1992

Rapid method for detection of muramic acid and cadaverine as indicators of microbial load on fresh meats

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Iowa State University, 1992

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Rapid method for detection of muramic acid and cadaverine as indicators of microbial load on fresh meats

by

Carlos Ivan Lebron

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Food Science and Human Nutrition
Major: Food Science and Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1992

DEDICATION

This work is dedicated to my parents, Guy and Norma and to my wife Lourdes, all who have supported me throughout my 10 years here in Ames, especially when the times were trying, and money short.

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INTRODUCTION

Meat is an excellent medium to sustain the growth of microorganisms. Given the right conditions, bacteria, yeast and molds will utilize the chemical components of meat to carry out their metabolisms and growth. The net result of that activity is, in most cases, meat spoilage.

The predominant flora of freshly slaughtered carcasses is mesophilic in nature and cannot grow at temperatures used for carcass chilling and holding. During refrigerated holding, the carcass flora begins to shift towards psychrotrophs of the *Pseudomonas-Acinetobacter-Moraxella* group. Growth of this group of organisms eventually spoils the meat. Usually, carcasses are cut into smaller portions in refrigerated work rooms. Since the majority of bacteria on processing equipment in refrigerated rooms is psychrotrophic in nature, this further assures the presence of these bacteria on the meat surfaces.

Spoilage by psychrotrophic bacteria is not hazardous to human health. It is rather a problem of aesthetics, product quality and economics. The shelf life of raw chilled meat and poultry is prolonged by those factors affecting the growth rate of the psychrotrophs: dry surface, low initial level of psychrotrophs, the pH of the meat, oxygen limitation and temperature.

In the meat industry there is a need for more rapid and better methods for the analysis and enumeration of bacterial in fresh meats. Classical plate counting methods many times understate the number of microorganisms when compared to counts made by direct microscopic examination. The purpose of this dissertation is to investigate the possibility of developing a rapid method to estimate total bacterial contamination on fresh pork, beef, and poultry based on bacterial cell wall components or by the analysis of a bacterial metabolite.

Explanation of Dissertation Format

This dissertation is arranged in two parts dealing with 1) the possible correlation between muramic acid and total bacterial numbers in meats, and 2) the possible correlation between cadaverine and total bacterial numbers in meats. The two parts are preceded by a literature review. Each part has an introduction, materials and methods and results and discussion section. All figures and tables are numbered independently in each part. Part 2 is arranged by experiments with materials and methods followed by results and discussion of each experiment. The reason for doing this is because each experiment is dependent on the previous one. All the literature cited was combined in a separate bibliography at the end. An overall summary and future work for additional research are in the last section.

LITERATURE REVIEW

The microbiology of meat and meat products is most often investigated in order to determine potential safety and keeping quality. Traditionally, the methods used involve counting and identifying the microbes present. This has been the basis of numerous studies on safety and quality which have influenced practical aspects of meat microbiology, including the development of criteria (guidelines, specifications and standards) for the manufacture and marketing of meat products. Generally, analyses have provided taxonomic descriptions of the components of the microbial populations present (e.g., enterobacteria, pseudomonads or staphylococci) rather than functional descriptions based on their physiological attributes which affect product properties. These attributes can include those products of metabolism that can affect flavor, color, pH or structure, or be toxic to the consumer. Since the enumeration of food-borne disease-causing bacteria is in most cases directly related to potential food-poisoning risk, the relation between microbial counts and spoilage is less well defined and it is important to recognize the imprecise nature of the traditional quality indicators when considering quality loss in meat products.

The characteristic microbial populations developing in meat products are the result of the effects of the prevailing

environmental conditions on growth of the types of microbes initially present in the raw materials or introduced by cross-contamination or processing. The types and range of chemicals, especially degradation products, formed by microbes and by meat enzymes, under particular storage conditions, will often lead to the development of undesirable flavors and texture but always will be ultimately associated with changes that the consumer recognizes as spoilage (Alford et al., 1971; Freeman et al., 1976; Ford and Park, 1980).

