THE DETERMINATION OF 5'-MONONUCLEOTIDES IN MEATS

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This is to certify that the

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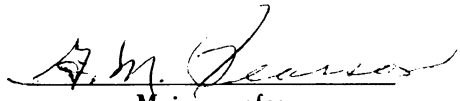

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ABSTRACT

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by Robert Dale Dannert

This study was designed to assess the level of 5'-mononucleotides in the muscle of meat animals. Following hot water extraction, the nucleotides were separated on a Dowex 1 X 8, 200-400 mesh (formate), ion-exchange resin column. As the nucleotides were eluted from the column, peaks were located with an Isco, Model UA, UV analyzer. Identification of the individual nucleotides was made with thin-layer and paper chromatography as well as from the absorption spectra and from the ratio of absorbance at 250/260 and 280/260 mμ. Quantitative evaluation of the 5'-mononucleotides present in each peak was accomplished by using a periodic acid - 2,4-dinitrophenylhydrazine (2,4-DNPH) color reaction, which effectively differentiates the 2'- and 3'-nucleotides from the 5'-isomer.

The first portion of this study dealt with the concentration of 5'-mononucleotides in three muscles from each of six pork carcasses. The muscles examined were the longissimus dorsi, biceps femoris, and semimembranosus. The concentration of inosine monophosphate (IMP) was 3.23, 3.12 and 2.98 μM./g. of tissue, respectively, for these muscles. The differences were not statistically significant. The standard deviation was 0.33, 0.42 and 0.40 μM./g. of tissue, for the longissimus dorsi, biceps femoris and semimembranosus muscles, respectively. Since the muscles had similar IMP concentrations and the longissimus dorsi had the smallest standard deviation, it was

used in all subsequent studies. It was also easily accessible and less damage was done to the carcass on removing the sample. The level of adenosine monophosphate (AMP) was considerably lower than the level of IMP, with values of 0.33, 0.90 and 0.33 μM./g. tissue being recorded for the longissimus dorsi, biceps femoris and semimembranosus muscles, respectively. Guanosine monophosphate (GMP) was not detected, while cytosine monophosphate (CMP) was found in trace amounts only. The level of uridine monophosphate (UMP) was 0.13, 0.20 and 0.11 μM./g. tissue, respectively, for the longissimus dorsi, biceps femoris and semimembranosus muscles. These values as well as all subsequent values were the average of duplicate determinations of the samples being analyzed.

Part two of this experiment was designed to elucidate the change in concentration of IMP and AMP in the longissimus dorsi muscle from the carcasses of six Holstein heifers during a 28 day aging period at 33-35°F. Samples were frozen in liquid nitrogen after removal at 0, 12 and 24 hours and at 4, 7, 14 and 28 days post-mortem. The level of IMP was 4.71, 5.44, 4.86, 4.47, 3.20, 2.17 and 0.75 μM./g. tissue, respectively, as the aging period was increased. The peak concentration of IMP occurred at 12 hours. The level then declined until at 24 hours the IMP content approximated that at 0 hours. It remained fairly constant until the 4th day, after which a linear decrease occurred until the 28th day of post-mortem cold storage, when less than 15% of the IMP remained. Statistical analysis revealed that all means were significantly different ($P < 0.01$) except for the values at 0 and 24 hours, and for 24 hours and 4 days. This suggests that, except for

the peak IMP concentration at 12 hours, only small changes occurred during the first four days of cold storage. Consistent decreases were noted thereafter. The concentration of AMP was much lower than that of IMP, with the 0 hour sample containing only, 0.94 μM./g. tissue. The AMP content then decreased to approximately 0.37 μM./g. tissue by 4 days storage. This value remained relatively stable during the remainder of the aging period. CMP and UMP were found in trace amounts and GMP was not detectable.

In a third study, the nucleotide content of the longissimus dorsi muscle of samples of pork removed at 0 and 48 hours, beef at 0 hours, bull and lamb carcasses at 7 days and pork heart muscle at 0 hours was compared. Due to the wide range in sampling and handling procedures, comparisons between species was difficult. The concentration of IMP was 4.38, 3.23, 4.71, 2.84, 2.24 and 0.13 μM./g. of tissue, respectively, for the above listed species. The level of AMP was 0.70, 0.33, 0.90, 0.76, 1.21 and 2.30 μM./g. tissue, respectively, for the same species. Low levels of CMP and UMP were observed, while GMP was not detectable.

In order to assess the flavor enhancing properties of IMP, MSG and IMP + GMP, these flavor potentiators were added to a standard frankfurter formulation. This formulation utilized pork heart muscle as the meat source, since it was demonstrated that pork heart contained little or no IMP. A 20 member taste panel evaluated the frankfurters using the 9 point hedonic scale. Scores of 6.40, 6.20, 5.55, 5.35, and 4.75 were recorded for formulas containing IMP + GMP, IMP, MSG, pork heart muscle alone (negative control) and all lean beef (positive control), respectively. Statistical analysis demonstrated that the

addition of IMP + GMP to frankfurters made from pork heart increased their acceptability over the beef frankfurters ($P<0.01$) and over the frankfurters containing heart muscle with no added flavor potentiator ($P<0.05$). When IMP was added to pork heart frankfurters, they were preferred over the all beef frankfurters ($P<0.01$). All other differences were not statistically significant, even though there was a trend which indicated that the panel preferred the frankfurters containing pork heart muscle with added flavor potentiators. Thus, results suggest that the flavor enhancers improved the flavor and texture of frankfurters made from pork hearts.

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INTRODUCTION

Since man first found he could improve the flavor of food, scientists the world over have been attempting to make our foods more palatable. Great strides have been made in this area. Pasteurization, aseptic canning, dehydration and freezing, and more recently freeze drying and irradiation, have all contributed in giving the food processor wide versatility in the products he manufactures. Thus, a variety of nutritious and flavorable products is available to satisfy the needs of the consumer.

Flavor is a primary factor in acceptance of food products. As a consequence, interest in isolation and characterization of flavor in various foodstuffs has grown rapidly. Even though meat is one of the basic commodities which has been extensively studied, relatively little is known about the flavor of meat due to the complexity of the chemical reactions occurring on heating. The elucidation of meat flavor is complicated by the fact that research has been directed toward both the volatile or non-volatile components. Further complications have occurred since experimental studies have been carried out on isolation of the flavor precursors of raw meat, as well as in studying the flavors developed during the cooking process.

The flavor industry has played a fundamental role in enhancing the acceptability of food products. Recent research efforts have pointed out that some compounds can blend or modify flavors and are known as "flavor potentiators", which may be defined as compounds that augment or suppress the response of the sensory organs to the stimulus of food. They are further characterized as being effective at very low levels and by

the fact that they add no flavor of their own. The presence of inosinic acid in meat was first reported in the classical work of Justus von Liebig (1803-73). He isolated and named inosinic acid after the Greek word meaning muscle. Disodium inositate, disodium guanylate and monosodium glutamate have proven useful in improving flavor scores of some meat and vegetable mixtures (Caul and Raymond, 1963, Kurtzman and Sjöström, 1963, Terasaki et al., 1965). The precise flavor effects obtained with inosinic acid depend to a large extent on the particular food to which it is added. In products such as soups, addition of inosinic acid results in a fuller flavor and an impression of increased viscosity. Disodium inositate also can suppress undesirable sulfide flavors in foods (Wagner et al., 1963) and radiation off-flavors associated with sulfur compounds (Merrit et al., 1959).

Until recently, methods of analysis made elucidation of the chemical components contributing to meat flavor a tedious and difficult task. Flavor studies have depended primarily upon subjective measurements of taste panels. However, recent developments in instrumentation provide both accuracy and sensitivity, which can be used to supplement standard organoleptic and chemical procedures. Physical methods have become indispensible to the food scientist. Volatile materials are now readily characterized by gas chromatography, infrared and/or ultraviolet spectroscopy and mass spectroscopy.

Separation of non-volatiles can be accomplished by dialysis through membranes of known pore size, ion exchange or molecular sieve chromatography. Ionophoresis is available for rapid separation of nucleotides (Bergkvist, 1959). Identity can be verified by paper or thin layer chromatography and by X-ray diffraction. Undoubtedly, new and more

accurate procedures will be evolved in the future to further classify meat flavor and its precursors.

EXPERIMENTAL OBJECTIVES

The primary objective of this experiment was to determine the concentration of inosine 5'-monophosphate (IMP) in muscle tissue. More specifically, the objectives were as follows:

1. To determine the concentration of IMP in different muscles from the same animal.

2. To determine the concentration of IMP in samples of beef muscle taken at varying times during aging at cooler temperatures.

3. To determine and compare the level of IMP found in samples of bull, heifer, lamb, pork, and pork heart muscle.

4. To assess the flavor enhancing properties of IMP.

LITERATURE REVIEW

Sjöström and Cairncross (1955) divided the term flavor into four broad groups. They designated the first as taste. It included the four well known basic sensations of sweetness, sourness, saltiness and bitterness. The second aspect, aroma, was used to describe sensations perceptible to the nose. The term body was reserved for texture, even though it makes no contribution toward taste or aroma. Finally, mouth satisfaction was characterized by increased salivation, pleasantness and smoothness of blending with very little contribution to taste or aroma.

Crocker (1937) defined flavor as that property of a food or beverage that excites the senses of taste and smell. He went on to state that a food might rate high in every aspect except one, but because of that fault might be hastily refused by the consumer. Kramlich and Pearson (1958) also emphasized the importance of flavor and indicated that little is known concerning the chemical components contributing to flavor. As a result many researchers have attempted to characterize the components of meat flavor.

5'-mononucleotides in flavor

The nucleotides involved in flavor are termed flavor enhancers and more recently flavor potentiators. This term, although new in the food industry is well known in some fields, such as pharmacy. A flavor potentiator has been described as a compound which augments or suppresses the responses of the sensory organs (odor, taste and mouth feel) to the stimulus of food. They are further characterized as being effective at

very low levels, and by the fact that they do not add any flavor of their own. This definition is in agreement with Sjöström and Cairncross (1955), who have defined a seasoning agent as a material, which, on being added to a food, alters and corrects the aroma and flavor, mainly by blending the character notes and by augmenting the total flavor impression without being necessarily detectable.

In 1913, Kodama first reported that the histidine salt of inosinic acid (IMP) was the major flavoring substance in dried bonito, an important seasoning material used in Japanese cookery. However, IMP was not produced as a seasoning ingredient on a large scale until quite recently, because both the biochemical background and the flavor enhancing properties of IMP are quite complicated (Kuninaka et al., 1964). Recently, remarkable advances in the biochemistry and organic chemistry of nucleic acids have made it possible to industrialize the production of the 5'-ribonucleotides in Japan. This has resulted in the application of these compounds to food as seasonings.

Caul and Raymond (1964) used samples of dried beef-flavored noodle soup to assess the flavor enhancing properties of inositate added at a level of 0.01% to the reconstituted soup. A flavor-profile analysis and a consumer home-use test were used for evaluation.

The flavor-profile analysis revealed the following:

a) The inositate sample created an impression of fuller flavor, which was attributed in part to the sensation of greater viscosity and in part to a higher degree of blending of the flavor notes. In contrast, the control soup seemed thinner, and the individual flavor notes were more discernable.

b) The inositate soup had a flavor which was more brothy, or more like meat stock, and which was less identifiable by its hydrolyzed vegetable protein character. The control soup gave the impression of a "bouillon cube".

c) The vegetables in the inositate soup had more individual flavor identity than in the control soup.

d) The noodles seemed to taste less starchy when inositate was added.

e) The salty tastes were different in that the control soup had a long lasting effect, while the soup containing inositate appeared to reach a peak and then the salty taste decreased rapidly.

Only 10% of the families failed to discern or report flavor differences between the 2 soups. In general, the panel recognized 1 or more differences corresponding to the flavor-profile analyses.

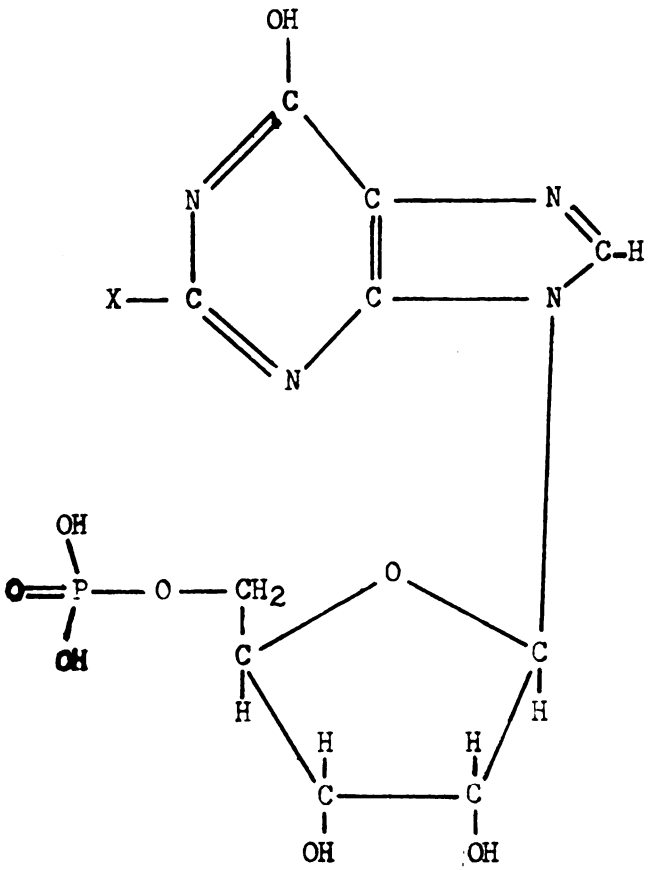
Wagner et al. (1963) reported that to a large extent the precise flavor effects obtained with disodium inositate depend upon the particular food to which it is added. They suggested that there are, however, certain effects which seem to be characteristic. These effects included a better blending of the individual flavor notes, a fuller flavor and an impression of increased viscosity on adding to soups. Furthermore, disodium inositate can also suppress undesirable sulfide flavors in food, as well as to modify sourness in many products, which results in an over-all decrease in harshness.

The flavor enhancing ability of the nucleotides is entirely dependent upon structure, which is very specific. In 1960 Kuninaka summarized the tastes of various RNA derivatives. In the process, he demonstrated that these compounds are made of 3 components, namely, a nitrogen base,

a ribose sugar and inorganic phosphate combined in a ratio of 1:1:1. Figure 1 shows the general formula for the 5'-ribonucleotides, which are effective as flavor enhancers. The base moiety is a purine containing a hydroxyl group at its number 6 carbon atom, while the 5'-position of the ribose is esterified to phosphoric acid. When inorganic phosphate and inosine or ribose 5'-phosphate and hypoxanthine were combined in equimolar mixtures no flavor enhancing ability could be detected. Kuninaka therefore concluded that ribosidic and phosphate ester linkages were essential for flavoring action. He was also able to demonstrate that the 2'- and 3'-mononucleotides imparted little or no flavor. Thus, according to his scheme, 3 compounds possessed flavor activity, namely 5'-inosinic acid, 5'-guanylic acid and 5'-xanthylic acid. Guanylic and xanthylic acid were found to be of lesser importance in muscle, since they occur in extremely low concentrations.

Toi et al. (1960) and later Wagner et al. (1963) reported that the recognition threshhold for disodium inositate was 0.02% or 200 ppm. Fujita et al. (1961) reported this level to be 0.012% or 120 ppm. and 0.0035% or 3.5 ppm. for disodium inositate and disodium guanylate, respectively.

Schimazono (1963) also reported that the threshhold values for disodium inositate and disodium guanylate in aqueous solution were 120 ppm. and 3.5 ppm., respectively. Disodium guanylate has about 2.1 to 5.5 times as much flavoring capacity as inositate according to Schimazono. He noted that this ratio varied with their concentrations and with the constituents in the food utilized.



If X = H = 5'-inosine monophosphate

X = NH_2 = 5'-guanosine monophosphate

X = OH = 5'-xanthine monophosphate

Figure 1. Structure of 5'-mononucleotides involved as flavor enhancers.

Synergistic action between MSG and 5'-nucleotides

In 1964, Kuninaka et al. pointed out that there is an additive effect when two acids or sugars are mixed. A contrasting effect occurs on adding a small amount of acid to a sugar. Kuninaka (1960) pointed out that there is an additive effect between guanylate and inositate, and that the taste of the two compounds is qualitatively the same. He also indicated that both these compounds have a synergistic effect with MSG.

Kuninaka et al. (1964) demonstrated that succinate and the basic amino acids, such as histidine, lysine and arginine, do not significantly enhance the flavor or taste of either inositate or guanylate. On the other hand, MSG greatly enhanced the flavor of these nucleotides. The reverse was also true. These authors went on to show that only small amounts of the nucleotides have remarkable capabilities for enhancing the flavor of MSG. In order to assess the flavor enhancing ability of the 5'-nucleotides on MSG, various ratios of IMP (or GMP) and MSG were prepared in a solution of 1.2% sodium chloride. The flavors of the resulting solutions were compared by paired comparison tests with salt samples containing 0.3% MSG as a standard. It was clearly demonstrated by this experiment that there is a significant synergistic effect due to IMP or GMP. For example, when MSG and IMP were combined in a ratio of 100:1, 50% less MSG was required to duplicate the taste of the standard solution containing 0.3% MSG. This work also indicated the synergistic action between MSG and GMP was about 4 times greater than that of MSG and IMP.

