), gives the higher value of 0.1 ppm for the same kind of beer.

With respect to the method employed and its utility, it must be observed that every regression point is composed of 40 sample points, and a regression line is determined by 200 judgments. Consequently, the material can be considered adequate. If the odor threshold values are criticized on the basis of the numerical values alone, some inaccuracy can be found, as there were regression lines with a slope which did not attain statistical significance under all circumstances. Nevertheless, the explanation of the model was quite good, and the regression lines of different substances differed from each other. So it seems possible that this method might be suitable for the determination of sensory thresholds and, at least, the relations between the substances can be considered to be significant.

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Physical and Chemical Characteristics of Free and Stretched Rabbit Muscle

SUMMARY—Shear strength, sarcomere length and protein solubility were studied in rabbit longissimus dorsi allowed to pass through rigor in free and stretched physical states. Stretched muscles were significantly more tender as evidenced by lower shear values. They also exhibited significantly longer sarcomeres as compared to their paired controls allowed to pass through rigor without restraint. Greater amounts of total protein were extracted from stretched muscles in all trials except one. Significantly greater amounts of actomyosin were extracted from stretched muscles in all trials. The actomyosin results were unexpected since it has been suggested by several workers that actomyosin formation is directly related to toughness in muscle. Some possible explanations for the results obtained in this study are discussed.

INTRODUCTION

MEAT, the post-mortem aspect of muscle, has been the subject of many investigations primarily because of its variation in tenderness. Efforts to elucidate causative factors have resulted in the study of various ante-mortem and post-mortem conditions known to influence tenderness.

Interest in the effect of the contractile state of muscle, during rigor, on ultimate tenderness was somewhat neglected until Locker (1960) reported that relaxed muscles were more tender than partly contracted muscles, and suggested that certain muscles might be improved in quality by altering contraction states during rigor. Subsequently there were several reports on the interrelationships of carcass position or restraint, sarcomere length, fiber diameter, cold shortening, and tenderness of muscle (Herring et al., 1965 a,b; Eisenhut et al., 1965; Marsh et al., 1966; Buck et al., 1967).

Changes in sarcomere lengths associated with different contraction states offer strong evidence that mechanical forces during rigor can result in a displacement of actin and myosin filaments. It has been suggested that post-mortem molecular alterations, as influenced by contraction state during rigor, may be of prime importance in the ultimate tenderness or toughness of meat (Herring et al., 1965 a,b; Buck et al., 1967; Howard et al., 1968).

Since the changes which take place in muscle after death favor the formation of a complex between the actin and myosin filaments, it might be concluded that the amount of actomyosin formed during rigor is related to the amount of interdigitation of filaments and, ultimately to toughness.

This study was designed to investigate the post-rigor differences in actomyosin content, sarcomere length, and shear strength of paired muscles allowed to pass through rigor in free and stretched states.

EXPERIMENTAL

Origin and preparation of samples

Five trials were conducted using five pairs of longissimus dorsi (LD) muscles obtained from 6-month-old, male, Dutch Belted rabbits.

Animals were anesthetized by exposure to chloroform vapors for 1–2 min. They were then immediately exsanguinated, skinned and eviscerated. One of the pair of LD muscles was removed from the area bounded by the 4th rib and the anterior edge of the hips. The excised muscle was placed on a moistened glass plate and covered with plastic film to prevent drying.

The rabbit carcass containing the remaining LD muscle was draped, ventral surface down, over a hook placed perpendicular to its long axis. The hook gripped the spinal column approximately behind the last rib. The front and rear legs were drawn together on the ventral side and tied in this position in order to maintain a positive stretch on the LD muscle.

The excised, free LD, and the stretched carcass were held at 1–2°C for 24 hr at which time it was assumed the rigor process was complete. Right and left LD's were alternated between the two treatments.

At the end of the storage period, the LD remaining on the carcass was excised using the same skeletal reference points mentioned earlier.

A 20-g sample was removed from the anterior end of each LD by making a cut perpendicular to the long axis of the muscle. This sample was divided, longitudinally, into two 10-g portions, one of which was used for the protein assay. The other 10-g portion was used in conjunction with another experiment, the results of which are not reported here.

A 3 mm thick slice was removed from the anterior end of each remaining muscle and was immediately fixed in a 10% formalin solution and stored for future microscopic analysis.