Factors affecting microbial growth in a food include both intrinsic and extrinsic factors (Mossel and Ingram, 1955). Intrinsic factors are predominantly chemical, including the concentration and availability of nutrients (which are often not replaced as they are metabolized), pH, redox potential, buffering capacity and the availability of water (water activity or a_w). The structure of meat and meat products is also an intrinsic property which affects diffusion through them and their thermal, mechanical and sorption properties. Although very little is known of the way many commercial meat handling processes, which modify these properties, influence microbial growth an exception is the effect of mincing which leads to increased surface area and nutrient availability, hence more rapid microbial growth (Ayres, 1955). The extrinsic factors are concerned mainly with storage and processing conditions and include storage temperature and the composition

and relative humidity of the gaseous atmosphere surrounding the meat. An extrinsic factor which is an important naturally occuring selective agent is carbon dioxide which is produced in the largest quantities during the growth of aerobic bacteria, and may be trapped by the gas-impermeable films used, in vacuum packaging. How quickly a piece of meat equilibrates with the extrinsic factors, such as temperature change or atmospheric modification, will be governed by its intrinsic structural properties. When meat is not at equilibrium with its surroundings, gradients of gas concentration, a_w and temperature may be formed within it, and these gradients may be responsible for differences between the types of bacteria growing at and beneath the product surface.

Microbes interact with each other and their environment and at the simplest level each species will increase its biomass at a certain rate which is a function of the physical and chemical environment. Usually characteristic microbes are associated with meat products having particular intrinsic properties, but their growth, death or survival is not governed only by these factors. Among additional factors are the direct or indirect effects of microbial competition and any accompanying changes in the chemical environment will result in microbial succession, so that in meat products stored for any length of time the most numerous species may change from time to time. When a substrate is growth-limiting, microbes can grow independently of each other

and eventually they will be inhibited by their own waste products, e.g., lactic acid or carbon dioxide, or by depletion of limiting substrate such as glucose or an amino acid, but where there is competition for a growth-limiting substrate such as oxygen or glucose, interspecies actions occur. It has been suggested that in chilled fresh meats, competition for various substrates determine the spoilage flora developing (Newton and Gill, 1980). Which species become dominant will be determined by their relative initial levels, relative affinity for the substrate, substrate availability, the relative growth rates of the competing species and the production of anti-microbial substrates (Wilkins, 1973).

Another type of interaction occurs when the metabolic product of one species acts as the growth-limiting substrate for another and a succession may be formed. In meat products, this may occur when one species enables another to develop by initiating the breakdown of otherwise unavailable substrate. Synergism may occur if one species alters the pH or redox and allows the growth of another species. Conversely, there is antagonism when the metabolic product of one species, or the environmental changes it causes limit the growth of another species. Microbial antagonisms are exploited by manipulating environmental conditions to achieve controlled competition and favor the growth of particular types, especially lactic acid bacteria, so that a succession of predictable sequence and composition is

produced. The technique is often used in the manufacture of fermented products such as salamis and its use has been further refined by the use of specific starter cultures to produce desirable flavor changes. The dominance of lactobacilli as spoilage organisms in many processed and fresh meats suggests that antagonism is of great importance in microbial selection in meat products. Some examples of limiting factors in action may be commonly seen in the meat industry. Lactic acid bacteria eventually inhibit their own growth by the consumption of carbohydrates and the production of lactic acid. The growth of pathogens, such as staphylococci, is thought to be inhibited in vacuum packaged bacon or mechanically recovered meat by the growth of the normal spoilage flora. On whole carcass meat, growth of aerobic spoilage bacteria (*Pseudomonads-Acinetobacter-Moraxella*) can be inhibited by lactic acid bacteria (Dubois et al., 1979).

Meat microbiology, like other branches of food microbiology, is concerned with the microorganisms found on the product and their effect on quality. The nature and level of microbial contamination has important consequences in relation to public health, storage life and the type of spoilage likely to develop. In the competitive markets of modern society, carcass meat is no longer a staple food to be purchased from the local butcher who personally selects and slaughters animals to suit his particular

trade. Changes in eating patterns coupled with technological developments in food processing and preservation have given consumers a much greater choice over the foods they can buy. Consequently meat eaters have become more selective, more critical and conscious of quality, particularly freshness, wholesomeness and palatability. At the same time, meat products may spend many months in storage and distribution before competing with locally produced fresh product.