Fujita et al. (1961) claimed that other compounds have a synergistic

effect on the taste threshhold of IMP. They demonstrated that the threshhold recognition values for disodium inositate in solutions of 0.1% sodium aspartate, MSG, beta hydroxyglutamate, sodium alpha amino adipinate and homocysteinate were reduced considerably, when compared with inosinic acid mixed with water. The threshhold levels for recognition of sodium aspartate, MSG, sodium beta oxyglutamate and sodium alpha amino adipinate in 0.01% of 5'-disodium inositate solution were reported to be 1/16, 1/15, 1/15 and 1/100 of those reported in water, respectively (Toi et al., 1960).

The interrelationships between disodium inositate, disodium guanylate, MSG and beef extract were examined by Schade et al. (1963a). Various levels and combinations of MSG, IMP and/or GMP were subjected to taste panels for evaluation by using ranking, triangle, multiple comparison preference and/or difference tests. When IMP (0.005%) was compared with MSG (0.15%) in commercial vegetable bouillon soup, which already contained MSG, the taste panel found a significant difference in flavor using a multiple comparison test. The untreated commercial product was used as a control. Triangle tests were conducted to compare bouillons containing high levels of MSG against bouillons containing low levels of MSG and 5'-nucleotides. Results indicated that replacement of 0.15% MSG by 0.005% IMP (30:1) or replacement of 0.20% MSG by 0.0015% GMP (133:1) gave significant and highly significant differences in flavor, respectively. In this study 50% of the panel detected the substitution by IMP. Of those finding the difference, 67% preferred the product with IMP.

Utilizing a multiple comparison preference test, beef extract at a level of 0.25% was compared by Schade et al. (1963a) with 0.025% IMP. Omission of 0.25% beef extract from the standard bouillon resulted in a product which was significantly less acceptable, whereas, the substitution

of 0.025% IMP for the beef extract resulted in a product which was significantly more acceptable. They concluded that IMP can be used to replace beef extract in a ratio of 1:10 and will still improve the acceptance of the product. However, the authors noted that the flavor of the product containing IMP was distinctly different from that containing beef extract. Therefore, the 5'-nucleotides may find their most advantageous use in extending the desirable effects obtained with beef extracts.

Effect of heat on nucleotide concentration

Schade et al. (1963b) examined the stability of IMP and GMP by using commercial food processing conditions of time and temperature of heating, pH and storage. Solutions of IMP and GMP in buffer solutions at pH 3.0, 3.5, 4.0, 4.5 and 5.0 were heated at 100°C. for 30 or 60 minutes and analyzed for nucleotide content to assess stability. Results are shown in table 1.

Table 1. Stability of inosine monophosphate and guanosine monophosphate at different pH values and temperatures

	% Recovery			
	30 minutes		60 minutes	
pH	GMP	IMP	GMP	IMP
3.0	95	92	90	88
3.5	95	100	91	95
4.0	97	100	89	97
4.5	97	99	89	99
5.0	100	100	94	98

The data indicate that near neutrality the nucleotides are quite stable, but as the pH is lowered into the acid range they become less stable. In addition, similar buffer solutions were sterilized and stored at room temperature for 3 to 6 months. Schade et al. (1963b) were unable to detect any loss of nucleotides during storage. This would indicate that the destruction of IMP and GMP in solutions is dependent on pH, heating time and temperature. Furthermore, results would suggest that IMP is quite stable in water extracts of meat tissue, since the pH would be above the low critical pH where destruction commences.

In order to investigate the effects of commercial processing and storage conditions on the flavor enhancing activity and chemical stability of IMP, 5 soups were prepared by Schade et al. (1963b). Each soup was prepared without adding IMP and at two levels of added IMP. The soups were stored for either 6 months at room temperature or for 5 months at 38°C. The soups were then analyzed for IMP content and evaluated organoleptically for flavor differences and preference rankings. The initial analysis indicated that the heat processed condensed soups showed an IMP loss, which varied from 12% to 35%, with the higher losses occurring in the samples with low IMP levels. There was no loss of IMP in dehydrated soups, since they were not heat processed. There was no appreciable IMP loss in any of the 5 soups following 5 months storage at 38°C. With one exception, this was also true for soups stored for 6 months at room temperature. The one loss observed occurred with heat processed condensed chicken soup. The author felt that reanalysis could solve the discrepancy in the data, since the level of IMP in this soup had not deteriorated after 5 months storage. According to taste panel results, all IMP treated soups were significantly different at the 1%

level from the untreated soups, initially and throughout the study. As a general trend, the panel preferred the soups containing the higher level of added IMP.

Fujita et al. (1961) reported similar findings. These authors heated IMP and GMP at 100°C. for 1 hour at pH values ranging from 4 to 6. No decomposition occurred with either product under these heating conditions. They also discovered that heating IMP or GMP at 100°C. at pH 5 for 30 minutes in the presence of oxidizing or reducing agents resulted in very little or no destruction, and that less than 10% destruction occurred during 1 hour of heating. Both IMP and GMP were quite stable in aqueous solutions during storage for 1 year at near neutral pH.

Various other workers have shown that relatively mild heat does not inactivate IMP. Hanaoaki and Higashi (1963) separated IMP from the boiling liquor of mackerel. They demonstrated that boiling for 1 to 2 hours caused no marked influence on the yield of IMP. Longer periods of boiling reduced IMP to inosine and phosphoric acid.

Take and Otsuka (1964) extracted samples of katsuobushi (dried bonito) under varied heating conditions. Although all samples showed the presence of IMP, a sensory panel found the samples heated to 60°C. for 5 minutes to be most acceptable. In 1965, these same authors prepared extracts by heating dried fish at 60°C. for 5 minutes, or by heating for 10 minutes at 100°C. They demonstrated the presence of IMP, inosine, hypoxanthine and adenine. However, they were unable to correlate organoleptic scores with the levels of IMP found in the extracts.

Shimazono (1963) found that hot water extracts of mushrooms (Cortinellus shiitake) contained significant amounts of guanylic,

adenylic, cytidylic and uridylic acids in contrast to the low levels found when the more conventional perchloric acid extraction procedure was utilized. Further investigations of this phenomenon revealed that the mycelium of the mushroom yielded a typical vegetable nucleotide pattern with no CMP or GMP and fairly low levels of AMP and UMP. During extraction with hot water, however, the 4 nucleotides were produced by the breakdown of intracellular RNA due to the action of the relatively thermo-stable RNA decomposing enzymes which were contained in the mushroom.

Effect of freezing on nucleotide concentration

Saito and Arai (1957a) examined the post-mortem changes in the content of polyphosphates in carp muscle stored at various temperatures (16°C., 0°C., -8°C. and liquid air). The rate of conversion of ATP appeared to be directly related to the temperature, with the slower rates of freezing resulting in more rapid breakdown of ATP. The ATP content was stable if frozen in liquid air. Later reports by the same authors (1958b, 1959) reported on the formation of IMP in carp muscle when frozen at -8°C. (18°F.). They found it was possible to classify the changes occurring in the nucleotide concentration of carp muscle into three stages. During stage I, which occurred during the first 3 hours after death, only small changes took place. Large amounts of ATP were converted to IMP during stage II (after 5 hours), while in stage III (after 28 hours) only small quantities of IMP were formed. From this work the authors concluded that the pathway to IMP formation was: ATP ---> ADP ---> AMP ---> IMP.

Another paper by Saito and Arai (1957b) traced the change in concentration of IMP in carp and squid muscle after storage for several months at -5°C. to -8°C. During prolonged storage, the IMP was gradually converted to inosine and finally to hypoxanthine. The authors reported that these changes were not microbial, but were due to enzymatic degradation. Squid muscle appeared to be lacking the enzyme, AMP-deaminase, since AMP was not converted to IMP at low temperatures.

Szentkiralyi (1957) used rabbit psoas muscle strips to demonstrate the effect of freezing on ATP concentration. The excised muscle was frozen immediately in an ether-dry ice mixture. The samples were thawed in Kreb's solution and the rate of conversion of ATP to AMP was determined. Other strips of muscle were analyzed immediately, while a third set of fibers were immersed in the oxygenated Kreb's solution as a control. Results demonstrated that freezing and thawing caused a rapid deamination of the adenosine nucleotides along with a simultaneous splitting of ATP. In most cases, 75% of the adenosine was deaminated after thawing for 3 to 4 minutes, while the control strips of muscle showed only a small change. From this experiment, which demonstrates thaw rigor, the authors concluded that inosinic acid is produced almost instantaneously, if there is maximal contraction and the recovery processes are inadequate. The work of Okamoto et al. (1957) is in agreement.

Jones and Murray (1961b) studied the effect of freezing on cod muscle stored at temperatures ranging from -14°C. (10°F.) to -30°C. (-14°F.). The authors found that changes varied with the temperature and time of storage. In general, the changes were very slow at lower temperatures, if ATP conversion to IMP was used as a criterion of

measurement. Although the concentration of these compounds was not markedly different at -14°C., the relative rate of change was more rapid. The level of inosine rose to 3.01 uM./g. after 62 weeks at -14°C., while at -30°C. there was little change. At -14°C., hypoxanthine increased from trace amounts to 0.569 uM./g., while no changes were observed at -30°C. Results suggest that samples stored at -30°C. are relatively stable for an indefinite period of time. Other papers, which report the effect of freezing, have been published by Sawant and Magar (1961a, 1961b) and Sameshima (1965).

Effect of enzymes on nucleotide concentration

The enzymes phosphatase and 5'-nucleotidase, which are found in animal and plant tissues, cause the phosphate group to be split off the nucleotide moiety (Chargaff and Davidson, 1955). When this occurs the flavoring activity is lost. Inactivation procedures before nucleotide addition, such as blanching, will prevent enzymes from causing nucleotide destruction. Shimazono (1963) also observed that 5'-ribonucleotides were rapidly decomposed when added to soybean sauce containing a high level of the enzyme phosphomonoesterase. This enzyme can be inactivated by heat treatment at 80°C. Consequently, soybean sauce is heated prior to the addition of nucleotides.

Fujita and Hasimoto (1960) found that fresh or frozen meat from marine vertebrates contained high concentrations of IMP, while seaweeds and invertebrate meats were quite low in IMP. Boiled, dried or canned fish, generally contained quite high levels of IMP. The authors assumed that the higher levels were due to heat inactivation of the enzyme

systems during processing. Results further substantiated the stability of IMP during heat processing. These authors also followed the degradation of IMP in jack mackerel. Although inosinic acid disappeared completely during autolysis, a considerable amount remained in dried fish of the same species. These results suggest that rapid dehydration may also be effective in preserving IMP.

Effect of stress on nucleotide concentration

According to Hedrick (1965), stress may play an important role in the acceptability of meat. Stress may be the result of hormonal or chemical injections, fatigue and exercise, excitement, preslaughter feeding or dietary restrictions. A major effect of stress is a decrease in the muscle glycogen reserve, which after death results in a lowered production of lactic acid and a higher ultimate pH. In turn, ATP and phosphocreatine are rapidly broken down, with the ATP being reduced to IMP. If the animal is stressed, the level of IMP at death will be higher.

Reay (1949) observed that fish differ from the muscle of meat animals in that the level of glycogen in resting muscle (0.8%) is considerably lower than for meat animals. Fish, which are normally caught by trawling, rapidly use up the glycogen reserve, and as a result the initial level of ATP is low while the IMP content is quite high. In addition, the pH is also quite high--usually in the range of 6.2-6.6. Reay suggested that the high pH may be responsible for the fact that fish are more susceptible to bacterial spoilage than meat animals, where the pH is usually lower.

Fujimaki and Kojo (1953) divided line caught frigate mackerels (Suxis tapeinosoma) into two groups; one which was killed instantly and

a second which were allowed to struggle until they died. As expected, ATP decreased very rapidly in stressed muscle in contrast to the slower rate in unstressed muscle. In addition, struggling markedly increased AMP and IMP and resulted in more ammonia production. The authors theorized that the early appearance of ammonia was a result of glutamine deamination, while during autolysis AMP was deaminated.

Guardia and Dollar (1965) in a comprehensive series of experiments attempted to elucidate the effects of exercise and feeding on the nucleotide concentration in English sole (Porophrys vetulus). The initial IMP level of the rested fish was 0.01 uM./g. and 0.08 uM./g. for fed and starved fish, respectively. This corresponds to values of 1.66 uM./g. and 2.11 uM./g. tissue, respectively, for fed and starved fish which had been exercised. The authors concluded that feeding did not cause any significant change in the total amount of nucleotides, although the amount of nucleotide did vary considerably with exercise. Active fish had lower levels of initial ATP and increased levels of IMP, but the difference was not significant after 7 days storage of ice.

Work by Zhivkov (1963) with chicken muscle seems to indicate that the season of the year may affect the concentration of nucleotides. He noted that there was a rise in the amounts of ATP and ADP in the spring of the year, which was accompanied by a decreased level of IMP.

Other papers concerning the biochemistry of fish and muscle, including effects of stress, nucleotide concentration and level and effect of free sugars have been published by Sharp (1935), Bate-Smith (1948), Tarr (1950, 1954, 1958), Jones and Murray (1957, 1961a), Jones (1958), Creelman and Tomlinson (1960), Fredholm (1960), Jones (1960), Jones and

Burt (1960), Tomlinson and Creelman (1960), Borgstrom (1961), Burt (1961), Tomlinson et al. (1961), Tomlinson and Geiger (1962b), Visioli (1964), Fraser et al. (1965) and Terasaki et al. (1965).

Effect of muscle on nucleotide concentration

The changes in concentration of ATP and its intermediate compounds were measured in pork and beef muscle (Millo, 1964). The samples were taken from four muscles during a 15 day aging period at 3-4°C. Results from the psoas major and lattissimus dorsi muscle were similar. ATP fell from 16.57 to 0.27 μM. of P/g. after 30 hours, and to 0 after 4 days. There was a simultaneous decrease of ADP and AMP, which were both still present in small amounts as long as 7 days. The concentration of IMP increased from 6 to 14 μM. P/g. in 7 hours, but commenced to decline after the 4th day post-mortem. A similar pattern of degradation was observed with the longissimus dorsi and pectoralis profundus muscles, but the rate of change was considerably faster. The muscles with the slower rate of metabolism were organoleptically superior. The author could not explain why these muscles differed, although he postulated that perhaps it was due to the physiological activity of the muscles in vivo.

Saito et al. (1959b) found a considerable difference in IMP level between the dorsal and red lateral muscle of rainbow trout. The dorsal muscle contained considerably higher (2 μM./g.) concentrations of IMP, and correspondingly lower levels for inosine and hypoxanthine. These authors also noted that the rate of change varied between muscles.

Arai (1960a, 1960b) and Arai et al. (1961) assessed the nucleotides content of the foot and adductor muscles of shellfish. The level of nucleotides was higher in the adductor muscles than in the foot muscle. This higher level was accompanied by a more rapid rate of decomposition. Generally, the authors found no hypoxanthine or inosine, which would indicate the absence of IMP in shellfish.

Kazeniac (1961), Minor (1964), Zhivkov (1964) and Terasaki (1965) have all reported that light muscles contain relatively larger amounts of inosinic acid than do samples from dark colored muscles. Minor went on to show that breast and leg muscles from heavy hens had the highest concentrations of IMP, while samples from light weight hens contained the lowest levels. He suggested that the higher inosinic acid content of the heavy hen samples probably contributed to their superior taste.

It is interesting to note that heart muscle contains no IMP. Visioli et al. (1964) found that there was a rapid decrease of all triphosphates in the myocardium of severely exercised rats. However, no IMP was detected. Also in 1964, Imai et al. explored the nucleotide content of skeletal and cardiac muscle from anemic rabbits. The level of 0.06 μM./g. for skeletal muscle appears very low. IMP, adenosine, inosine and hypoxanthine were all conspicuously absent from cardiac muscle.

Effect of storage on nucleotide concentration

Fish

Jones and Murray (1957) followed the autolysis of nucleotides in the muscle of cod stored for 6 days at 2°C. The levels of ATP, ADP, and AMP fell and became stable at 0.10, 0.14, 0.14 μM./g., respectively. In

exhausted muscle, the level of IMP decreased constantly from an initial level of 4.34 μM./g. to 0.66 μM./g. after 6 days. These authors also noted the effect of exercise. There was over an 80% conversion of ATP to IMP if the cod were exhausted by struggling prior to killing. Jones and Murray in a later paper (1962) on cod muscle again demonstrated the effects of storage and stress on nucleotide concentration during a 12 day storage period. Results presented in this paper were difficult to analyze. Values for fish caught in February differed from those caught in July, which might indicate a seasonal effect. In both cases, the maximum concentration of IMP occurred within one day of killing. There was then continual IMP degradation, until none remained after 12 days storage. In this paper Jones and Murray postulated that the major breakdown pathway of ATP was as follows: ATP ---> ADP ---> AMP ---> IMP ---> Inosine ---> hypoxanthine.