The unused portions of LD were used for shear strength measurements.

Sarcomere length determination

A 3 × 3 mm strip was removed from the formalin fixed slice and blended for 5½ min with distilled water in a chilled blender. The suspension of myofibrils was examined directly in a phase contrast microscope, and sarcomere length was determined as an average of 25 myofibrils for each of the muscle samples.

Shear force measurements

The sample for shear force determination was clamped loosely between ¼ in. thick aluminum plates and immersed in a bath of corn oil held at 127°C. Sample temperature was determined continuously by means of a thermocouple, placed in the center of the sample, and a recording potentiometer.

The sample was removed from the oil bath when the internal temperature reached 71°C. Maximal internal temperature reached after removal from the oil bath was 90°C.

The sample was allowed to cool to room temperature and strips for shearing were prepared with a device consisting of two surgical scalpels taped together so that the distance between the cutting edges of the blades was exactly 7 mm. Beginning at the posterior end of the muscle, five 7 mm thick slices were removed by cutting perpendicular to the long axis of the muscle. A 7 mm strip was then removed from the center of each slice by cutting in a dorsal-ventral direction. This produced a strip with muscle fibers diagonal to the long axis of the strip.

An Allo-Kramer Shear Press, equipped with a single-bladed meat shear cell, was used to determine the force required to shear the strips perpendicular to their long axes. Operating parameters were as follows: proving ring, 100 lb; pressure, 90 lb; ram speed, 21.5 sec. full stroke. The recorder range switch was set at 20% so that full scale on the recorder chart equaled 20 lb.

Protein extraction and fractionation

Actomyosin and myosin were determined by a modification of the method developed

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by Weinberg et al. (1960). Ten g of sample were homogenized in 200 ml of a solution of KCl, 0.4*M*, and potassium phosphate buffer, pH 7.5, 0.05*M*. This solution had an ionic strength of 0.55.

The KCl extracted samples were centrifuged at 750 × G for 5 min and the sediment was discarded. An aliquot of the supernatant fraction was removed for protein determination. A second aliquot was diluted to an ionic strength of 0.225 by the slow addition of distilled-deionized water to precipitate the actomyosin. The suspension was centrifuged at 1000 × G for 15 min and the actomyosin precipitate was resuspended in KCl and saved for protein analysis.

An aliquot of the supernatant fraction was diluted to an ionic strength of 0.05 and the myosin fraction removed by centrifugation as indicated above. The protein content of the myosin fraction was also determined as was that in the final supernatant or non-contractile protein fraction. All extractions were conducted at 0–5°C. The protein content of samples was estimated by a biuret method (Gornall et al., 1949.)

Table 1—Protein extractability of paired muscles allowed to pass through rigor in free and stetched conditions.

	Free					Stretched				
Trial no.	Total[1] protein	ACTO[2]	MYO[3]	NCP[4]	ACTO. as % of total protein	Total protein	ACTO	MYO	NCP	ACTO. as % of total protein
1	48.1	14.9	6.4	17.5	31.0	87.8	53.8	1.2	24.6	61.0
2	64.6	28.9	3.3	29.5	45.0	98.8	49.0	18.7	31.8	50.0
3	86.4	38.8	3.9	19.0	45.0	80.4	43.5	1.4	22.1	54.0
4	62.6	25.6	2.4	37.0	41.0	88.9	52.0	1.7	35.6	58.0
5	66.3	26.8	2.7	25.2	40.0	82.6	43.8	0	23.7	53.0
x's	65.6	27.0	3.7	24.0	40.0	87.7	48.4	4.6	27.6	55.2

[1] All figures, except those indicated as percentages, reported as mg/g of fresh tissue.
[2] ACTO = Actomyosin.
[3] MYO = Myosin.
[4] NCP = Non-contractile protein.

RESULTS & DISCUSSION

THE MUSCLES allowed to pass through rigor without restraint were approximately 17 cm in length at the time of excision. These muscles did not show any measurable cold shortening or cold contraction at the end of the storage period. This is in agreement with Locker et al. (1963) who reported a cold shortening effect for certain beef muscles but reported the cold contraction was absent in rabbit LD and psoas. The post rigor excised muscles were approximately 20 cm long when measured after excision. The carcass stretching technique employed was effective in increasing muscle lengths $2^1/_2$ to 4 cm over paired controls.