The microbiology of carcass meats is highly dependent on the conditions under which animals are grown, slaughtered and processed. Three important factors determine the microbiological quality of meat. These are the condition of the animal at slaughter; the spread of contamination during slaughter and processing; and the temperature, time and other conditions of storage and distribution. The prevalence of particular animal diseases may vary from country to country but differences in contamination are likely to be more in degree than in kind. While the primitive slaughterhouse in some developing country may bear little resemblance to the complex facilities of a large export meat works in a meat producing country like United States, the basic principles determining contamination and microbial growth apply whether the daily kill is 1 or 20,000 animals.

Slaughter and dressing

In the normal, healthy animal most of the tissues which ultimately become meat or meat products, including muscle, fat and various edible organs like heart, liver, kidney, and brain, are sterile. A few products such as tongue and tripe naturally carry microbial contamination on the surface. During slaughter and processing all potentially edible tissues are subject to contamination from a variety of sources within and outside the animal (Ayres, 1955). Fresh meat presents an environment which is ideal for the growth of many microbes; thus contamination can easily result in spoilage or hazard to the health of the consumer. Meat hygiene aims to reduce contamination and prevent the proliferation of harmful organisms.

Knowledge of the sources of infection, means of spread and control of microbial growth is essential for the effective implementation of hygienic measures. Without identification of sensitive materials, critical process points and relevant human factors, much effort and expense may be used without resulting in a more hygienic product.

Pathogens of concern

Meat is frequently implicated in the spread of food-borne disease. The virtual elimination in many parts of the world of serious epidemic disease like typhoid, cholera and smallpox has

directed attention to food-borne diseases. The widespread distribution of meat products in national and international trade has made the consequences of disease spread through consumption of meat more serious. This has stimulated demands by consumer groups for microbiological standards for meat products at retail level.

In considering the microbiology of meats, it is necessary to distinguish between animal diseases such as anthrax, brucellosis, cysticercosis, trichinosis and tuberculosis, which can be detected by ante-morten or post-mortem inspection, and infections causing food poisoning in humans, which may be present in symptomless carrier animals or reach the product from other sources. Ante-mortem inspection cannot detect all animals which could constitute a hazard to health as certain infections and other conditions are not clinically apparent. These include acute diseases in the very early stages, localized and chronic diseases such as liver abscesses, and the symptomless carrier state in animals which have recovered from disease but remain excretors of the causative organisms. The presence of an unknown proportion of symptomless carriers of food-borne disease among apparently healthy stock presented for slaughter is probably the most serious problem in meat hygiene. Stress during transport may cause a breakdown of the carrier state producing overt disease with a greatly increase excretion of the organism. The close contact

between animals in the holding area then facilitates the spread of the infection. However, because the extent of the problem is unknown, precautionary measures may, at times be excessive involving additional costs and unnecessary condemnation of an otherwise satisfactory product.

Salmonella is one of the major concerns. Food-poisoning outbreaks caused by salmonellae in meat and meat products are common in many countries. *Salmonella* infection is often spread among animals through the use of contaminated foodstuffs and the incidence tends to be highest where intensive stock raising is practiced but the disease may also occurs in animals raised on open pasture. *Salmonella* serotypes of concern in relation to food poisoning may not be those which have the greatest effect on the animal (Nottingham et al., 1972). Serotypes that are carried by the animal without producing obvious symptoms present more difficulty in the maintenance of hygiene than those with greater virulence, as the meat processor has no indication of their presence. Any drug treatment or immunization program that reduces the disease symptoms without affecting the growth of the organism responsible may increase the risk of spreading food poisoning. Where the infection is present the proportion of infected animals generally increases markedly by the time the animals are slaughtered through spread of infection during transportation and

holding at the slaughterhouse (Nottingham and Urselmann, 1961; Robinson, 1966).