Kassemsarn et al. (1963) followed the autolysis of nucleotides in three species of fish; i.e., haddock, lemon sole and plaice. The fish were eviscerated, stored in ice at 2°C. and held for periods up to 24 days. Samples were extracted with perchloric acid and separated on Dowex-1 X 8 columns. The authors found that the initial ATP level was low, while IMP was present in higher amounts in all three species. IMP completely disappeared in plaice in 11 days and more slowly in lemon sole. A slight increase occurred in haddock prior to degradation. The course of IMP degradation varied widely between species. Inosine concentration reached a peak in 10 days for haddock, 7 days for plaice and was present in traces only in lemon sole. Hypoxanthine reached peak values at 17 days, 12-14 days and at 14 days for haddock, lemon sole and plaice,

respectively. Kassemsarn postulated a breakdown pathway for ATP, which corresponded to that suggested by Jones and Murray (1962).

Takeda and Shimeno (1964b) found that the nucleotide degradation of Auxis tapeinosoma was similar to that for other species of fish. They also found that inosine predominated in fish, while hypoxanthine predominated for the other species, which have been studied.

Meats

Dvorak (1958) studied the degree of nucleotide decomposition in meat from beef bulls aged at either 37°C. or 5°C. Samples were taken from the psoas major. The muscles were irradiated and stored at 100% relative humidity at either 37°C for 20 hours, or at 5°C. for 15 days. Results are summarized in table 2.

Table 2. Nucleotide content for beef muscle held at 5°C. and 37°C-μM./g. tissue

	Temperature						
	37°C.			5°C.			
Nucleotide	3 hrs.	10 hrs.	20 hrs.	1 day	2 days	10 days	15 days
ATP	1.5	0	0	1.5	0.1	0.2	0.2
ADP	0.9	0.7	0.2	0.9	0.7	0.8	0.7
IMP	1.7	2.9	0.9	1.7	3.7	1.9	1.7
Hypoxanthine	1.7	2.9	4.5	1.7	1.8	3.4	4.5

The authors suggested that at 5°C., the remaining ADP was probably bound to the myofibrils. They postulated that the enzyme systems were less active at the lower temperature, and as a result the ADP remained intact. This agrees with Perry (1951). He hypothesized that the acid

labile phosphorous, arising mainly from the ADP in rabbit muscle, was bound to the myofibril in such a way that it is not acted upon by the enzymes present in the myofibrils. He did not speculate as to what factors could protect the bound nucleotides.

Tomlinson and Geiger (1962a) reported that there were no differences in the quantity of adenine nucleotides bound in washed muscle extracts from freshly frozen (pre-rigor) lingcod and in those from muscle severly denatured during freezer storage. However, when similar samples of lingcod muscle were heated, almost complete release of the bound nucleotides occurred. Jones and Murray (1961a) reported similar values. The stable ADP level was higher in rested fish muscle (pH 6.3), while the levels of ATP and AMP were lower than for the exhausted codling (pH 6.8). These stable values were achieved in the presence of high enzymatic activities capable of degrading free nucleotides, since added nucleotides were rapidly degraded in an analogous homogenate system. It was therefore apparent to the authors that the nucleotides were present in post-rigor muscle in some protected form. The effect did not appear to be that of a simple equilibrium of "free" nucleotides, under the action of the enzyme systems to which they were subjected, as the level of IMP produced by the action of AMP deaminase was falling rapidly throughout the duration of the experiment due to the action 5'-nucleotidase. Rather, it seemed likely that the stable fraction of adenine corresponded to the "bound" nucleotide content of the myofibrils.

Jones and Murray (1961a) suggested that the proportion of the different adenine nucleotides bound to the myofibrils are determined by pH dependent enzymatic activities, since myokinase is most active at pH 7.3. This in

turn may be part of the reason for the lower levels of ADP at the higher pH (6.8) of exhausted muscle as compared to rested muscle (pH 6.3).

Rhodes (1965) followed the autolysis of nucleotides in beef and lamb irradiated at 0.4 Megarads during storage for 70 days at 2°C. Irradiation effectively prevented bacterial spoilage throughout storage, without affecting the nucleotide content or natural enzyme activity of the meat. He found that IMP was completely reduced to hypoxanthine in 30-40 days in some beef cuts, but more slowly in others. In addition, the rate of IMP degradation was considerably slower in leg of lamb, although the total nucleotide content was similar. Attempts to correlate levels of IMP and flavor scores for beef and lamb were unsuccessful. The author therefore concluded that his results do not support the theory that IMP plays a major role in meat flavor, either as a precursor or as a potentiator. Furthermore, he suggested that measurement of IMP or its breakdown products appears to offer little or no promise for measuring the freshness of red meats.

Terasaki et al. (1965) also examined the rate of nucleotide reduction in chicken and pork stored at 4°C. In addition, the authors determined the level of IMP in breast muscle of lamb and thigh muscle of horse meat. Analysis revealed that the maximum concentration of inosinic acid occurred about 3 hours post-mortem with chicken and between 2-3 days post-mortem with pork. However, it was noted that the maximum concentration of IMP in chicken breast muscle was approximately twice that of the longissimus dorsi muscle from pork. The IMP concentration of lamb and horse was 2.36 and 3.07 μM./g. muscle respectively. The validity of these last 2 values may be questioned since the samples utilized were taken from

frozen lamb and horse meat imported from Australia and Argentina, respectively. These authors utilized a paired comparison sensory test to demonstrate that pork samples, which contained maximum levels of IMP were preferred over samples taken when the IMP content was less than maximum.

Ando et al. (1965) reported that 50% of the IMP in carp muscle was degraded in 2 days, while it required 2 weeks storage at 1°C. for beef or chicken muscle to be degraded to the same extent.

In 1963, Fredholm studied the rate of nucleotide degradation in beef and pork stored at 2°C. for 5 days. The nucleotides were separated by the ionophoresis method of Bergkvist (1957) and quantitative measurements were made from the color intensity of the resulting spots. Fredholm's results (table 3) differ appreciably from previous work in this field, since he found the nucleotide content to be relatively

Table 3. Quantity of adenosine phosphate after storage in percent of quantity present immediately after slaughter

Species	Storage time (hours)	% remaining in comparison to 0 hours		
		Compound		
		ATP	ADP	AMP
Beef	24	100	75	100
	72	50	30	60
	120	23	23	40
Pork	24	100	50	77
	72	35	50	31
	120	17	30	19

stable. Both beef and pork ATP levels were practically the same after 24 hours post-mortem as immediately following slaughter, and even after 3 days

these values were reported to be 50% and 35%, respectively. The rate of degradation for ADP and AMP were similar. The nucleotides appeared to be more stable in beef than in pork.

Nucleotide concentration in other foodstuffs

Fujita et al. (1959) evaluated the nucleotide concentration of various foodstuffs. Samples were separated by ion exchange chromatography and the concentration of each nucleotide was determined spectrophotometrically from their molecular extinction coefficients. Jack mackerel and bonito contained 737 and 853 mg.% of IMP on a dry basis, respectively. Ham contained 67 mg.% on the same basis, while IMP levels were not detectable in dried mushrooms or miso (bean paste). Using similar techniques, Nakajima et al. (1961a, 1961b) also demonstrated that dried mushrooms and aquatic invertebrates contained no IMP. The IMP content of dried bonito was 18.1 μM./g., and dried sardines contained 3.73 μM./g. The authors suggested that IMP acted as a flavor component in fish. Saruno and Sasaki (1955) precipitated the nucleotides of fermented sake with uranium acetate, converted the precipitate to the copper salt, decomposed the salt with sulfuric acid to liberate the bases and then identified the bases with paper chromatography. The presence of hypoxanthine indicated that inosinic acid was present in fermented sake. These authors then postulated that IMP together with succinic acid are the chief components of sake flavor.

Pathway of inosine monophosphate formation and degradation in muscle

Bendall and Davey (1957), Saito and Arai (1958a, 1958b, 1959), Sawant and Nagar (1961b), Jones and Murray (1962), Kassemsarn (1963),

Shimazono (1963), Tarr and Leroux (1963), Kuninaka et al. (1964) and Jones and Murray (1964) have speculated on the most probable post-mortem pathway of IMP formation in muscle. The general consensus is that the ATP present in muscle at death is degraded by successive steps to ADP and AMP by the enzymes ATPase and myokinase. AMP is rapidly deaminated by the enzyme AMP-deaminase to IMP, which is then reduced to inosine. Inosine in turn breaks down to hypoxanthine, which in the presence of bacterial oxidases is degraded to xanthine.

Bendall and Davey (1957) examined the changes occurring in rabbit muscle during the course of rigor mortis at 0°C., 17°C. and 37°C. The authors concluded that the appearance of ammonia, which was liberated by deamination of the adenine nucleotides, was directly related to their disappearance and to the formation of inosine nucleotides. The disappearance of the adenine nucleotides and the appearance of the inosine nucleotides occurred in nearly equimolar proportions. Two atoms of labile-P disappeared for each mole of ATP degraded or for each mole of IMP that appeared. At 37°C., the authors indicated that ATP reduction occurred in three stages as shown below:

Reaction	Enzyme involved
a) $ATP \longrightarrow ADP + IP$	ATPase
b) $2\ ADP \rightleftharpoons ATP + AMP$	Myokinase
c) $AMP \longrightarrow IMP + NH_3$	Deaminase

At 17°C. small amounts of IDP and ITP probably originate from two reactions as suggested by Bendall and Davey (1957) and as outlined below:

Reaction	Enzyme involved
a) $ADP \longrightarrow IDP + NH_3$	Bound deaminase
b) $IDP + ATP \rightleftharpoons ITP + ADP$	Transphosphorylase

Bendall and Davey (1957) postulated that at higher pH values ADP is tightly bound to the myofibrils and is therefore resistant to attack. As the pH falls during development of rigor, it becomes progressively more vulnerable to deamination by some bound deaminase, which is more active at lower pH's.

Tarr and Leroux (1963) attempted to elucidate the major pathway of glycolytic intermediates in salmonoid fish. Intravenous introduction of C^{14}-labeled glucose or glucose 6-phosphate resulted in rapid labeling of such intermediates as fructose 6-phosphate, fructose di-phosphate, and lactate in both muscle and liver. On using C^{14}-labeled muscle glycogen, results suggest that the glycogen is degraded to maltose and glucose post-mortem, both by an amylase and the Embden-Meyerhof system, and that very little glucose arises by hydrolysis of glucose 6-phosphate. When C^{14}-labeled ATP was introduced into fish muscle post-mortem, it yielded radioactive IMP and ribose, but glucose 6-phosphate and glucose were unlabeled. Radioactive ribose-5-phosphate (or ribose) was never found in fish muscle injected with C^{14}-labeled glucose or C^{14}-labeled glucose 6-phosphate, indicating that these compounds are not formed via the hexosemonophosphate shunt system. Ribose-5-phosphate was not found in the muscle of living fish, and only in comparatively small amounts post-mortem. From the evidence these authors presented, it is quite obvious that ATP is degraded to IMP post-mortem, and that IMP is reduced further to inosine and then to ribose and hypoxanthine.

Saito and Arai (1958a) found that the reduction of ATP was related quantitatively to the formation of IMP. The authors demonstrated this by measuring the levels of ATP and AMP, both before and after homogenizing

fresh carp muscle for 10 minutes in a 0.13 M. NaCl solution with ice. During the homogenizing procedure, 2.90 µM./g. of AMP was decomposed while 2.09 µM./g. of IMP was formed. The deamination of the AMP did not appear to be affected by the low temperature. Keir and Davidson (1958) and Davey (1961) have also published papers dealing with the metabolism of IMP.

Jones (1961) reported a high inosine 5'-monophosphate content in fresh fish and showed that it was one of the major flavoring components. He desribed IMP as possessing a strong salty-acid flavor with overtones described as "meat extract," "yeasty" or "almonds". He went on to describe the taste of hypoxanthine as strongly bitter at the level at which it occurs in fish after 10 days cold storage. He also stated that the removal of glucose, the major hexose phosphates and inosine 5'-monophosphate account for part of the loss of the initial sweet, meaty flavor in fish. Realizing that the concentration of hypoxanthine increased with cold storage, Jones and Murray (1964) and Jones et al. (1964) postulated that procedures for following the course of nucleotide degradation would seem to be an excellent opportunity for obtaining valid useful indices of quality. Most of the currently available chemical indices are measuring bacterial spoilage as pointed out by these workers. By assaying for hypoxanthine, certain initial autolytic phases of deterioration can be observed. As a result, this assay could be quite useful in assessing the quality of fish or meat stored at cooler temperatures. This measure would probably be of more value in the fish industry, since nucleotide decomposition occurs at a faster rate than microbial spoilage. Several researchers have used this hypothesis and

developed rapid and accurate methods for determining the freshness of stored fish muscle. Among these, Saito et al. (1959a), Spinelli et al. (1964), Jones and Murray (1964) and Jones et al. (1964) have published papers in this field.

Methods of evaluating nucleotide concentration

Nucleotides are generally separated by ion-exchange chromatography and then quantitatively evaluated from their molecular extinction coefficient. In general, only the ion-exchange resins or the elution system being utilized are changed to meet a particular researchers need. Most methods for separation of nucleotides by ion-exchange are patterned after that of Bergkvist and Deutch (1954) and Hurlbert et al. (1954). Other variations have been employed by Saito and Arai (1958b), Jones and Murray (1961a), Nakajima et al. (1961a) and Takeda and Shimeno (1964a).

Cohn (1950) discussed conditions which are necessary for successful ion-exchange chromatography. He noted that the net charge per ion was the most important factor in determining the ion affinity for an ion-exchange resin, when a closely related family of compounds was being separated. Since both acid (phosphate) and basic (amino) groups are present on a nucleotide, the pH of the medium determines the net charge by affecting the degree of dissociation of these groups. Cohn further noted that a sample of nucleotides could be adsorbed onto an ion-exchange resin at pH values greater than 6 and could then be removed by lowering the pH in a stepwise fashion. He suggested increasing the anion concentration by adding salt near the end of elution to prevent hydrolysis of the nucleotides.

In 1957, Bergkvist noted that ion-exchange chromatography has several advantages, since it is capable of handling both large and small quantities of material. In addition, it is suitable for preparative work, great resolving power is attained and contaminants are of little importance. Bergkvist cited two disadvantages, first, it is a very slow procedure, and second, large volumes of liquids are required during elution.

Numerous workers have reported other more conventional procedures for the separation of nucleotides. Bergkvist (1957) developed a method based on chromatography together with ionophoresis on filter paper. Smillie (1959) described a procedure for separating nucleotides on ion-exchange paper. More recently, Strickland (1965), Randerath (1965), Randerath and Randerath (1965), and Grippo et al. (1965) have described procedures for the separation, identification and quantitative measurement of nucleotides using thin-layer chromatography. Weinstein (1964) described an automated method of analysis for individual samples as they are eluted from ion-exchange columns. Macy and Bailey (1966) developed a rapid procedure for separation of nucleotides on a Dowex ion-exchange resin column. They successfully shortened the time for complete resolution of a sample from 12 to 5 hours.

Water soluble flavor precursors

In a review of the literature on meat flavor, Doty et al. (1961) observed that the purely subjective approaches which were used in earlier flavor research have been replaced by modern objective procedures. They stated that two approaches have been used in meat flavor research. One

technique involves the isolation and identification of flavor components, both volatile and nonvolatile, from cooked meat. The second method depends upon the isolation and characterization of flavor precursors from raw meat. There are obvious advantages and disadvantages inherent in either approach. They suggested that many compounds may be isolated from cooked meat, yet there is no way of determining the relative importance of these compounds to overall meat flavor. Thus, if isolation of flavor components from cooked meat is utilized, the various components must be estimated quantitatively and then recombined in the correct concentration to again achieve the original flavor. Doty further pointed out that if one isolates and identifies the precursors of cooked meat flavor from raw meat, it is still necessary to elaborate the chemical changes which are responsible for the development of typical cooked meat flavor.

With reference to precursors, first Crocker (1948) and then Kramlich and Pearson (1958) and Hornstein et al. (1960) demonstrated that flavor precursors can be leached from raw beef, although none of these workers attempted to establish their identity. In 1948, Crocker stated that the typical meaty flavors apparently were contained in the fibers of cooked meat rather than in the expressible juices, and were released by chewing. Barylko-Pikielna (1957) by taste panel evaluation demonstrated that the typical flavor of roast beef was present in the water insoluble residue, whereas, the water soluble fraction had a very intense but atypical beef flavor. Kramlich and Pearson (1958) worked with press fluids from raw and cooked beef. Their results indicated that press fluids of raw meat had a highly concentrated flavor on cooking. Cooking prior to

extraction increased the flavor, indicating that the full flavor development may be due to heating the juice and the fiber together.

Using paper chromatography, Wood (1956) demonstrated the presence of inosine and hypoxanthine in commercial meat extracts. This would suggest the presence of inosinic acid, even though it was not identified. It was interesting to note that no sugars or carbohydrates were detected in the extract. Wood surmised that they may have combined with some nitrogenous constituent in the extract during browning.

In 1957, Wood and Bender undertook the formidable task of isolating and identifying the constituents of commercial ox-muscle extract. In the process, they detected over 100 compounds. This list included, inosinic acid, inosine and hypoxanthine. In a later report, Bender et al. (1958) compared the constituents of an extract of fresh ox-muscle with commercial beef extract. Despite different methods of extraction and subsequent treatment, the composition of the two extracts was remarkably similar. Inosine and hypoxanthine were present in both extracts. The most notable difference was the loss of approximately 80% of the amino acids in the commercial product as well as 100% of the reducing sugars. The authors postulated that these losses were related to the higher yield of colored pigments isolated from the commercial extract, such as would be the case with the Maillard-type browning reaction.