Sarcomere lengths of free and restrained muscles

Several reports have been published demonstrating a relationship between state of contraction during rigor and sarcomere length (Herring et al., 1967; 1965 a,b; Howard et al., 1968). In this study, sarcomere length was determined to measure the effectiveness of the stretching treatment to be sure that the paired muscles did have different average amounts of overlap of the actin and myosin filaments.

The average sarcomere lengths of the muscles used in the five trials are shown in Figure 1. The sarcomere lengths of the free muscles were relatively uniform ranging from 1.74 to 1.95μ. These values are similar to those reported by Paul (1965) who found an average sarcomere length of 1.76μ for raw rabbit LD after 24 hr cold storage.

The effectiveness of the stretching treatment employed was variable, producing sarcomere lengths in post-rigor excised muscles ranging from 2.27 to 3.17μ. This represents increases in sarcomere lengths, over paired free muscles, ranging from 16 to 82%. The paired differences were tested for significance by means of the paired t-test (Snedecor, 1956) and were found to be significantly different ($P < 0.02$).

Shear force measurements

The average force required to shear 5 strips prepared from each of the free and stretched muscle samples used in the five trials is shown in Figure 2.

Greater force was required to shear samples prepared from free muscles in all trials. Application of the paired t-test revealed that the differences in shear force between free and stretched muscles in this study were statistically significant ($P < 0.01$).

The differences in shear force between muscle samples were consistently large with the exception of Trial 2. It will be recalled, however, that the stretching treatment employed was least effective in this trial producing only a 16% increase in sarcomere length.

The interrelationships of shear force, sarcomere length and muscle restraint reported in this study were expected and have appeared in the literature previously

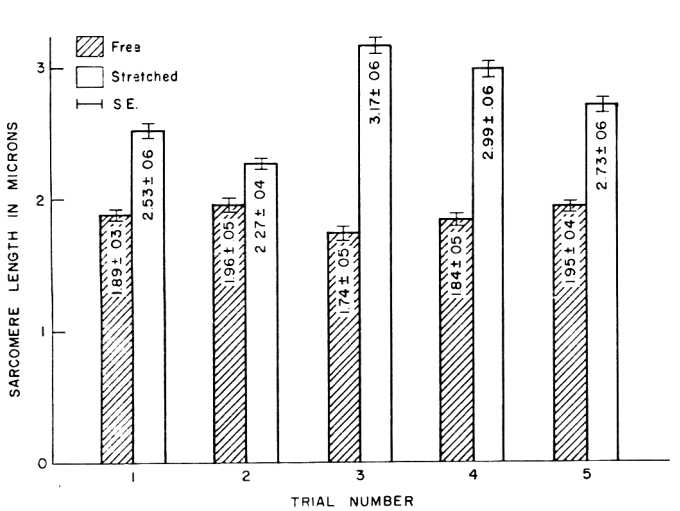

Fig. 1—Average sarcomere length of free and restrained longissimus dorsi muscles.

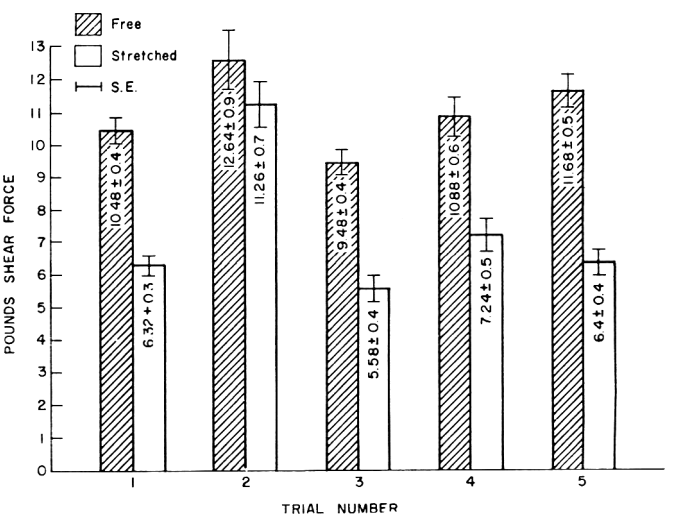

Fig. 2—Average force required to shear cooked muscle strips prepared from free and restrained longissimus dorsi muscles.