Other pathogens which may be present but remain undetected in slaughter animals include Clostridia, Staphylococci, Streptococci, Leptospira, Corynebacteria, *Yersinia enterocolitica* and *Toxoplasma gondii. Clostridium perfringens* is common in animal feces and small numbers of this organism are frequently found on meat (Smart et al., 1979). *Clostridium botulinum* does not appear to be very common in animals although outbreaks of botulinum have been associated with the consumption of meat products. A 1976 report (Robert and Smart) suggests that, with better methods, isolation of *Cl. botulinum* may increase and this organism may at times be relatively common in pigs. Meat animals are subject to a number of clostridial diseases and these bacteria may be present in carcasses passed for human consumption but it is not clear whether those infections have any significance in respect to foodborne disease. Food poisoning Staphylococci are widely distributed and meat can become contaminated from animal sources as well as human. It has been reported that the proportion of enterotoxin producers is lower among Staphylococci isolates of animal origin than among those from humans (Hajek and Marsalek, 1973; Sinell et al., 1975) but more recent work suggests that this may not always be the case (Nottingham et al., 1972). Bruising has been regarded as a likely focus of

staphylococcal infection (Roskey and Hamdy, 1972) and it has been suggested than in commercial practice bruised tissue is more heavily contaminated than undamaged tissue (Petersen, 1978). However, other studies have failed to detect any difference in the microbial quality of bruised and unbruised tissue (Gill and Penney, 1979). Outbreaks of skin sepsis caused by group A *Streptococci* among meat workers has been reported (Fraser et al., 1977) but the significance of this in relation to the consumption of meat is unclear. The association of group D *Streptococci* with food poisoning has not been confirmed but these organisms are sometimes used as indicators of fecal pollution.

With meat processed under hygienic conditions the number of pathogens coming from sources outside the animal are usually very small and the microflora consist of mainly saprophytic species. The skin or hide of the particular animal and others being dressed in its close proximity is probably the major source (Ayres, 1955; Fournand et al.,1978; Grau, 1979). Much of this contamination is originally of fecal origin but it will include the normal flora of the skin, *Staphylococci*, micrococci, *Pseudomonads*, yeast and molds as well as organisms from soil and water. Many of these organisms are psychrotrophs, able to grow at low temperatures and are potential spoilers of chilled meat (Newton et al., 1978; Patterson and Gibbs, 1978).

The extent to which contamination occurs and the composition of the flora that results reflects the standard of hygiene in the slaughterhouse. In some parts of the world, animals are killed and dressed under most unhygienic conditions (Mann, 1960). Although contamination is high, generally little harm results as the meat is consumed without much delay so that the organisms do not have sufficient time to grow to infectious levels or to produce toxins. Animals may be killed singly on a farm under relatively primitive conditions yet receive very little contamination because of the skill of the slaughterman and the reduced opportunity for cross-contamination. Commercial slaughter procedures have developed from one-man, one-animal operation to a labor-intensive chain system in which every carcass is handled many times. Unfortunately, this increased handling may nullify any benefits in reduced contamination that might be anticipated from the chain to a more modern system (Nottingham et al.,1974). It is difficult to skin carcasses without spreading contamination through direct contact between dirty and clean surfaces, or by airborne particles and aerosols. With large-scale operations there is great pressure to maintain maximum throughput, and consequently cross-contamination becomes a major problem. Improved mechanical systems for skinning sheep and cattle may help in this regard. It must be noted that increased inspection for disease in itself increases handling and the opportunity for spread of

infection. It is very difficult to obtain clean meat from dirty animals; thus the state of the animal at slaughter is important. The cleanliness of livestock depends on a number of factors including location of the farm, method of transport and holding conditions at the slaughterhouse. Cattle from feed lots may carry more fecal bacteria and fewer soil organisms than those from pastures. An increase of fecal contamination could increase the risk of pathogenic contamination (Linton et al., 1977). The nature and amount of fecal contamination is affected by dietary and other factors (Grau and Smith, 1974). Contamination of animals from dry dusty areas will differ from that of livestock grazed on pasture under wet and muddy conditions. Feces from animals grazing on lush pastures are likely to be semi-fluid making stock particularly prone to gross contamination during transportation. Organisms from the soil are more likely to be able to grow at low temperatures and thus spoil meat. The prevailing temperature of soils has some effect on the proportions of psychrotrophic organisms in contamination from this source. Tropical soils contain fewer cold-tolerant bacteria than soils from temperate zones and the organisms on the skin of cattle and meat appears to follow a similar trend (Empey and Scott, 1939).