The American Meat Institute Foundation has studied the flavor precursors from raw beef and their studies have been summarized by Doty et al. (1961). The authors selected this approach after observing that the fat fraction from the third acetone extract of raw ground beef gave a

typical broiled steak odor on heating. Subsequent research on this fraction revealed that the flavor was not due to the fatty material per se, but was caused by substances that diffused through a semi-permeable membrane upon dialysis with water. On separation of this fraction, they isolated a white granular material, which was unstable and became a brown, tarry mass if stored under vacuum. Ammonia and/or amines were released as browning progressed, and the material assumed a characteristic stale meat odor. Wood (1961) reported that a similar acetone-soluble material from ox-muscle extract decomposed in the same manner. He went on to state that nucleic acid decomposition was an important factor in browning and development of meat flavor. He isolated inosinic acid and ribose-5'-phosphate as the active ingredients. Wood described the taste of inosinic acid as meaty.

Batzer et al. (1960) undertook the task of trying to characterize and identify the components in the water-soluble fraction that yielded the typical meaty odor on heating. Several fractions were obtained by the extraction procedure utilized. Fraction A, which contained the flavor components, was obtained by filling cellulose dialysis tubing with distilled water and inserting the tubes into the water-soluble extract of beef. Upon further fractionation of the diffusate by dialysis through a sausage casing, two fractions were obtained. The material remaining in the sausage casing (Aa) was primarily protein in nature, while the material that diffused through the sausage casing (Ab) was composed primarily of small molecular weight compounds, such as sugars, amino acids and small peptides. Two more fractions resulted when fraction Aa was separated on a Sephadex G-25 column. One was a protein

fraction, and the other was characterized as the basic meat flavor precursor in beef. Chicken or pork loin tissue extracts treated in an identical manner resulted in similar fractions. These fractions had almost the same basic odor as that isolated from beef muscle. In a classical series of experiments, the same investigators showed that the basic meat flavor precursor was a glycoprotein, which gave a strongly positive carbohydrate reaction prior to hydrolysis with perchloric acid and a strongly positive phosphate test after hydrolysis. Ultra-centrifugation at 60,000 rpm. resulted in no distinct peak, suggesting that a relatively low molecular weight compound was involved. Ninhydrin tests were positive after 8 spots had been separated by paper chromatography of the acid hydrolysate. Two of the ninhydrin positive spots could not be positively identified, but the others revealed the presence of leucine, proline, isoleucine, alpha alanine, valine, serine and beta alanine, with trace amounts of glycine and glutamic acid. When the diffusate fraction of the secondary dialysis (Ab) was separated on Dowex-50 ion-exchange resin and eluted with acid, a spectra of nucleotide peaks matching published results for hypoxanthine, inosine and inosinic acid were obtained. Further studies by Batzer et al. (1962) resulted in the identification of inosinic acid, inosine and a glycoprotein having a glucose moiety. These authors concluded that these simple water soluble compounds in combination with certain amino acids are important components of basic meat flavor.

Wasserman and Gray (1965) reported data that varied significantly from results obtained by Batzer et al. (1960, 1962) on utilizing the same fractionation procedure for isolating precursors of beef flavor.

In contrast to Batzer, these authors were unable to effect a second separation of Fraction A, when it was subjected to dialysis in Visking sausage casings. Instead, there was essentially an equal distribution of the casing contents across the membrane. Three fractions rather than the two reported by Batzer et al. were obtained when Aa was separated on Sephadex G-25. Only one fraction (Aa_2) had the characteristic aroma of commercial meat extract. Fraction Ab was separated on Dowex-50 to yield three fractions. After freeze drying, the meaty aroma was found only in the heat decomposed solutions of Ab_2. They demonstrated that this fraction contained only amino acids, hypoxanthine and a trace of inosine. Inosinic acid was conspicuously absent. These authors also went on to show that fraction Ab_2 contained no glucose or ribose sugars as claimed by Batzer et al. (1960, 1962), even though a meat-like aroma existed.

Macy et al. (1964a, 1964b) demonstrated the presence of inosine and hypoxanthine in water-soluble extracts of beef, lamb and pork. They went on to suggest that inosine may play a major role in the browning of meat, and that it could be important as a flavor and/or odor precursor in meat products.

In 1961, Kazeniac discussed chicken flavor and some of the components responsible for flavor. He noted that hypoxanthine and inosine have a bitter taste, whereas, inosinic acid makes a major contribution to mouth satisfaction by intensifying the flavor effects of other compounds. He attributed the fact that light meat broths from chicken have a stronger taste and mouth satisfaction to their higher inosinic acid content.

EXPERIMENTAL PROCEDURE

This investigation was necessarily broken down into several phases. The first study was designed to find the concentration of nucleotides in each of three muscles from pork carcasses. The second phase dealt with the nucleotide levels in samples taken from beef carcasses during post-mortem aging. The third phase investigated the concentration of nucleotides from various species of meat animals. The final investigation was designed to assess the flavor enhancing properties of inosine monophosphate.

Experimental material

Muscle investigation

Six Hampshire barrows with an average weight of 210 lbs. were used for this portion of the study. Three muscles were sampled from the right side of each carcass. They were the 10th to 13th rib section of the longissimus dorsi muscle of the loin, and the semimembranosus and biceps femoris muscle from the ham. The samples were removed from the carcasses approximately 48 hours post-mortem, in an attempt to approximate commercially existing conditions. All fat and connective tissue were removed, and the samples were ground twice through a 2 mm. plate. The samples were placed in sample bottles, frozen and stored at -20°F. until further analysis.

Beef aging study

Six Holstein heifers, approximately 24 months of age were used in this investigation. Samples of the longissimus dorsi muscle were removed

according to the schedule shown in table 4.

Table 4. Sample time and location for beef aging study

Sampling time post-mortem	Sample location
0 hr.	12th rib section
12 hr.	11th rib section
24 hr.	10th rib section
4 days	9th rib section
7 days	8th rib section
14 days	7th rib section
28 days	6th rib section

The wholesale rib from the right side of each carcass was removed and stored at 33-35°F. The sample at 0 hour was removed as rapidly as possible following stunning. In all cases, no more than 15 minutes elapsed before the initial sample was removed. All 0 hour and subsequent samples were immediately frozen in liquid nitrogen and stored at -20°F. until removed for analysis. Before freezing, all fat, connective tissue and any exposed lean areas were trimmed away in order to avoid contamination or excessive moisture loss. Considerable trimming was necessary on all samples aged for either 14 or 28 days, because they were contaminated by molds and other microorganisms.

Species investigation

Samples were taken from pork, lamb and bull carcasses for this portion of the study.

Six pork carcasses weighing approximately 220 lbs. were utilized in this experiment. Samples were removed from the longissimus dorsi muscle at the 10th rib within 15 minutes of stunning. Samples were excised after removing the backfat from the loin in the 10th rib area

and then by using a cork borer to remove the sample. After removal of the connective tissue the samples were frozen in liquid nitrogen and stored at -20°F. until analysis. Heart samples from the same carcasses were removed, frozen and stored in the same manner. In addition, heart muscle was sampled at 12 and 24 hours post-mortem. This series of samples was also utilized to study different extraction procedures.

The entire longissimus dorsi muscle from the wholesale loin section of 6 lamb carcasses was sampled. The lambs had been used for class evaluation, and as a result were not kept under constant storage conditions. Samples were taken approximately 7 days post-mortem, ground through a 2 mm. plate, frozen and stored at -20°F.

Samples from 6 bull carcasses, which had been used for another study, were analyzed. These samples had been taken from the longissimus dorsi muscle of the 10th rib section of the wholesale rib. They were ground through a 2 mm. plate, frozen and stored at -20°F. These samples had been previously thawed for proximate analysis, refrozen and stored before being analyzed for nucleotides.

Methods for evaluation of nucleotide content

Extraction

Samples were not thawed prior to analysis. Approximately 10 grams of tissue was weighed into a Virtis homogenizer flask. A total of 25 ml. of distilled water was added, and the sample was homogenized for 2 minutes at maximum speed. The sample was then quantitatively transferred to a 100 ml. volumetric flask and boiled for 5 minutes. It was allowed to cool before bringing the volume of the extract up to 100 ml. with distilled water. Following centrifugation for 20 minutes, the extract was filtered

through a Whatman GF/B glass fiber filter pad. The resulting extract was measured and adjusted to pH 8.0 by adding 1 N. sodium hydroxide. A 10 ml. aliquot of the extract was then separated with an ion-exchange column. The remainder of the extract was frozen and stored at -20°F.

Chromatographic separation

The method of Lento et al. (1963) was used to separate the nucleotides. Dowex-1 X 8 anion exchange resin (200-400 mesh) was washed with water to remove the fines and suspended solids. It was then packed as a wet slurry in a 12 mm. (inside diameter) glass column to a height of approximately 160 mm. The column was fitted with a stopcock and a plug of glass wool in order to retain the resin. The resin was then converted to the hydroxyl form by adding three bed volumes of 1 N. sodium hydroxide. After washing the resin free of excess sodium hydroxide, it was converted back to the formate form by the addition of 3 bed volumes of 6 N. formic acid. The excess reagent was again removed by washing with distilled water. At this point, an aliquot of the test solution was added to the the top of the column and passed through at the rate of 1 ml. per minute. The resin was washed free of UV absorbing materials with water until the effluent gave a reading of approximately 100 percent transmission. The nucleosides, bases and free sugars, which were removed from the column in the wash water, were saved for further analyses. The resin columns were reuseable on recharging them as described above. Cohn (1950) reported that some columns were used as many as 30 times without any loss of capacity or exchanging power.

Elution

The nucleotides were eluted by a 3 phase gradient system consisting of water, 0.5 N. formic acid and 0.2 N. sodium formate. Three aspirator bottles were aligned above one another as shown in figure 2. The lower bottle contained 250 ml. distilled water, the middle bottle contained 250 ml. 0.5 N. formic acid and the upper bottle contained 250 ml. 0.2N. sodium formate. Magnetic stirrers mixed the solutions in the lower 2 bottles during elution.

To begin the elution, the screw clamps were removed from the Tygon tubing connections between the aspirator bottles. It was necessary for this tubing to be airtight so repeatable gradients could be obtained during the elution process. The solution was allowed to percolate through the column at a flow rate of 1 ml. per minute. As the effluent flowed from the column it passed through an Isco, Model UA, UV analyzer, which automatically recorded the absorbance at 254 mμ. The fraction collector was wired to the monitor so that each time it moved it was recorded on the chart paper. This system made it possible to locate any single fraction containing the 5'-nucleotides for further analysis.

Quantitative evaluation

Since only the 5' isomers of the nucleotides are important as flavor enhancers, the method of Lento et al. (1963), which is specific for the 5'-ribonucleotides, was employed. This is in contrast to earlier procedures described by Bock et al. (1956), in which the quantitative determination of the nucleotides is based upon their molar extinction coefficients.

One ml. of 0.01 N. periodic acid (HIO_4) was added to a 5 ml. aliquot

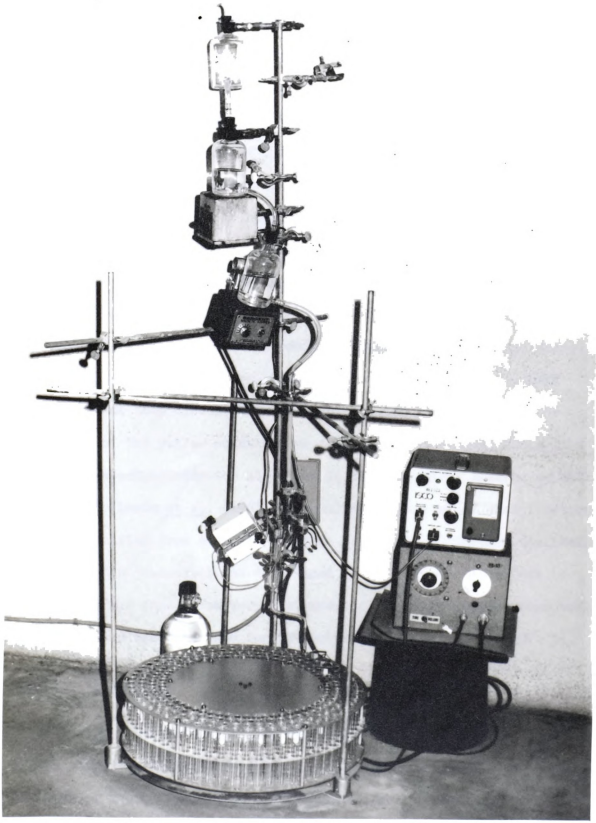


Figure 2. Diagram of the gradient elution apparatus

of the sample. The mixture was heated for exactly 3 minutes at 70°C. This step oxidizes the ribose moiety of the nucleotide between the number 2 and 3 carbon atoms and is specific for the 5' isomers. Oxidation was stopped by the addition of 1 ml. of 0.05 N. sodium arsenite (Na_2HAsO_3). This was followed immediately by the addition of 1 ml. of concentrated hydrochloric acid and 1 ml. of 2,4-dinitrophenylhydrazine (2,4-DNPH) in 2 N. hydrochloric acid. After mixing, the tubes were placed in a boiling water bath for 10 minutes to hydrolyze the oxidation products, which facilitated precipitation of the bis-hydrazone. It was necessary to add the reagents in the order described to prevent interaction of periodic acid and 2,4-DNPH, which results in high reagent blanks. The precipitate was then collected on a medium porosity fritted glass filter and washed with distilled water to remove any unreacted 2,4-DNPH. The 2,4-DNPH derivative was then dissolved in acetone, diluted to volume, and the absorbance of the yellow solution was read against a reagent blank in a Beckman DU spectrophotometer at 435 mμ in a 10 mm. pyrex absorbance cell. According to Lento et al. (1963) the nucleotide content of each fraction can be calculated from a single standard, since the extinction coefficient of each of the 5'-nucleotides determined by this colorimetric test is essentially the same. However, a standard curve using inosine monophosphate was developed and used for the actual determinations (Figure 3).

Qualitative analyses

Thin-layer chromatography was employed to aid in the identification of the eluted nucleotides by comparing the R_f values of unknown compounds to those of known standards, which were run concurrently. Chromatography was carried out using Desaga equipment purchased from the Desaga Company,

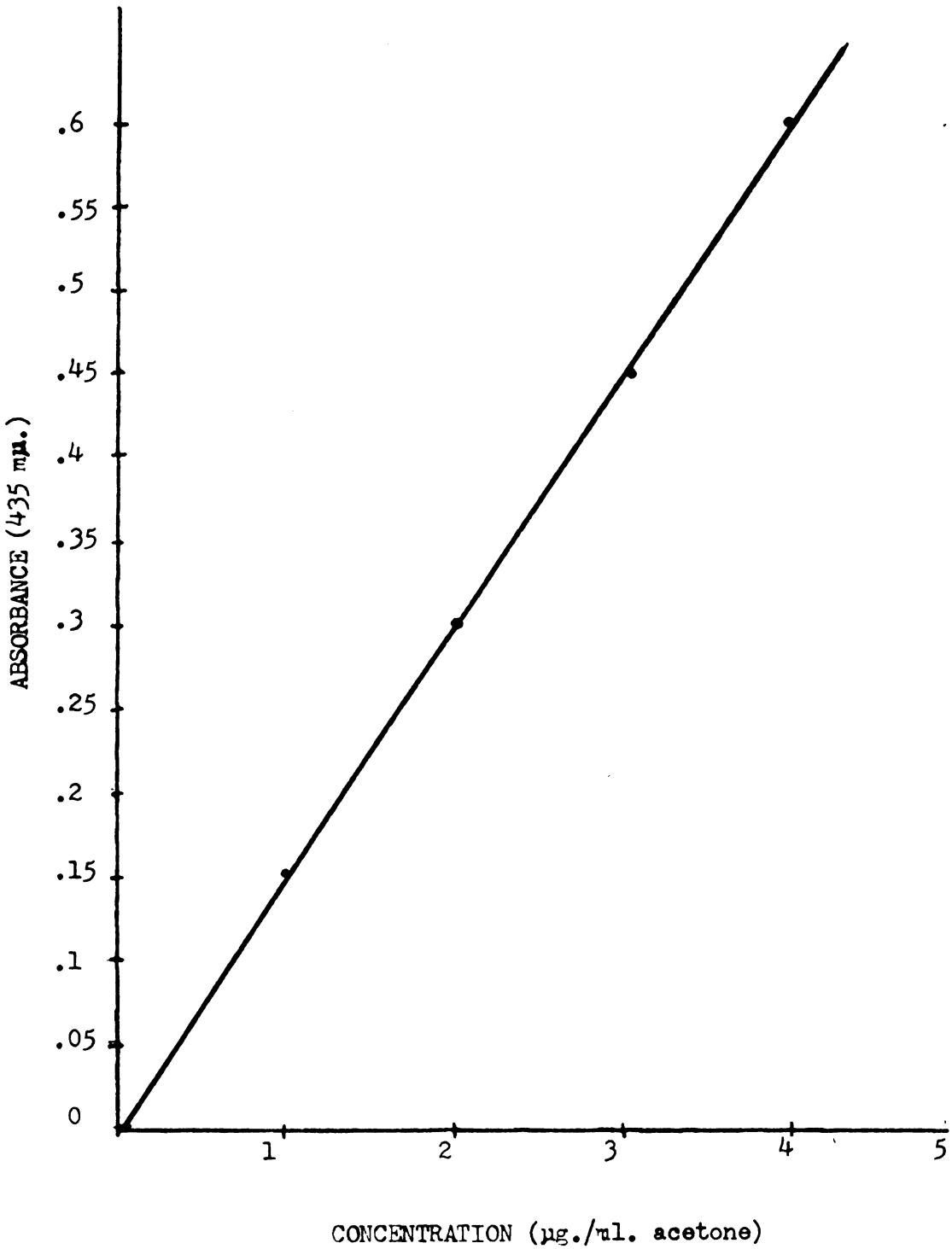


Figure 3. Standard curve for inosine 5'- monophosphate.