(Herring et al., 1965 a,b Herring et al., 1967; Cook, 1967; Buck et al., 1967).

Protein extraction

The results of the protein extractions are shown in Table 1. Greater amounts of total protein were extracted, post-rigor, from stretched muscles in all trials except Trial 3. Although these differences did not prove to be statistically significant, they were approaching significance ($P < 0.1$) and are similar to those reported by Cook (1967) who found stretching significantly increased the post-rigor extraction of protein from bovine muscle.

The increased protein extraction associated with the stretched, more tender, muscles offers support to the work of Hegarty et al. (1963) who found a positive relation between myofibrillar protein solubility and tenderness of bovine muscle. These results are not in agreement, however, with Goll et al. (1964) who stated that protein solubility did not appear to be related to tenderness.

The longer sarcomeres associated with the stretched muscles from which greater amounts of total protein are extracted offer additional support to Cook (1967) who suggested a relationship between protein solubility and the contractile state of the proteins.

The results of the actomyosin extraction shown in Table 1 were completely unexpected. Greater total amounts of actomyosin were extracted from stretched muscles in all trials. This was associated with the greater amounts of total protein extracted from stretched muscle. However, when actomyosin is expressed as a percent of the total protein extracted, it is still greater in stretched muscles. These differences proved to be statistically significant ($P < 0.01$).

It is generally agreed that linkages are formed between actin and myosin filaments during rigor. The amount of actomyosin formed during rigor may be affected by state of contraction and tenderness could be improved by physically inhibiting the formation of actomyosin during rigor mortis (Herring et al., 1965 a,b; Buck et al., 1967; Cook, 1967).

The results of this study do not offer any support to this theory and it would appear that stretching increases the actomyosin content of muscle and that increased tenderness is associated with increased amounts of actomyosin rather than decreased amounts.

One must interpret these results cautiously, however, since the extraction techniques employed may not present a true picture of the proteins as they exist in muscle prior to extraction.

One possible explanation for the unexpected results of this study is that stretching may stimulate muscle so that it uses ATP more rapidly and more completely, forming actomyosin which does not dissociate upon extraction. Free muscle, on the other hand, may contain greater amounts of residual ATP which would tend to dissociate the actomyosin during extraction.

Evidence has been offered by several workers (Valin, 1968; Davey et al., 1968) that the tenderizing effects of aging are associated with the weakening and final dissolution of the Z-band structures. They also found that extraction of myofibrillar protein increased as aging progressed. Another possible explanation for the greater extraction of total protein and actomyosin from stretched muscle, therefore, may be that the stretching causes a similar disruption, perhaps even detaching actin from the Z-band membranes, resulting in the extraction of greater amounts of complex.

The results reported in this investigation do not necessarily contradict those who have suggested that actomyosin formation is related to tenderness. They do, however, point out the importance of understanding more clearly the changes taking place in muscle during rigor and the reliability of present procedures for actomyosin extraction.

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Institute of Food Technologists

The Institute of Food Technologists is a professional society of scientists, engineers, educators and executives in the field of food technology. Food technologists are professionals who apply science and engineering to the research, production, processing, packaging, distribution, preparation and utilization of foods. Individuals who are qualified by education, special training, or experience, are extended an invitation to join in professional association with the select group of the food industry's scientific and technological personnel who are Institute members. Membership is worth many times its modest cost, reflecting positive benefits, stimulation and opportunities for the individual in his business or profession.

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The Institute, as a non-profit, professional, educational society, has several major aims: to stimulate investigations into technological food problems; to present, discuss and publish the results of such investigations; to raise the educational standards of Food Technologists; and to promote recognition of the scientific approach to food and the basic role of the Food Technologist in industry. All of these activities have the ultimate objective to provide the best possible foods for mankind.

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Organized July 1, 1939, at Cambridge, Mass., with a membership of less than 100, the Institute has grown to more than 10,000. It is world-wide in scope, with members in the Americas, Scandinavia, England, Holland, Germany, France, India, Australia, New Zealand, and Japan, among others.

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IFT INTERNATIONAL AWARD. Purpose of this award (Silver Salver sponsored by Australian Institute of Food Science and Technology, and $1,000) is to recognize an IFT Member for promoting international exchange of ideas in Food Technology.

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