The gastro-intestinal and respiratory tracts, urine and milk are other important animal sources of infection. With care, evisceration can be carried out without contaminating the carcass

(Nottingham et al., 1974; Grau, 1979) but if the intestinal tract is burst or punctured during evisceration the carcass can become highly contaminated. Generally *Escherichia coli* comprises a greater proportion of the total aerobic flora of the intestine than of the hide or hair. Thus the ratio of *E. coli* to total aerobic count can be used as an indicator of whether the major source of carcass contamination is the intestinal tract or the hide. Milk from the udder of lactating animals is likely to contain a variety of bacteria including micrococci, bacilli and organisms causing mastitis, so care must be taken when skinning to avoid spilling milk on the carcass.

Other sources of contamination include the hands, knives and clothing of workers, processing equipment such as saws, boning tables and conveyors, and the water used to wash the carcasses, hands and equipment (Ayres, 1955). Some microorganisms of human origin may reach the meat in this way but if a reasonable standard of hygiene is maintained, contamination from these sources is likely to be negligible compared with that from the animal itself.

Under good hygienic conditions, beef carcasses are likely to have surface aerobic bacterial counts of 10^2 to $10^4/cm^2$ (Notingham et al., 1974). Aerobic counts at 25°C tend to be 50% higher than those at 37°C indicating that a sizeable proportion of the microbial flora is psychrotrophic. Meat prepared under less

hygienic conditions can be expected to have higher microbial counts than those reported above (El Mossalami and Wassef, 1971; Kotula et al.,1975) and the composition of the flora may differ. Sheep carcasses usually have higher level of contamination than beef with bacterial counts of 10^2 to 10^5/cm2 (Nottingham, 1979). Coliforms also tend to be more numerous on mutton than on beef. The level of contamination is usually lower in the body cavity than on the external surface (Ingram and Roberts, 1976). Pig carcasses are normally not skinned before chilling and the level of surface contamination can vary widely depending on the efficiency of the scalding, hair removal and singeing procedures. Carcass scalding appear to be the major factor determining the level of bacterial contamination remaining on the surface. The effect on microbial counts is related to time and temperature of scalding.

Chilling of meats

Up to the end of dressing, microbiological concerns are mainly with contamination. At this stage the flora comprises a mixture of mesophiles and psychrotrophs, the proportion of each depending on the origin of the contamination. Further contamination can occur during chilling through contact with the walls or floor, by splashing if cleaning is carried out in a loaded chiller and from the air. For maximum storage life it is essential to

prevent the build up of a psychrotrophic flora in chillers by regular cleaning. It is necessary to fumigate chillers periodically to destroy organisms in hard-to-clean areas with ducts and small spaces. At a level of 100 organisms per m^2/min aerial contamination contributes about 14 cell/cm^2 to the surface flora in 24 hours. This level of contamination is not likely to have serious effects.

After dressing, growth of microorganisms becomes important. The surface of a freshly dressed carcass is usually wet and warm, ideal conditions for microbial growth; thus if there is to be any delay before consumption of further processing it is necessary to cool the carcass. In practice, cooling of carcasses after dressing takes place under a variety of conditions depending on the facilities available and the storage life required. At one extreme, carcasses may be held at ambient temperatures of 20°C or higher for short periods before cutting. In these circumstances the meat is usually consumed within 36 hours of slaughter (Buchanan, 1986).

The growth of mesophiles, particularly food-poisoning bacteria, is the major microbiological concern at higher temperatures (Ingram and Dainty, 1971).

At the other extreme, carcasses proceed immediately from the slaugtherline into blast freezers operating at temperatures down to -40°C with air velocities of 5 m/s and capable of reducing the internal temperature of a 15 kg lamb carcass to -12°C within six

hours. The rapid cooling achieved in the blast freezer cold-shocks bacteria on the surface, thus reducing the bacterial count, and at the usual temperature of frozen storage (-12 to-18°C) bacterial growth does not occur. Between these extremes, chilling may take place at a variety of rates. If the meat is intended for consumption within or two weeks it is sufficient to use chilling conditions which restrict bacterial growth, but if a long storage life in the chilled state is required it is necessary to use more stringent conditions which reduce the number of viable bacteria on the surface of the carcass.