Heidelberg, Germany. It consisted of a plastic aligning tray, which held 5 plates (20 X 20 cm.), a spotting template, an applicator and a rectangular chromatography developing tank. MN Cellulose Powder 300 with no binder was purchased from the Brinkman Instrument Company, Westbury, New York. A total of 15 grams of absorbant was slurried with 90 ml. distilled water and applied to the clean glass plates in a uniform layer about 250 microns in thickness.

The plates were allowed to dry at room temperature for 15 minutes and then activated in an oven at 100°C. for 30 minutes. The activated plates were then stored in a desiccator until used.

For identification of nucleotides, the chromatoplates were spotted with 10^{-3} to 10^{-2} µM. of the known standards and the unknown compounds. Care had to be exercised since tailing occurred if over 10^{-2} µM. of the nucleotides were added to the plates. They were then developed for 80 minutes in an equilibrated chromatographic tank. The solvent system consisted of n-butanol/acetone/acetic acid/5% ammonium hydroxide/water in a 4.5:1.5:1:1:2 ratio. After development, the plates were allowed to dry at room temperature. Spots were observed by visualization under an ultraviolet lamp.

Nucleosides and bases were identified in a similar manner. The same cellulose plates were utilized. The plates were spotted with 10^{-2} µM. of the substances involved and developed for 45 minutes in a solvent system of distilled water. The plates were then air dried and observed under ultraviolet light. After observing under UV lights, tracings of all plates were recorded on acetate paper.

Paper chromatography on Whatman No. 1 filter paper was used as a second method of analysis. Solvent systems utilized were adapted from

those described in the Pabst Circular OR-10 (1956). Sheets, 21 X 16 inches, with a base line drawn 1¼ inches from the bottom were spotted with approximately 150 µg. of the nucleotides. They were then immersed in covered jars and developed for 16 hours at 27°C. The chromatograms were then air dried and read under ultraviolet light. The solvent system consisted of isobutyric acid/concentrated ammonium hydroxide/ water (66:1:33) at pH 3.7.

Finally, the nucleotides were characterized from their ultraviolet absorption spectra and the ratio of absorption at 250/260 and 280/260 mµ. Spectras were determined at pH 2, 7 and 11 and compared with known spectras given in the Pabst Circular OR-10 (1956) or Pabst Circular OR-17 (1961). After several determinations with known samples, the elution sequence of the nucleotides was established. The same was true of unknown samples. Thus, it was not always necessary to make qualitative determinations on all samples added to the column.

Sensory evaluation

Preliminary work in this study indicated that the concentration of IMP in heart muscle is negligible. This agrees with the findings of Imai *et al.* (1964) and Visioli *et al.* (1964), who reported finding no IMP in heart muscle of rabbit and rat, respectively. The lack of IMP occurs since the enzyme AMP-deaminase seems to be lacking in cardiac and smooth muscle of the body. For the above reason it was decided to use heart muscle for sensory evaluations in this experiment. It was felt that due to the lack of IMP better control could be exercised, and the flavor enhancing ability of the nucleotide could be more clearly demonstrated.

Pork heart muscle was incorporated into a standard formulation of cooked frankfurters in order to assess the effect of added flavor enhancers. The recipe used for these frankfurters is shown in table 5.

Table 5. Recipe for frankfurters

Ingredients	Amount
Pork heart muscle	6.5 lb.
Pork fat	3.5 lb.
Salt	136.0 g.
Sugar	23.0 g.
White pepper	17.5 g.
Ginger	3.5 g.
Coriander	5.0 g.
Allspice	1.0 g.
Garlic powder	0.8 g.
Sodium ascorbate	12.5 g.
Sodium nitrate	4.0 g.
Sodium nitrite	1.0 g.
Dried skimmilk	226.0 g.
Shaved ice	3.0 lb.

Five formulations of frankfurters were prepared, one utilizing beef muscle as in commercial practice and four other formulas utilizing heart muscle and varying levels of flavor potentiators as shown in table 6.

Table 6. Level of added flavor potentiators

	Formulation				
Ingredients	1	2	3	4	5
Meat source	Heart	Heart	Heart	Heart	Beef muscle
MSG	0	0.2%	0	0	0
IMP	0	0	0.05%	0	0
IMP + GMP	0	0	0	0.025%	0

The trimmed heart muscle and fat were ground separately through a ½ inch plate. The meat was then placed in a Silent Cutter and chopped for about 5 minutes to a smooth consistency. The spices and most of the ice were added and mixed. At this point, the dried skimmilk was added. The fat was added and chopping was continued for approximately 3 more minutes until the consistency was smooth. The temperature was maintained below 55-60°F. by the addition of shaved ice.

The finely chopped mixture was stuffed into artifical casings and linked on the linking rack. They were then smoked for 35 minutes at 175°F. The smokehouse temperature was lowered to 165°F. and smoking was continued until the internal temperature of the frankfurters reached 152°F. The frankfurters were removed from the smokehouse and showered for 5 minutes in hot water and 10 minutes in cold water. After cooling, the frankfurters were evaluated organoleptically with a consumer type taste panel utilizing the 9-point hedonic scale.

EXPERIMENTAL RESULTS AND DISCUSSION

Evaluation of techniques and procedures

Known and unknown samples were qualitatively and quantitatively evaluated before beginning work with regular samples, in order to assess the effectiveness of the procedures involved. The chromatographic separation of a synthetic mixture of 5'-mononucleotides and the order in which these compounds are eluted by a three phase gradient elution system is shown in figure 4. The elution order of these compounds was 5'-CMP, 5'-AMP, 5'-UMP, 5'-IMP and 5'-GMP. The order of elution was similar to that of Lento et al. (1963). In addition, Cohn (1950) reported that the order of elution at pH values of 2.5 to 3 should be CMP, AMP, UMP and GMP according to his theory that the compounds with the lowest net negative charge would have the least affinity for an ion-exchange resin and as a result would be eluted first. He did not use IMP in this theoretical discussion or in the series of experiments he used to demonstrate his postulation. The shape of the eluted nucleotide peaks was interesting. The first peak was always very sharp and well defined, while each succeeding peak decreased in height and increased in width. Even so, resolution was definite and it was easy to identify each compound.

Figure 5 illustrates the chromatographic resolution of a sample of unknown nucleotides from the biceps femoris muscle of pork. It is quite evident that very good separation was obtained when this system of gradient elution was employed, as no overlapping of the various compounds occurred. The separation pattern and peak size shown in figure 5 were

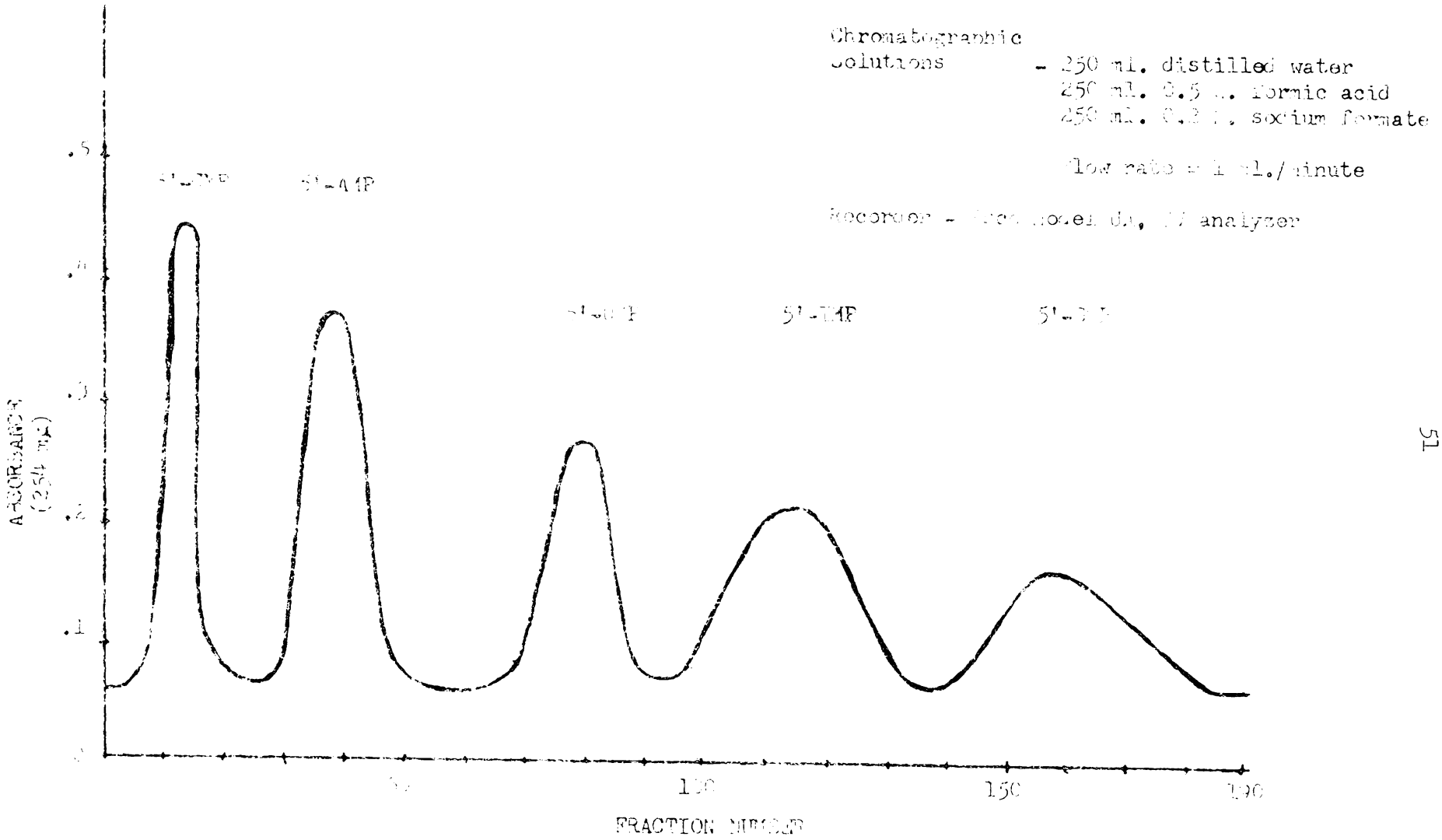


Figure 8. Chromatographic separation of a synthetic mixture of 5'-mononucleotides.

Column - Dowex-1 X 8, formate
200-400 mesh, 12 X 160 mm.

Chromatographic
Solutions - 250 ml. distilled water
250 ml. 0.5 N. formic acid
250 ml. 0.2 N. sodium formate

Flow rate = 1 ml./minute

Recorder - Isco Model UA, UV analyzer

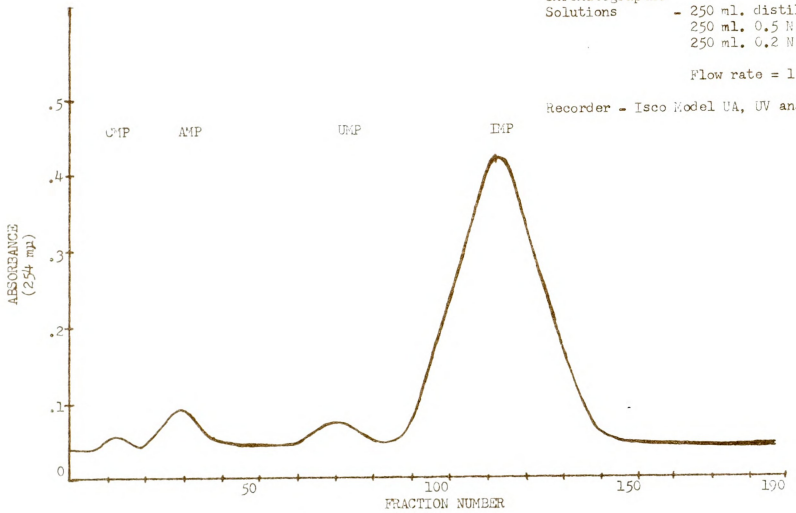


Figure 5. Chromatographic separation of 5'-mononucleotides in an unknown samples of Biceps femoris muscle from pork.

typical for other pork muscles, as well as for samples from bull, lamb and beef from the beef aging study. Even though IMP was the predominant nucleotide, it was always well separated from UMP.

The recovery of known amounts of 5'-nucleotides following separation and colorimetric analysis is shown in table 7. Recovery rates ranged from

Table 7. Recovery of 5'-nucleotides by ion exchange chromatographic separation and colorimetric analysis.

Nucleotide	Added µg.	Recovered µg.	%	Added µg.	Recovered µg.	%
5'-CMP	300	741	92.6	1300	1329	102.2
5'-AMP	700	720	102.8	1300	1296	99.6
5'-UMP	700	733	104.7	1500	1470	98.0
5'-IMP	700	742	106.3	1400	1360	97.1
5'-GMP	300	311	103.6	1300	1311	100.8

92.6% to 106.3% and from 97.1% to 102.2% for low and high levels of added nucleotides, respectively. These are excellent recovery rates, especially when talking in terms of µg. added and recovered. A very small weighing error could drastically effect the results since 100 µg. is equal to 0.0001 g. and many analytical balances are accurate only to 0.0001 g. Some difficulty was encountered when analysis were first started. However, after a few trial runs excellent recovery and repeatability was obtained. Known samples were added to the column and analyzed throughout the duration of the experiment in order to assess the accuracy of the techniques and the resolving power of the ion exchange resins, which were being recharged and reused. In no case was a column used over 20 times, even if the

resolving power of the Dowex appeared to be adequate. Cohn (1950) reported using columns up to 30 times with no loss of capacity or resolving power.

Results of a pre-trial study on the effectiveness of two extraction procedures is shown in table 8. Analysis of the data indicated that the

Table 8. Effect of extraction procedure on inosine 5'-monophosphate content

	Extraction Procedure	
Sample	Perchloric acid	Boiling water
1	4.80	5.50
2	4.44	4.33
3	4.51	4.57
4	3.11	3.01
Mean	4.21	4.35

difference between treatments was not statistically significant on using Duncan's New Multiple Range Test for evaluating means (Steel and Torrie, 1960, Snedecor, 1956). This indicates that either the perchloric acid or the hot water extraction procedure could be utilized without seriously affecting experimental results. These results are logical since work by Schade et al. (1963b), Fujita et al. (1961), Hanaoki and Higashi (1963) and Take and Otsuka (1964) indicated that nucleotides are stable at 100°C., as long as the pH is maintained near neutral. Hot water extraction was used to inactivate any enzyme systems present which might dephosphorylate 5'-inosine monophosphate and thereby render it ineffective as a flavor potentiator (Chargoff and Davidson, 1955, Shimazono, 1963 and Fujita and

Hasimoto, 1960). The hot water extraction procedure was used not only to extract the nucleotides and to inactivate the enzymes but also because it was simpler and less time consuming.

Qualitative determinations of the nucleotides were made spectrophotometrically and with thin-layer and paper chromatography. This was necessary in order to identify and establish the order of nucleotide elution. Since samples were diluted during elution, the individual peaks were collected and freeze dried to concentrate them. Freeze dried peaks from several successive runs were then composited until enough material was available for characterization. Generally, UMP from one run was adequate for qualitative analysis, while several runs were required to accumulate ample AMP, CMP and GMP for characterization. Once the order of elution was established as well as the approximate position or time of elution, it was not necessary to carry out qualitative characterization on every sample. However, qualitative and quantitative analyses were made whenever a new column was being used or when a different series of samples was being analyzed.

Results of the spectrophotometric analysis of known and unknown nucleotide peaks are shown in table 9. Excellent results were obtained in identifying the individual nucleotide peaks with this method. The ratio of absorbance at 250/260 and 280/260 mμ was faster and simpler to obtain than was the complete spectra of a compound. In addition, these results were equally as reliable. Absorption spectras were tedious and time consuming to measure with a Beckman DU spectrophotometer, since the blank reading required adjustment each time the wave length was changed during the determination.

Table 9. Spectrophotometric analysis of known and unknown nucleotide peaks - pH 7

Maximum absorbance			Ratio of absorbance				
			250/260		280/260		
Peak	Unknown	Known	Unknown	Known	Unknown	Known	Identity
1	271	271	.88	.84	.93	.98	CMP
2	259	259	.82	.79	.29	.16	AMP
3	262	262	.76	.73	.38	.39	UMP
4	249	248	1.65	1.67	.27	.25	IMP
5	251	252	1.18	1.16	.63	.66	GMP

Table 10 shows the results of thin-layer and paper chromatography for identification of the nucleotides, while figure 6 illustrates the migration pattern of the nucleotides during thin-layer chromatography. Positive identification of the nucleotides was made when the R_f values of the unknowns were compared to the standards, which were analyzed concurrently. As can be seen in table 10, the relative rate of separation of the nucleotides was greater if thin-layer chromatography was utilized. As shown in table 10, the rate of migration of UMP, IMP and GMP was similar. Separation of these three nucleotides would not have been possible if all three compounds had been applied to the same spot at the origin. In the present study, separation was achieved by ion exchange chromatography, so that paper chromatography was utilized only for identification. Thin-layer chromatography was superior to paper chromatography, since it was a faster procedure (80 minutes vs. 16 hours), less tailing of the spots occurred, less material was required for spotting the chromatograms and

Chromatoplate - MN cellulose powder 300

Solvent system - n butanol/acetone/acetic acid/5% ammonium hydroxide/water 4.5:1.5:1:1:2

Development time - 80 minutes

Detection - Ultraviolet light

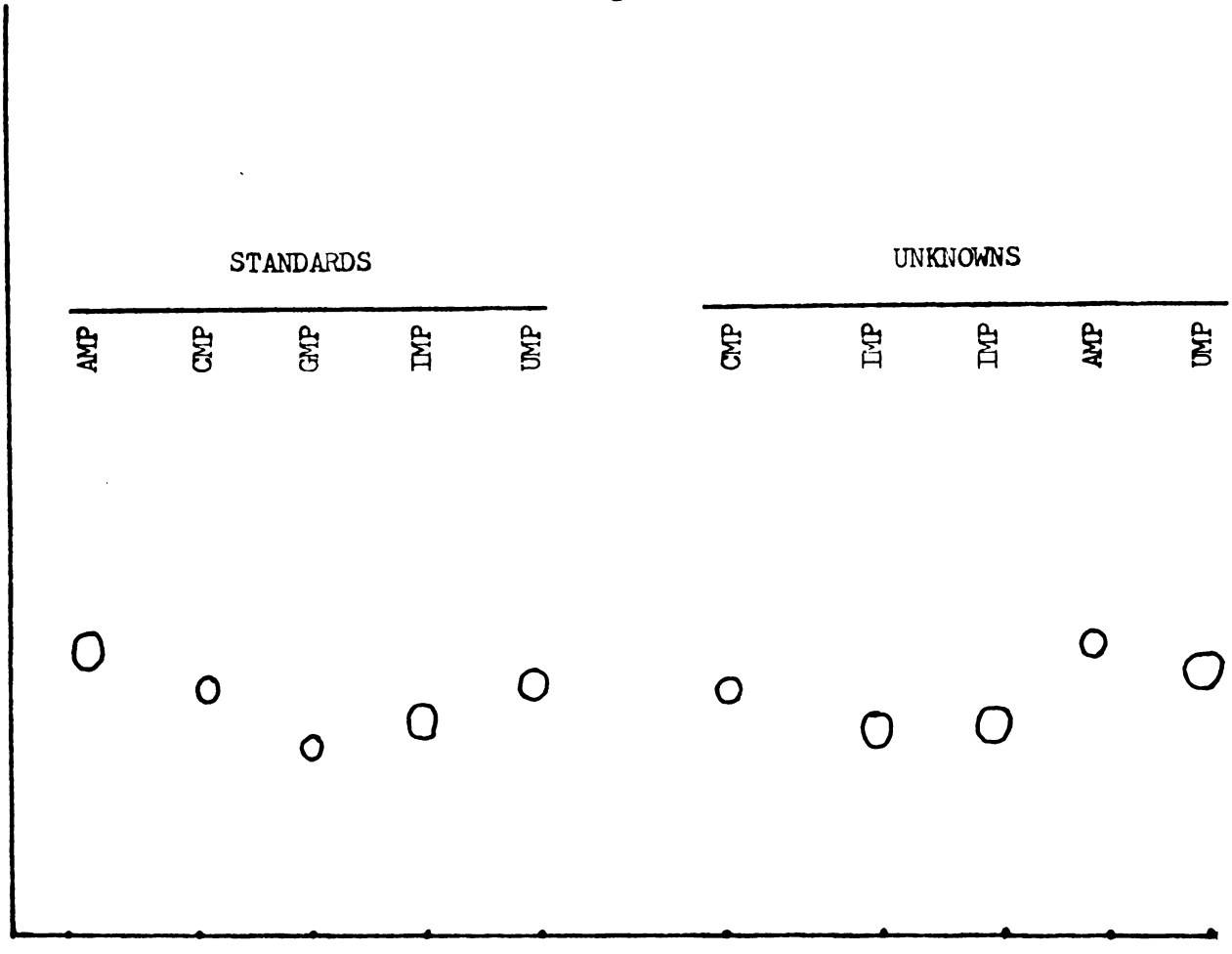


Figure 6. Thin-layer chromatogram of nucleotides.

Table 10. Results of thin-layer and paper chromatography analyses of nucleotides

Peak Number	Thin-layer Chromatography R_f Unknown	Thin-layer Chromatography R_f Known	Paper Chromatography R_f Unknown	Paper Chromatography R_f Known	Identity
1	.33	.33	.24	.24	CMP
2	.38	.37	.47	.49	AMP
3	.36	.35	.15	.15	UMP
4	.30	.30	.14	.135	IMP
5	.26	.25	.13	.126	GMP

repeatability was better. It can be seen from figure 6 that resolution of the nucleotides was quite good. Longer development time further increased the resolution.

From the results of these three qualitative evaluation procedures positive identification of the eluted nucleotides from samples of known nucleotide mixtures, as well as from unknown meat samples was accomplished. In addition, the elution order and approximate elution position were established.

The periodic acid - 2,4-DNPH color reaction employed for quantitative determination of the nucleotides can be used to distinguish the 5'-nucleotides from their 2'- and 3'-isomers. This can be accomplished by selective oxidation of adjacent hydroxyl groups attached to the number 2 and 3 carbon atoms of the ribose moiety. The products of this oxidation are then reacted with 2,4-DNPH and estimated colorimetrically. This scheme is very effective for assessing the level of the flavorful 5'-nucleotides in foods. Any

compound, which reacts with periodic acid and/or 2,4-DNPH, could conceivably interfere in the colorimetric determination of the 5'-nucleotides. However, the periodate - 2,4-DNPH color reaction incorporates the use of anion exchange chromatography as a prerequisite to colorimetric analysis, and all anionic compounds are retained by the resin. Interference from carbonyl compounds, carbohydrates, nucleosides or other nonionic substances is eliminated, since these compounds are washed from the resin prior to gradient elution.

Other compounds of biological origin with anionic groupings, such as phosphorylated sugars and the 5'-di- and tri-phosphonucleotides, interfere with this color reaction due to the presence of the vicinal hydroxyl group. In the latter case, however, the di- and tri-nucleotides are not eluted from the column by the gradient system employed. The phosphorylated sugars are eluted in the vicinity of the 5'-nucleotides but are located in separate fractions. Further, these compounds do not absorb in the ultraviolet region, and would therefore not be observable by the UV scanner on elution from the column.

Muscle investigation

This portion of the experiment was designed to assess the nucleotide concentration in each of three muscles from six pork carcasses. The muscles involved were the biceps femoris, longissimus dorsi and semimembranosus. Samples were separated by ion-exchange chromatography and quantitatively evaluated with a periodic acid - 2,4-DNPH colorimetric test. Results of the IMP determination from this portion of the investigation are given in table 11 while the complete mononucleotide content of the three muscles is shown in Appendix table I.

Table 11. Inonsine 5'-monophosphate content of 3 pork muscles - μM./g. tissue

Animal Number	Muscle		
	Biceps femoris	Longissimus dorsi	Semi membranosus
1	3.40	3.03	2.59
2	3.59	3.31	3.18
3	3.45	2.91	3.58
4	2.65	3.60	2.54
5	2.99	2.92	2.81
6	2.65	3.63	3.17
Mean	3.12	3.23	2.98

The average inosinic acid content of the longissimus dorsi, biceps femoris, and the semimembranosus muscles was 3.23, 3.12 and 2.98 μM./g. tissue, respectively. Statistical analysis of the muscle means indicated that significant differences did not exist at either the 1% or 5% level, when Duncan's New Multiple Range Test was utilized (Steel and Torrie, 1961, Snedecor, 1956). Even though the data were not statistically significant, a slight trend appeared to be evident. The semimembranosus muscle contained the lowest level of IMP in four of the six pigs examined, which accounts for its low average value. The biceps femoris contained the highest IMP level in three of the pigs tested. Even though the longissimus dorsi muscle had the highest concentration of IMP from only two pigs it still had the highest average IMP content. This indicates considerable variation between muscles from pig to pig. The variation is substantiated by the fact that the standard deviation for the longissimus dorsi, biceps

femoris and semimembranosus muscles was 0.33, 0.42 and 0.40 μM./g. tissue, respectively. The wide range between high and low values, of course, agreed with the relatively high standard deviations. However, standard deviation is a rather poor measure in this case, as there are many factors which could cause wide variation from pig to pig and as a result increase the standard deviation. Hedrick (1965) in a review article indicated that stress could have an effect on the ATP level. As a result of stress, the ATP level could be altered, which in turn would affect the IMP level. Hedrick suggested that stress may be a result of hormonal or chemical injections, fatigue, exercise, excitement, preslaughter feeding and dietary restrictions. When evaluating the data in this study, it should be remembered that they were taken 48 hours post-mortem. Consequently, inosinic acid degradation may have already begun. It should be pointed out that there may be two possible mechanisms for the degradation of inosinic acid. First, a constant amount of IMP may be decomposed during a given time period. In this case, the range between values for two muscles would remain constant as IMP was decomposed. Secondly, the rate of decomposition may occur on a percentage basis. In this case, the range between values for two muscles would decrease as degradation proceeded. If we had two muscles, for example, with an IMP content of 5 and 4 μM./g. tissue, respectively, and a 5% reduction in IMP content occurred, the new IMP values would be 4.75 and 3.80 μM./g. tissue. The difference between the two muscles would now be 0.95 μM./g. tissue rather than the 1.0 μM./g. tissue, as was the case in the original samples.

The IMP content of these three muscles agrees well with results of Terasaki et al. (1965), who reported the IMP content of the longissimus

dorsi muscle from 154 pound Yorkshire barrows to be approximately 3.0 μM./g. tissue at 48 hours post-mortem. Hillo (1965) found that the IMP content was similar for the psoas major, lattissimus dorsi, longissimus dorsi and pectoralis profundus muscles from beef and pork, although the rate of decomposition varied between muscles during a 15 day storage period at 3-4°C.

Differences between muscles have been observed by Saito et al. (1959b), who indicated that the IMP content of the dorsal muscle of the rainbow trout is considerably higher than the red lateral muscle. Arai (1960a, 1960b) and Arai et al. (1961) reported differences between the foot and adductor muscles of shellfish. Minor (1964), Zhivkov (1965), Kazeniac (1961) and Terasaki et al. (1965) have all reported that samples of chicken breast muscle contained relatively higher concentrations of IMP than did the darker colored muscle.

One objective of this portion of the study was to determine if the inosinic acid concentration varied from muscle to muscle. Even though statistical analysis revealed that there was no difference between the means of the three muscles examined, it must be remembered that relatively small numbers were involved in this investigation. If large numbers of determinations had been carried out, it is possible that some of the trends observed might have been statistically significant. Since the muscles examined contained essentially the same IMP concentration, it was decided that the longissimus dorsi muscle would be utilized for further investigations. The longissimus dorsi muscle was more accessible, less damage was done on removal of the sample and the standard deviation of the longissimus dorsi muscle from the six pork carcasses studied was smaller, thus indicating that less animal to animal variation existed.

GMP was not detectable, while CMP was present in only trace amounts in all three muscles tested (Appendix table I). The level of UMP was only slightly higher. The longissimus dorsi, biceps femoris and semimembranosus muscles contained 0.12, 0.20 and 0.11 μM./g. tissue, respectively. At such low concentrations, accurate determinations of the nucleotides are difficult to obtain since it is not easy to ascertain from the recording contained on the chart paper the exact point at which the peak was eluted.

The AMP concentration in pork muscle was 0.33, 0.90 and 0.33 μM./g. tissue for the longissimus dorsi, biceps femoris and semimembranosus muscles, respectively. These values are considerably higher than other values reported in the literature. Nakajima et al. (1961) reported AMP values of 0.19, 0.06-0.33 and 3.1-5.9 μM./g. tissue for fish, meats and invertebrate animals, respectively. Terasaki et al. (1965) listed the levels of AMP for mutton, horse, broilers and pork at 0.19, 0.07, 0.15 and 0.15 μM./g. tissue, respectively. Jones and Murray (1961a) found a level of 0.01 μM./g. of codling muscle.

The high average value (0.90 μM./g. tissue) obtained for the biceps femoris muscle was due primarily to the exceptionally high level in one sample (Appendix table I). Due to the one high value, the standard deviation for the biceps femoris muscle was extremely high, being 0.64 μM./g. tissue. The reason for the high level in the one case cannot be explained. All values in this investigation are the result of averaging the concentration from duplicate determinations. All duplicate measurements included separate extraction, ion exchange chromatographic separation and quantitative evaluation, which was also carried out on

duplicate samples of each peak. Standard deviations for the longissimus dorsi and semimembranosus muscles were 0.08 and 0.17 μM./g. of tissue, respectively, indicating considerably less variability between animals for these muscles than for the biceps femoris.

Several workers (Perry 1951, Dvorak, 1958, Jones and Murray 1961a, Tomlinson and Geiger 1962a) have indicated that some nucleotides are bound to the myofibrils of muscle. These adenine nucleotides appear to be protected in some manner from enzymatic breakdown at pH values which normally occur in post-rigor muscle. Tomlinson and Geiger (1962a) demonstrated with lingcod muscle that the bound nucleotides were released from the myofibrils by heating, but they were not released if the muscle was simply frozen and analyzed. Dvorak (1958) suggested that temperature might cause the release of bound nucleotides, since the adenine nucleotides were stable at 5°C., while at 37°C. they were not stable. If this is the case, extraction with boiling water may release the bound adenine nucleotides and at the same time inactivate the AMP-deaminase enzyme system, so that the nucleotides are not converted to IMP. This would partially explain the reason for finding higher levels of AMP in these three muscles in the present study than have been reported by other workers, when they used the more conventional perchloric acid extraction procedure. Jones and Murray (1961a) and Bendall and Davey (1958) both suggested that nucleotide binding is dependent upon pH and concluded that less nucleotides are bound at higher pH values.

Fredholm (1963) suggested that the level of the adenine nucleotides was more stable than previously believed. He indicated that the level of AMP remaining at 24, 72 and 120 hours was 100%, 60% and 40% for beef

duplicate samples of each peak. Standard deviations for the longissimus dorsi and semimembranosus muscles were 0.08 and 0.17 μM./g. of tissue, respectively, indicating considerably less variability between animals for these muscles than for the biceps femoris.

Several workers (Perry 1951, Dvorak, 1958, Jones and Murray 1961a, Tomlinson and Geiger 1962a) have indicated that some nucleotides are bound to the myofibrils of muscle. These adenine nucleotides appear to be protected in some manner from enzymatic breakdown at pH values which normally occur in post-rigor muscle. Tomlinson and Geiger (1962a) demonstrated with lingcod muscle that the bound nucleotides were released from the myofibrils by heating, but they were not released if the muscle was simply frozen and analyzed. Dvorak (1958) suggested that temperature might cause the release of bound nucleotides, since the adenine nucleotides were stable at 5°C., while at 37°C. they were not stable. If this is the case, extraction with boiling water may release the bound adenine nucleotides and at the same time inactivate the AMP-deaminase enzyme system, so that the nucleotides are not converted to IMP. This would partially explain the reason for finding higher levels of AMP in these three muscles in the present study than have been reported by other workers, when they used the more conventional perchloric acid extraction procedure. Jones and Murray (1961a) and Bendall and Davey (1958) both suggested that nucleotide binding is dependent upon pH and concluded that less nucleotides are bound at higher pH values.

Fredholm (1963) suggested that the level of the adenine nucleotides was more stable than previously believed. He indicated that the level of AMP remaining at 24, 72 and 120 hours was 100%, 60% and 40% for beef

and 77%, 31% and 19% for pork, respectively, when compared to the value at 0 hours. Fredholm used inophoresis to separate the nucleotides in this experiment. However, his quantitative evaluation was simply the measurement of the color intensity of the resulting spots. Since the adenine nucleotides occur at such low levels in meat, it appears likely that this method of quantitative measurement would be inadequate and as a result these values might be questioned.

Beef aging study

The aging study was designed to assess the nucleotide content of the longissimus dorsi muscle of beef during a 28 day storage period at 33-35°F. Samples were analyzed at 0, 12 and 24 hours and at 4, 7, 14 and 28 days post-mortem. The concentration of inosinic acid throughout aging is shown in table 12, while Appendix table II lists the

Table 12. Inosine 5'-monophosphate content of the longissimus dorsi muscle in beef aging study - μM./g. tissue

Animal Number	Time 0 hours	12 hours	24 hours	4 days	7 days	14 days	28 days
1	4.73	5.50	4.89	4.84	3.46	2.28	0.84
2	5.32	5.63	4.39	4.12	3.45	2.57	1.00
3	4.30	4.85	4.03	3.81	2.93	2.27	0.79
4	4.11	5.14	5.03	4.07	2.61	1.86	0.46
5	4.90	5.40	5.47	5.09	3.22	2.10	0.54
6	4.34	5.12	5.32	4.89	3.51	1.94	0.87
Mean	4.71	5.44	4.86	4.47	3.20	2.17	0.75

concentration of ATP during this same period. Figure 7 schematically shows the formation and degradation of IMP.