Prior to the discovery of the cold-shortening phenomenon, the contraction and toughening of muscle produced by rapid cooling prerigor (Locker and Hagyard, 1963; Locker et al., 1975) the emphasis in chilling was in rapid cooling achieved by high air velocities and low temperatures. This was aimed at preventing the growth of pathogens and the development of bone-taint. It also reduced weight loss during chilling. However in most slaughterhouses rapid chilling was an ideal that was rarely, if ever, achieved (Malton, 1972). Even where chillers capable of attaining sufficient low temperatures were available, poor air circulation caused by overcrowding often reduced the cooling rate well below what was expected. It is no wonder that many butchers believed that overnight holding in a well ventilated cooler was preferable to chilling and the theory of animal heat trapped within the carcass by early chilling was responsible for bone-taint was

widely accepted. As it happened, the old-time butcher was right in opposing rapid chilling but for the wrong reasons. The realization that rapid cooling could produce an irreversible toughening of the meat has prompted another look at the optimum conditions for chilling (Haughey and Frazerhurst, 1972; Locker et al., 1975). To promote tenderness and ease of cutting, slower chilling rates at temperatures of 7-10°C can be used. Under these conditions bacterial growth on the surface can then be controlled by appropriated conditions of humidity and air flow.

Spoilage of chilled meat

Once the temperature at the surface of the carcass falls below 10°C growth of mesophiles will no longer occur and a psychrotrophic flora will gradually displace the micrococci and the coliforms which are common in freshly dressed carcasses. Most pathogens are mesophiles and with a few exceptions growth of these organisms on chilled meat is not a problem. *Salmonellae* do not grow at temperatures below about 6°C (Matches and Liston, 1968; Angelotti et al., 1961) and on meat at 10°C the generation time is about 10h.

The development of a psychrotrophic flora is shown by an increase in the 25°C counts compared with those obtained by incubation at 37°C. Strains of *Pseudomonas, Moraxella, Acinetobacter, Lactobacillus, Brochothrix thermosphacta,*

Alteromonas and certain genera of the family *Enterobacteriaceae* are the most common psychrotrophs found on chilled meat. Isolation of *Flavobacterium, Alcaligenes, Vibrio, Aeromonas* and *Arthrobacter* is reported less frequently (Ingram and Dainty, 1971; Sutherland et al., 1975; Hanna et al., 1977). The composition of the flora will depend on the time and condition of storage. On meat held under aerobic conditions, *Pseudomonas* species have a marked advantage in growth rate compared with other genera and this advantage tends to increase with decreasing temperature (Gill and Newton, 1977, 1978). No interactions between species were observed until the maximum bacterial cell density was attained when *Pseudomonas* reduced both the growth rate and maximum cells densities attained by competing species. Growth rates and final numbers of *Pseudomonas* species were unaffected by maximum numbers of other species. The inhibition of other organisms by *Pseudomonas* appeared to be due to their inability to compete with *Pseudomonas* for the available oxygen. *Pseudomonas* is less tolerant of reduced water activity than lactobacilli and *B. thermosphacta*, and these organisms may become dominant on the dryer part of the carcass. *Acinetobacter* can become a major constituent of the flora in circumstances where meat of high ultimate pH contains a relatively high proportion of this bacterium in the initial flora. Molds and yeasts grow more slowly than the bacteria but they are more tolerant of

reduced water activity and consequently appear frequently on the surface of chilled beef sides after 4-6 weeks of aerobic storage (Law and Vere-Jones, 1955). Psychrotrophic molds found on chilled meat include species of *Penicillum, Cladosporium, Thamnidium, Chrysosporium, Mucor* and *Rhizopus*. Yeasts include *Torulopsis, Rhodotorula, Candida* and *Cryptococcus* (Ayres, 1955; Baxter and Illston, 1976).