Inosinic acid reached its highest concentration 12 hours after slaughter (table 12). The level then began to decline so that by 24 hours post-mortem it was approximately the same as the value at 0 hours. The concentration of IMP then remained relatively stable until the 4th day, after which a gradual but steady decline was observed. At 14 days post-mortem, the IMP level was less than 50% of the value of 5.44 μM./g. found at 12 hours. At 28 days the average IMP concentration was 0.75 μM./g., or less than 15% of the maximum concentration occurring at 12 hours. The decrease in IMP content from 4 days to 28 days appeared to be almost linear in nature (figure 7).

Table 13 shows the results of the statistical analysis of the means in this study when Duncan's New Multiple Range Test was utilized (Steel and Torrie, 1961, Snedecor, 1956). The differences between means

Table 13. Statistical analysis of the inosine monophosphate concentration means of beef aging study.

	Time period						
	12 hrs.	24. hrs.	0 hrs.	4 days	7 days	14 days	28 days
Mean	5.44	4.86	4.71	4.47	3.28	2.17	0.75

Any two means not underscored by the same line are significantly different at the 1% level.
Any two means underscored by the same line are not significantly different at the 1% level.

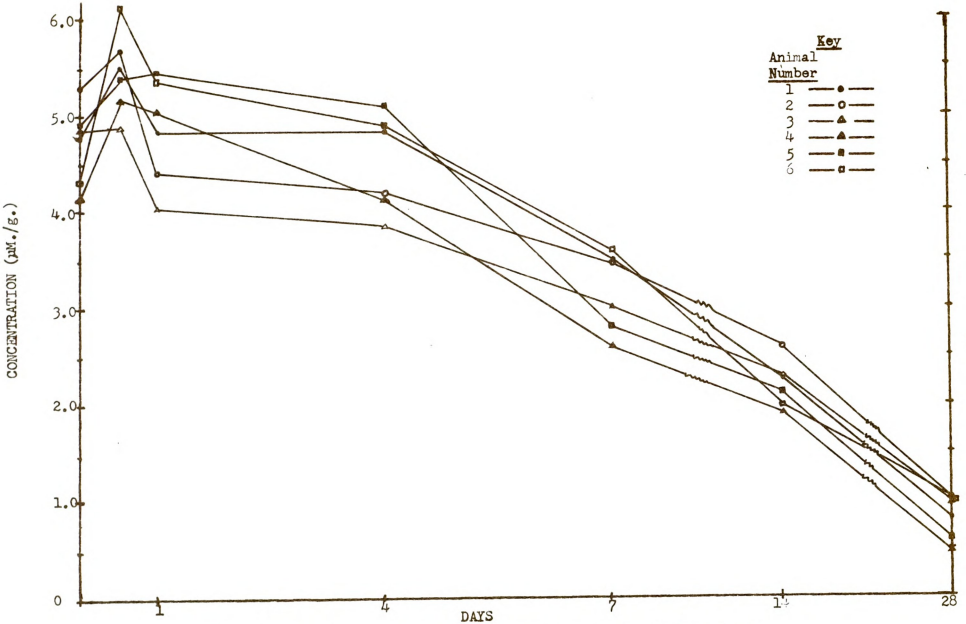


Figure 7. Level of inosine 5'-monophosphate in the longissimus dorsi muscle of beef during a 28 day storage period at 33-35° F.

were statistically significant in all cases except for values at 24 hours and 0 hour and at 0 hour and 4 days. This suggests, that except for the peak IMP concentration at 12 hours post-mortem, there is very little change in the level of IMP during the first 4 days of cold storage. The differences between means after 4 days storage were statistically significant at the 1% level, which would indicate that consistent changes in the level of IMP were taking place.

The rate of IMP degradation found in the present investigation agrees with that reported by Terasaki et al. (1965). These authors found that the peak concentration of IMP in pork was not observed until the 3rd day post-mortem and that the level was 3 mM./g. tissue. The level gradually decreased until the 10th and last day of the study, when approximately 1/3 of the inosinic acid had been decomposed. Terasaki et al. demonstrated that chicken breast muscle had a peak inosinic acid content at 8 hours post-mortem with a gradual degradation through the 8th day, when the test was concluded. Although nucleotide decomposition rates were generally the same, the actual nucleotide content could not be validly compared since different species were involved in the studies. Endo et al. (1966) demonstrated that 50% of the IMP concentration of beef and chicken was decomposed in two weeks at 0-1°C. Dvorak (1958) found that the IMP concentration in samples of bull muscle was the greatest at two days post-mortem and then fell until 15 days when it was approximately the same as after one day of cold storage.

Rhodes (1965) observed that inosinic acid was completely degraded in some beef cuts after 30 - 40 days, while the rate of decomposition occurred more slowly in other cuts of beef and in lamb. The data are in general agreement with the relatively low levels of IMP found following 28 days of cold storage in the present study.

The initial level of inosinic acid in beef in the present study appeared to be high. This could be the result of two factors. First, the samples were not taken at exactly 0 hours. Samples were, however, taken within 15 minutes of stunning and frozen immediately in liquid nitrogen. Even in this brief period of time, there could have been a rapid dephosphorylation and deamination of the adenine nucleotides. Secondly, if the animal were excited to any degree before slaughter, the ATP supply could have been partly depleted and as a result a relatively high concentration of inosinic acid would be present. This latter phenomena has been noted by numerous scientists, namely, Terasaki et al. (1965), Jones and Murray (1957, 1961a, 1961b), Fraser et al. (1965), Fujimaki and Kojo (1953) and Guardia and Dollar (1965). It is probable that the high initial level of inosinic acid could be partially overcome by sampling immediately after bleeding and by using care not to excite the animals prior to slaughtering.

Results from this experiment also indicate that it may be of value to take more samples earlier in the storage period so that the exact time of peak IMP concentration could be pinpointed. This could be accomplished by taking samples at 0, 4, 8, 12, 16, 20 and 24 hours, as well as at 36 and 48 hours. This sampling plan would comprehensively cover any critical changes in the level of inosinic acid during early storage when the greatest changes are apparently occurring.

The level of ATP was highest at 0 hour and decreased constantly until the 5th day (Appendix table III). At this time, the concentration stabilized in the neighborhood of 0.37 μM./g. tissue. As with the pork muscle study, these values again are higher than other values reported

in the literature. From knowledge of adenine nucleotide degradation, it appears logical that the level of AMP would be highest at 0 hour and then decrease thereafter as was found in the present investigation. The AMP level would likely be lower due to the activity of AMP-deaminase, unless the adenine nucleotides were protected in some manner as was previously discussed. The pattern of AMP degradation noted in this study is similar to that demonstrated by Terasaki et al. (1965), who showed that the level of AMP reached a relatively stable level of about 0.15 μM./g. after 48 hours cold storage. Jones and Murray (1957, 1961a), Dvorak (1958) and Kassemsarn et al. (1963) have also demonstrated a similar pattern of AMP degradation.

Figure 8 shows a typical separation pattern for nucleosides and bases. Table 14 gives the R_f values and identity of the nucleosides and bases from a sample of the longissimus dorsi muscle of beef. Hypo-

Table 14. Thin-layer chromatography of nucleosides and bases from a sample of the longissimus dorsi muscle of beef

Unknown R_f	Known R_f	Identity
0.74	0.73	Inosine
0.57	0.59	Hypoxanthine
0.35	0.34	Adenine
0.85	0.84	Uridine

xanthine, inosine, adenine and uridine were present in the effluent, which resulted from washing the ion-exchange column with distilled water prior to elution with the three phase gradient system. Their presence

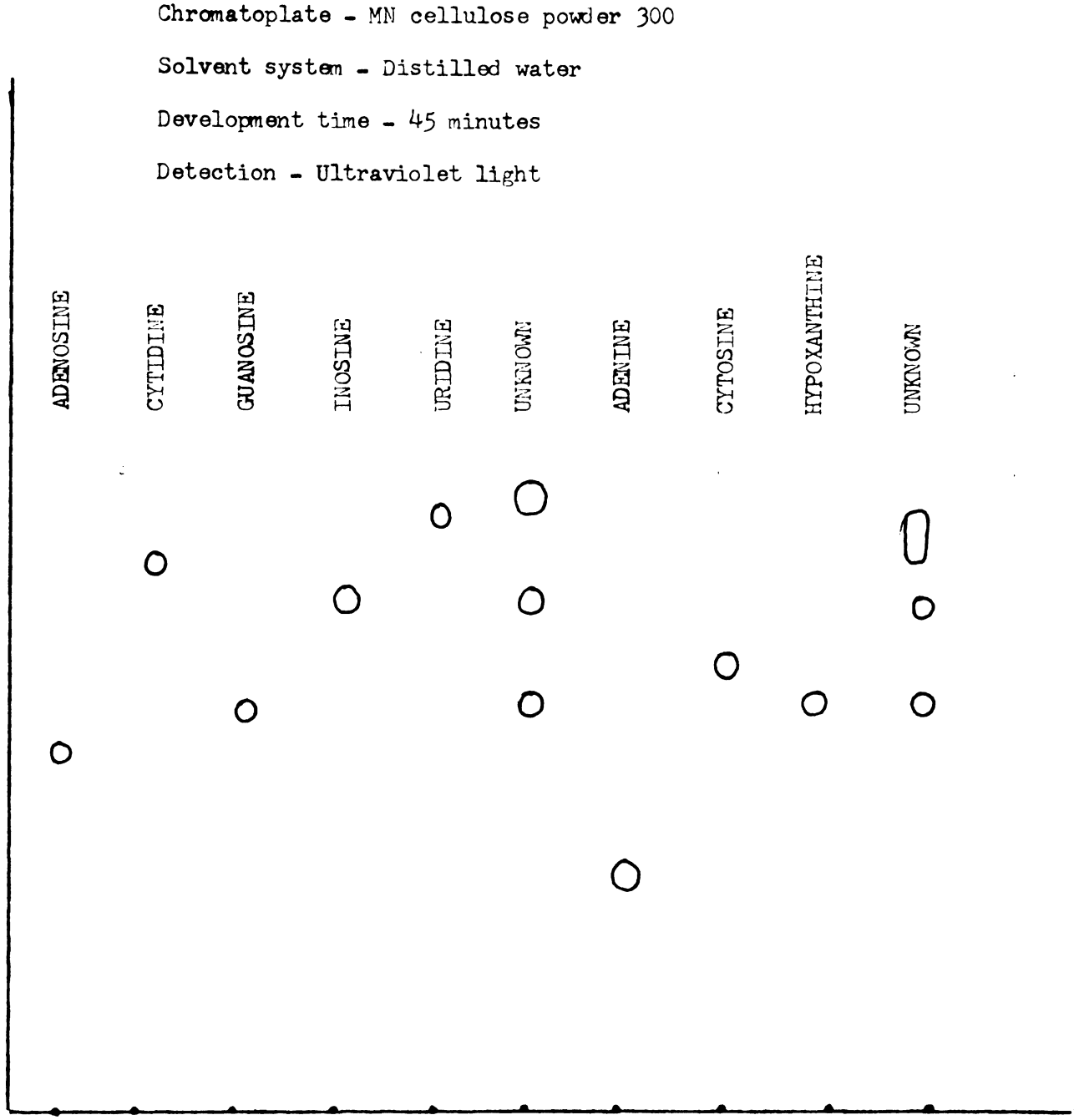


Figure 8. Thin-layer chromatogram of nucleosides and bases.

was demonstrated when thin-layer chromatography was used to separate the components in the effluent (figure 8). Inosine and hypoxanthine were present due to the degradation of inosinic acid, while adenine and uridine undoubtedly resulted from the decomposition of AMP and UMP which had previously been shown to be present in muscle (Appendix table I). Cytosine may not have been observed since CMP is present in trace amounts only. From figure 8, it can be seen that good separation of nucleosides and bases was effected by using thin-layer chromatography. The ease and simplicity of the test is evident since the solvent system consisted of distilled water and only 45 minutes were required for the development of the chromatograms, which are then air dried and read under ultraviolet light.

Although the level of hypoxanthine and inosine were not quantitatively measured in this experiment, it was readily evident that their concentration was increasing steadily as IMP was degraded during the storage period. The increase was evident as shown by the gradually increasing peak size obtained when the nucleosides and bases were scanned by the ultraviolet scanner as they left the column. There was only a very minute hump on the base line from samples taken at 0 hours. In many cases the peak was absent entirely. An optical density reading of over 0.5 was noted after 28 days storage, which corresponded to the maximum reading on the chart paper. It is evident from these observations that the accumulation of hypoxanthine from IMP degradation might be used to assess the freshness of muscle tissue. Jones and Murray (1964), Jones et al. (1964), Spinelli et al. (1964) and Saito et al. (1959) have all demonstrated that the freshness of stored fish can be effectively

measured by the level of hypoxanthine. These procedures are based on the assumption that the degradation of IMP to hypoxanthine is a linear function. This being true, one need only to measure the hypoxanthine content of fish muscle, compare this value to a known curve or insert it into a standard formula and evaluate the freshness of the product. However, Rhodes (1965) concluded that the measurement of IMP or its breakdown products appeared to offer little or no promise for measuring the freshness of red meats. There may be a greater number of problems in developing a reliable method for assessing the freshness of red meats, since the rate of IMP degradation appears to proceed at a considerably slower rate in meat than in fish muscle. Secondly, spoilage does not occur so rapidly and is more readily discernible in meat than in fish, where the method was evolved out of necessity of evaluating freshness.

Species investigation

The nucleotide content of the longissimus dorsi muscle of bull, beef, lamb, pork and of pork heart muscle were determined and compared. The inosinic acid concentration of these muscles is shown in table 15. Appendix tables III, IV, and V list the total nucleotide content for bull, lamb, and pork muscle at 0 hours and for pork heart muscle. Beef muscle at 0 hours contained the highest level (4.71 μM./g.) of IMP, while pork heart muscle contained the least (0.13 μM./g.). The concentration of IMP in other species fell between these values. Pork muscle at 0 hours, pork muscle at 48 hours, bull muscle and lamb muscle contained 4.38, 3.23, 2.84 and 2.24 μM./g. of tissue, respectively.

Table 15. Inosine 5'-monophosphate content of the longissimus dorsi muscle of bull, beef, lamb and pork and of pork heart muscle - μM./g. tissue.

	Species					
Animal Number	Bull	Beef (0 hour)	Lamb	Pork (0 hour)	Pork (48 hours)	Pork Heart (0 hour)
1	3.04	4.78	2.82	4.10	3.03	0.27
2	2.89	5.32	2.12	4.26	3.31	0.10
3	2.95	4.80	1.68	4.35	2.91	0.03
4	2.65	4.11	2.66	3.84	3.60	0.13
5	3.05	4.90	1.86	5.35	2.92	--
6	2.45	4.34	2.31	--	3.61	--
Mean	2.84	4.71	2.24	4.38	3.23	0.13

It is rather difficult to make comparisons between the species involved in this portion of the study as wide variations existed in the time of sample procurement. In addition, samples were not all treated in a similar manner. However, the samples of beef and pork taken at 0 hours should be comparable. Although beef contained 0.33 μM./g. more inosinic acid than pork, this difference was not significant, when species means were analyzed using Duncan's New Multiple Range Test (Steel and Torrie, 1961, Snedecor, 1956). Although the mean difference between samples of beef and pork at 0 hours was not statistically significant, a trend for beef carcasses to have higher levels of inosinic acid appeared to be evident (table 15).

The samples of pork taken at 0 hours contained 1.15 μM. more IMP per gram of tissue than samples removed after 48 hours of cold storage. This is over a 25% reduction in IMP content. Although this value may seem somewhat high, different pigs were being compared at the two periods and the handling procedures may have varied slightly. The concentration of inosinic acid in the pork at 0 hours differs from the results obtained by Terasaki et al. (1965), who reported the IMP level to be approximately 1.0 μM./g. tissue. The difference between values in the two studies would be explainable if the samples utilized by these workers had been removed from the animals immediately after stunning and bleeding. However, it does not seem likely that Terasaki et al. could have obtained the pork samples immediately upon death, since the pigs were stunned, bled and scalded prior to sampling. It should also be noted that the number of animals utilized was not mentioned in this paper. The 0 hour samples in the present experiment were taken within 15 minutes of stunning, and this slight delay could explain the relatively high concentrations at 0 hours. Terasaki et al. reported a level of 3.0 μM./g. tissue at 48 hours post-mortem, which corresponds quite closely to the value of 3.23 μM./g. tissue in the present experiment.

The concentration of IMP in the longissimus dorsi muscle from bull carcasses was 2.84 μM./g. of tissue (table 15). The values from the six bulls, all fell into a very narrow range, which extended from 2.45 to 3.05 μM./g. tissue. The standard deviation of these samples was 0.24 μM./g. tissue. No control could be exercised over the treatment of samples in this study since the samples were part of another investigation. The samples were taken on the 7th day post-mortem, ground and frozen. In

addition, these samples had been thawed for proximate analysis and then refrozen before being analyzed for inosinic acid concentration. The beef aging study revealed that the IMP level after 7 days was 3.20 μM./g. tissue which is comparable to the value found for the bulls. It was also found in the beef aging study, that the range between the high and low values tended to decrease as the length of the cold storage was increased. This would partially explain the narrow range of values found for the bulls. If the thawing and refreezing had any effect on the concentration, it would be likely to cause a further degradation as well as to further decrease the range of values. This would agree with Guardia and Dollar (1965), who stated that differences in IMP content were evident between exercised and unexercised fish, but that this difference became less as the length of cold storage increased, until no significant differences were observed after 7 days of post-mortem storage. The levels of IMP found in the bull samples were comparable to values for beef bulls reported by Dvorak (1958). He reported that the concentration of inosinic acid after two days storage was 3.7 μM./g. tissue and that it had decreased to 1.9 μM./g. tissue after 10 days, when the samples were aged at 5°C.