The effectiveness of current practices for meat processing in protecting the consumer from microbiological hazards has been widely questioned, and in particular, doubt has been cast on the value of their strict enforcement in reducing the risk of food poisoning (Fruin, 1978). Usually, it is not possible to formulate satisfactory single microbiological criteria and provide a precise assessment of the safety of either raw materials or finished product (ICMSF, 1980). Microbiological limits cannot be set without adequate reference to the other factors which may or may not be under the manufacturers control, e.g. raw material quality, processing, distribution and storage conditions, and those factors controlled by consumers who often do not have a full appreciation of the effects of their actions. Adequate control of the temperature and storage time are the most important means of minimizing health hazards associated with raw meat (Mackey et al., 1980). After dressing or other processing, carcasses and other raw meat products should be chilled promptly to 5-7°C, then transported,

stored and displayed at this or lower temperatures (Mackey et al., 1980). For cooked meats the thermal process used should be reliably controlled to ensure that the slowest heating points reach a minimum temperature in the range 65-75°C and the product as a whole receives a cooking time/temperature combination giving the required amount of heating to ensure vegetative cells are killed (Leistner and Rodel, 1976). Because pasteurization will substantially reduce the number of microbes in a product, effective cooling and processing hygiene after the last cooking step play a major role in determining product safety and quality of those cooked products because few competitive flora are present. The production of some processed meats may not include a heating step, and many of these products, and some cooked ones too, are preserved in such a way that the normal spoilage flora and any pathogens introduced by faulty handling are unable to grow (Leistner and Rodel, 1976). Therefore effective control of the formulation of cured products is most appropriate to minimize the food-poisoning risks of these products.

Indicator organisms such as *E. coli,* and other Enterobacteriaceae and the coliforms are generally sought in raw and cooked meats as indicators of its possible contamination with gut contents or fecal material (Buttiaux and Mossel, 1961; Speck, 1976; Notermans et al., 1977). Their use as indicators of this type of contamination has been widely suggested because their ability

to survive in the environment mimics that of *Salmonellae* and also because they will grow under similar conditions to Salmonellae and hence they can indicate unsatisfactory, warm storage. In addition they are usually present in the gut and feces of animals in far greater numbers than *Salmonellae* and may be less-variably distributed. However, there is no good evidence that number of indicator organisms at a particular point in time can be related to the extent of the original fecal contamination (Notermans et al.,1977) or to the risk of enteric pathogens being present (Cox et al., 1975). Ideally meat should be free of pathogens, such as salmonellae and the spores of pathogenic *Clostridia*, and although in many cases these can be reduced in numbers, excluded, inhibited or killed by proper processing, their complete absence from raw meat is not feasible. Specifications requiring complete absence cannot be effectively monitored and enforced because detection methods are not sufficiently sensitive and sampling techniques have certain built-in tolerances, determined by sample size, the number of samples that can be handled per batch and the sensitivity of any testing done and can specify the probability of accepting or rejecting batches above or below a fixed limit, thus providing a quantitative means of assessing the safety and quality of food.

The consumer and the microbiologist do not, of course, use the same criteria for assessing the safety and quality of meat.

Microbiologists use microbial counts, processing and hygiene data, and other criteria such as toxin presence or chemical changes to asses the bacterial status of meat. The consumer uses the presence of mold colonies, slime, color change or other visual or olfactory evidence of spoilage as a cause for rejection. The significance of high counts of non-pathogenic microbes in raw meat cannot be assessed by anyone without knowledge of processing or handling. Often meat with total counts under 10^8/g will give an acceptable product, if it has been vacuum packaged, stored chilled and will be cooked before eating. If prolonged storage of meat containing large numbers of bacteria is contemplated then concern about their metabolic activities and products should occur as these can lead to premature product deterioration. It is possible to produce under ideal commercial abattoir and butchery conditions, meat containing 10^4 or fewer bacteria per gram (Roberts et al., 1980). Although raw meat containing large numbers of non-pathogenic bacteria does not present a health hazard, it should be viewed as having been produced unhygienically or poorly stored or contaminated during processing. Meat mishandling in any of these ways present different problems of data interpretation to the quality controller or microbiologist. Meat stored under warmed conditions may present a safety risk because it contains mesophilic bacteria which will increase in number during storage. It is a particular risk if it is eaten

raw or if worked without further cooking or handled in such a way that it can cross-contaminate other foods. Similarly meat which has been contaminated at a point during processing will contain an inoculum of unknown composition, possibly including pathogens. Meat which has been stored under chilled conditions for a long period or under warm conditions for a shorter time will usually only present a quality risk, because it contains both metabolic products and active enzymes from the microbial population (Branem, 1979). Remnants of "high-count" meat can remain in storage or production areas and complete the cycle of contamination. Hence, regular surveillance and control of hygiene of production and storage areas and attention to temperature control and stock rotation can minimize problems for both the producer and the consumer.