Nakajima et al. (1961b) reported the IMP content of beef to be 3.07 μM./g. and the value for pork to be 3.51 μM./g. He also reported the combined level of inosine-hypoxanthine to be 2.90 μM./g. tissue, which would indicate considerable IMP degradation. This suggests that the meat samples were fairly old before analysis, although he did not report the length of aging or sample source.

Terasaki et al. (1965) reported the inosinic acid content of mutton (9 month old ewes) to be 2.36 μM./g. tissue. This corresponds closely to the value of 2.24 μM./g. tissue found in this experiment (table 15). The values reported by Terasaki et al. for mutton are difficult to interpret, as the meat had been imported from Australia in the frozen state. As a consequence, nothing was known about the post-mortem treatment received by the samples. In addition, the length of the aging period and the freezing temperature at which the carcasses were held during shipment from Australia were not known. If the freezing temperature was maintained at -30°C. or lower IMP degradation was probably negligible, while at higher temperatures some IMP decomposition probably would have occurred. Jones and Murray (1961b) reported that IMP was stable in cod fish muscle when stored at -30°C. The authors measured the level of inosine accumulation as a criterion of IMP degradation during cold storage. At -30°C. no inosine was detectable, while at -14°C. the inosine content of cod fish muscle was 3.01 μM./g. tissue following 62 weeks of storage. This corresponds to an inosine content of 3.23 μM./g. tissue for lamb muscle as reported by Terasaki et al. (1965). This value would indicate that considerable IMP had been degraded in the lamb samples prior to being utilized for their investigation.

The lambs in the present experiment had been used for classroom carcass evaluation. As a result, the lambs were not held at a constant temperature during the seven day holding period prior to the time that the samples were removed. Some of the variation in IMP content can probably be explained by the fact that part of the lambs were held continually at low temperatures and part were held at varying periods of time at cold and warm temperatures.

Dvorak (1958) reported that the rate of IMP decomposition varied with storage temperature. He demonstrated that the rate of nucleotide degradation was more rapid in bull carcasses held at 38°C. than when aged at 5°C. Although, the temperature extremes in the present study were not so severe, variation in the holding temperature may suggest reasons for such differences.

The concentration of inosine 5'-monophosphate in heart muscle was 0.13 μM./g. tissue (table 15). This value is the average determination of four heart muscles and would have been approximately 25% lower except for the 0.27 μM./g. tissue found in one heart. The comparatively low level of IMP found in this study is in agreement with the results of Visioli et al. (1964) and Imai et al. (1964). Visioli et al. demonstrated that little IMP was produced in the myocardium of severely exercised rats, while Imai et al. noted that IMP was absent in heart tissue from anemic rabbits. It is also interesting that many invertebrate sea animals do not possess IMP. Saito and Arai (1957a) reported that squid contain no IMP, while Fujita and Hasimoto (1960), Arai (1960a, 1960b) and Arai et al. (1961) and Nakajima (1961a, 1962b) verified the fact that invertebrate sea animals contain little or no inosinic acid.

The AMP content in the pork heart muscle samples was 2.29 μM./g. tissue (table 15). The high concentration of AMP may indicate that the enzyme, AMP-deaminase, is absent in heart muscle. Suelter (Unpublished data) has found that the enzyme AMP-deaminase is present in heart muscle. However, the enzyme is not normally active since ATP is required to activate the system. This would explain the reason for the high AMP content and the low concentration of IMP in heart muscle. Davey (1961)

was able to demonstrate that dialyzed extracts of skeletal muscle were capable of synthesizing adenylosuccinic acid by the condensation of inosine monophosphate and aspartic acid, from which it is converted to adenine nucleotides. This enzyme was shown to be absent in heart, lung and kidney tissue. It is interesting to speculate that the absence of this amination enzyme may be the reason why deaminase is not present in heart muscle. For example, if AMP was deaminated to IMP during periods of severe exercise, there would be no mechanism present for the reamination of IMP. It has not been demonstrated whether the deamination-reamination reaction is involved in the cycle of chemical events associated with muscular activity, although skeletal muscle contains both enzymes.

Guanosine monophosphate was not detectable in the present series of samples, while CMP was present in only trace amounts as shown in Appendix tables III, IV and V. Since these compounds are present in such small quantities, more accurate measurement of their concentrations could be made by adding larger samples to the ion-exchange columns. The level of UMP was trace amounts, 0.06 and 0.02 μM./g. of tissue, respectively, for pork sampled at 0 hours and for bull and lamb carcasses sampled at 7 days post-mortem (Appendix tables III, IV and V). The concentration of adenylic acid for these samples was 0.70, 0.76 and 1.21 μM./g. of tissue, respectively.

Sensory evaluation

The final portion of this experiment was designed to assess the flavor enhancing properties of the flavor potentiators. It was shown earlier in this study that the level of inosinic acid in pork heart muscle was very

low. Thus, a frankfurter formulation using pork heart in place of lean beef or pork was utilized. Five different recipes were prepared. The first was a control and contained heart muscle only, the second contained heart muscle plus 0.24% MSG, while the third recipe consisted of heart muscle plus 0.05% inosinic acid. The fourth formulation consisted of heart muscle and 0.025% Mertase (IMP + GMP, 1:1 ratio). A fifth formula was made with beef muscle in order to give a positive control. Evaluation of the frankfurters was made by using a 9 point hedonic scale, where 9 was the highest score and 1 the lowest. The panel members were instructed to rate each sample for flavor only.

Table 16 shows the average values for the 20 member taste panel, while Appendix table VI summarizes the results for each panel member. The average values from the hedonic scale preference ratings indicate

Table 16. Results[1] of taste panel evaluation of added flavor potentiators.

	Formulation				
	Pork Heart				Beef
	1 Control	2 MSG	3 IMP	4 IMP + GMP	5 Control
Mean	$5.35^{b,c}$	$5.55^{a,b,c}$	$6.20^{a,b}$	6.40^{a}	4.75^{c}

[1] Any means with the same superscript are not significantly different from each other at the 5% level.

that the panel preferred the sample that contained IMP + GMP. The value of 6.40 is midway between "like moderately" and "like slightly" on the hedonic scale. The other values were 6.20 for the sample with added IMP, 5.55 when MSG was added to heart muscle, 5.35 when pork heart was used

as the meat source with no added potentiator and 4.75 for beef muscle (table 16). The value for beef falls below the median on the hedonic scale and is between "dislike slightly" and "neither like nor dislike." Analysis of variance (Appendix table VII) indicated that the average values obtained from taste panel evaluation of frankfurters were significantly different at the 1% level of probability.

Since analysis of variance indicated that significant differences existed, Duncan's New Multiple Range Test (Steel and Torrie, 1961, Snedecor, 1956) was utilized to determine the significance between means. The analysis revealed that the addition of Mertase (IMP + GMP) to pork heart frankfurters significantly increased the acceptability of this product over the beef frankfurters at the 1% level and over the all pork heart frankfurters with no added flavor enhancer at the 5% level of significance. When IMP was added to pork heart frankfurters, they were significantly preferred over beef frankfurters at the 1% level. The differences between the other means in this study were not significant at either the 1% or 5% level of probability. Although the data are rather limited, results suggest that the addition of IMP + GMP or IMP to pork heart frankfurters significantly increased the acceptability of these products. In addition, the frankfurters containing IMP or IMP + GMP generally tended to be more acceptable to the panel members than the formulation containing heart muscle alone or the one with added MSG (table 16).

Several problems were encountered in this portion of the experiment. First of all the stability of the heart muscle emulsion was not very good. These samples required stuffing and processing in a relatively short time

so that the emulsion did not break. Even so, some fat caps were observed in this study. Secondly, the peelability of the frankfurters made with pork heart was poor and as a result removal of the casings was difficult. Third, the texture of the pork heart frankfurters was poor, being soggy and rather coarse. It was noted, however, that the addition of any of the three flavor potentiators, and especially MSG, tended to produce a firmer product. The beef, which was incorporated into the frankfurters, had been frozen for approximately one year. This may partially explain the poor acceptability of the beef frankfurters. A second factor, which may have detracted from the beef frankfurters, was the relatively high fat content. A preliminary study had demonstrated that due to the high moisture content of heart muscle some additional fat in the recipe was needed to make a firmer product. The increased fat content was not so noticeable in the frankfurters containing heart, and as a result the panel may have been influenced.

It should be pointed out that this was only a preliminary test and that only one level of the flavor enhancer was used. In additional testing, various levels of the flavor potentiators should be added and their effect evaluated in order to define optimum levels. The flavor enhancing compounds could also be added to frankfurter formulations where red meats are being utilized. In actuality, the latter test may have more commercial application since the amount of available heart muscle is limited.

SUMMARY AND CONCLUSIONS

Known and unknown nucleotide samples were separated on Dowex 1 X 8, 200-400 mesh, (formate) ion-exchange resin columns and quantitatively measured utilizing a periodic acid - 2,4-DNPH color reaction. Recovery rates and repeatability were excellent. Qualitative evaluation of the eluted nucleotide peaks was accomplished by using thin-layer and paper chromatography and by measuring absorbance spectras and the ratio of absorbance at 250/260 and 280/260 mμ. Of the three methods utilized, thin-layer chromatography was the simplest and fastest to use, and in addition gave excellent results.

The longissimus dorsi, biceps femoris and semimembranosus muscles from six pigs were analyzed for their nucleotide content. The concentration of inosine monophosphate in the three muscles was 3.23, 3.12 and 2.98 μM./g. of tissue, respectively, which was not statistically significant. The standard deviation was 0.33, 0.42 and 0.40 μM./g. of tissue, respectively, for the longissimus dorsi, biceps femoris and semimembranosus muscles. Since the three muscles were similar in their nucleotide content and the longissimus dorsi had the smallest standard deviation, it was used in all subsequent studies. It was also easily accessible and less damage was done to the carcass on removing the sample.

The level of AMP in pork was 0.33, 0.90 and 0.33 μM./g. of tissue for the longissimus dorsi, biceps femoris and semimembranosus muscles, respectively. The level of UMP was 0.13, 0.11 and 0.20 μM./g. of tissue for the same three muscles, respectively, while CMP was present in traces only and GMP was not detectable. The fact that GMP and CMP were not present in measurable levels suggests that larger samples should be added

to the ion-exchange column for separation.

The second part of this study was designed to determine the level of IMP and AMP from six beef carcasses stored at 33 - 35°F. for 28 days. Samples were removed and analyzed at 0, 12 and 24 hours and at 4, 7, 14 and 28 days post-mortem. The peak concentration of IMP occurred at 12 hours. The level then declined until at 24 hours the IMP content approximated that at 0 hours. It remained fairly constant until the 4th day, after which a linear decrease occurred until the 28th day of post-mortem storage, when less than 15% of the IMP remained. The concentration of AMP was highest at 0 hours and was followed by a steady decline until the 4th day, after which it remained relatively constant.

Phase three of the present experiment was conducted to evaluate the nucleotide content of various species. The longissimus dorsi muscle removed from bull and lamb carcasses after 7 days post-mortem storage was compared with pork sampled at 0 and 48 hours, beef at 0 hours and pork heart muscle at 0 hours. The concentration of IMP for these samples was 2.84, 2.24, 4.38, 3.23, 4.71 and 0.13 μM./g. of tissue, respectively. Results were difficult to compare since a wide range of sampling times and handling procedures were involved. The level of AMP in the above samples was 0.76, 1.21, 0.70, 0.33, 0.90 and 2.30 μM./g. of tissue, respectively. Low levels of CMP and UMP were observed while GMP was not detectable.

In order to assess the flavor enhancing properties of IMP, IMP + GMP and MSG, these potentiators were added to a standard frankfurter formulation which contained pork heart muscle as a meat source. A 20 member taste panel evaluated the frankfurters on the 9 point hedonic

scale. Scores of 6.40, 6.20, 5.55, 5.35 and 4.75 were obtained for recipes containing 0.025% IMP + GMP, 0.05% IMP, 0.24% MSG, heart muscle alone and all lean beef (control), respectively. Statistical analysis revealed that the addition of IMP + GMP to pork heart frankfurters increased their acceptability over the beef frankfurters ($P < 0.01$) and over the frankfurters containing heart muscle with no added potentiator ($P < 0.05$). On addition of IMP to pork heart frankfurters, they were preferred over the beef frankfurters ($P < 0.01$). All other differences were not statistically significant. Thus, results suggested that the flavor potentiators improved the acceptability of the frankfurters containing pork hearts.

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APPENDIX

Table I. Nucleotide content of 3 pork muscles (48 hours post-mortem) - μM./g. tissue

Animal Number	CMP	AMP	UMP	IMP
		Biceps femoris		
1	Trace	0.30	0.06	3.40
2	Trace	0.68	0.27	3.59
3	Trace	0.96	0.13	3.45
4	Trace	2.09	0.23	2.65
5	Trace	0.41	0.28	2.99
6	Trace	0.96	0.21	2.65
Mean		0.90	0.20	3.12
		Longissimus dorsi		
1	Trace	0.34	0.09	3.03
2	Trace	0.37	0.12	3.31
3	Trace	0.45	0.18	2.91
4	Trace	0.23	0.13	3.60
5	Trace	0.35	0.13	2.92
6	Trace	0.26	0.15	3.61
Mean		0.33	0.13	3.23
		Semimembranosus		
1	Trace	0.43	0.12	2.59
2	Trace	0.22	0.12	3.18
3	Trace	0.55	0.12	3.58
4	Trace	0.47	0.08	2.54
5	Trace	0.13	0.13	2.81
6	Trace	0.19	0.10	3.17
Mean		0.33	0.11	2.98

Table II. Adenosine 5'-monophosphate content of the longissimus dorsi muscle in aging study - μM./g. tissue

	Time						
Animal Number	0 hours	12 hours	24 hours	4 days	7 days	14 days	28 days
1	0.85	0.42	0.32	0.22	0.65	0.31	0.23
2	1.00	0.63	0.30	0.39	0.35	0.45	0.26
3	0.62	0.59	0.73	0.61	0.61	0.42	0.32
4	1.04	1.15	0.71	0.32	0.22	0.28	0.31
5	1.14	0.63	0.52	0.36	0.24	0.47	0.23
6	1.02	0.82	0.36	0.28	0.50	0.41	0.45
Mean	0.94	0.71	0.49	0.36	0.43	0.39	0.30

Table III. Nucleotide content of the longissimus dorsi muscle of bulls - μM./g. tissue

Animal Number	CrP	ATP	UTP	ITP
1	Trace	1.46	0.06	3.04
2	Trace	0.51	0.08	2.89
3	Trace	0.86	0.05	2.95
4	Trace	0.39	0.08	2.65
5	Trace	0.47	0.06	3.05
6	Trace	0.89	Trace	2.45
Mean		0.76	0.06	2.84

Table IV. Nucleotide content of the longissimus dorsi muscles of lambs - μM./g. tissue

Animal Number	CMP	AMP	UMP	IMP
1	Trace	1.41	0.03	2.82
2	Trace	0.35	0.03	2.12
3	Trace	0.98	0.05	1.68
4	Trace	2.01	Trace	2.66
5	Trace	0.61	Trace	1.86
6	Trace	1.90	0.02	2.31
Mean		1.21		2.24

Table V. Nucleotide content of the longissimus dorsi muscle of pork (0 hour) and of pork heart - μM./g. tissue

Animal	Longissimus dorsi				Heart	
Number	CMP	AMP	UMP	IMP	AMP	IMP
1	Trace	0.70	Trace	4.10	2.52	0.27
2	Trace	0.86	Trace	4.26	2.60	0.10
3	Trace	0.70	Trace	4.35	1.75	0.03
4	Trace	0.44	Trace	3.84	2.34	0.13
5	Trace	0.82	Trace	5.35	--	--
Mean		0.70		4.38	2.30	0.13

Table VI. Results of individual tasters from taste panel evaluation of flavor potentiators - (9 = highest value)

Taster Number	Formulation: Heart 1	MSG 2	IMP 3	IMP + GMP 4	Beef 5
1	3	7	7	6	4
2	3	5	4	4	5
3	4	4	4	7	4
4	6	5	7	6	4
5	6	7	7	8	6
6	8	7	7	8	6
7	5	7	7	6	5
8	7	3	4	7	6
9	6	7	5	6	2
10	5	6	7	8	4
11	4	4	7	6	2
12	4	4	6	6	8
13	7	6	6	7	5
14	5	4	4	5	4
15	6	6	6	6	5
16	5	7	7	6	4
17	5	6	6	7	5
18	6	6	8	7	5
19	7	6	8	6	6
20	5	4	7	6	5
Mean	5.35	5.55	6.20	6.40	4.75
S.D.	1.35	1.31	1.32	0.98	1.37

Table VII. Analysis of variance of panel evaluation

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Treatment	4	36.0	9.0	5.52**
Error	95	154.75	1.63	
Total	99	190.75		

** Significant at 1% level of probability