A large number of different sampling techniques have been applied to meat carcasses and meat. The choice of a technique is mainly a matter of personal preference and convenience, although over-riding this are considerations of the specific need to obtain samples that most accurately and consistently reflect the true microbiological status of any meat under examination.

Samples taken from the surface of a carcass provide the most useful index of the extent of microbial contamination of the carcass, because the surface is the area most likely to be affected by high humidity or any short-term temperature fluctuations during

storage, and also because the interior of muscle is sterile or very nearly so.

The surface microbial count of stored carcasses increases as a function of time and storage temperature. Useful information concerning the keeping qualities at a given temperature can be obtained by surface counts and in certain cases by visual inspection, if either can be correlated reliably with quality. A number of different destructive and non-destructive methods may be used to estimate number of microbes present on any surface and they provide different types of information about it (Kitchell et al., 1973). Some non-destructive methods break up colonies releasing large numbers of free bacteria into the eluting fluid (swabbing or rinsing); others only replicate the surface clones (contact plate, direct agar plating) producing apparently lower counts which may be erroneously misinterpreted as indicating cleaner meat. When choosing a method it is important to understand what information it gives, and to assess its sensitivity and reproducibility. In general, results indicate that destructive methods, involving sample removal and maceration, give higher counts and less variable results than contact plate and swab methods. However, swabbing techniques are used widely for examining the surface of red meats. One method employs a template to delineate the area to be swabbed and thorough rubbing with wet and/or dry swabbing provides the abrasion to

dislodge microbes from the surface. The method of Kitchell et al., (1973) is one of the most-commonly employed. Scraping is also a useful method of sampling and has been suggested for poultry, cattle and sheep carcasses and meat cuts (Patterson, 1971). It has been suggested a surface sampling method involving pipetting into a hollow cylinder which is held with its open end tightly against the surface (Williams, 1967). The meat surface below the diluent is scraped with a sterile spatula to obtain maximum suspension of bacteria in the solution; physical abrasion of the surface increased the count 10-fold.

The use of a spray gun and collecting device such as described by Clark (1965) and improved by Leistner (1979) and Thran (1979) is potentially a useful method for rapid sampling of large carcasses but requires further evaluation before it is used as a routine method. The method of Leistner (1979) is of interest because it exploits a simple method of measuring the turbidity of washings to estimate the microbial counts on the surfaces of carcasses. For sampling frozen boneless meats and poultry, the use of an auger attached to a variable-speed electric drill is probably the most-commonly used and convenient sampling technique (Barnes et al., 1973). Swab techniques, when correctly applied, give a reasonable estimate of the number of micro-organisms on a meat surface and have the value that they are non-destructive. For investigative work the best arrangement is to

collect samples from the largest number of carcasses and sites on the carcass as possible, e.g., 10 (Olgaard, 1977) and to sample a reasonably large area, e.g., 100 cm^2 at each site examined. However, this is very tedious and time-consuming and, as Ingram and Roberts (1976) point out, the detailed examination of a few carcasses gives no reliable indication of the hygienic status of operations in an abattoir, unless the progress of any particular carcass is followed through processing. Thus the use of microbial counts on carcasses to check routinely compliance with a code or directive relating to hygienic abattoir practices is unreliable and may therefore be misleading (WHO, 1979). The reliability of the non-destructive methods also depends on the size of the microbial population. Generally, the contact plate, agar sausage, and the swab/agar plate method can only be used effectively on relatively clean carcass surfaces, as these methods give only a picture of the number of micro-colonies, not their size, and their upper limit of sensitivity is defined by the confluent growth of large colonies (10^8 cfu/cm^2) and the topography of the surface (e.g. follicles, hairs, creases and roughness) which affects the spatial distribution of the bacteria and the area contacted (Olgaard, 1977).

Microbiologists have struggled to find some general or specific quantitative relationship between levels and types of microbes on carcasses or within comminuted meats and the storage life or quality of meat for further processing or sale to the

consumer. Although for most meat products there is an inverse relationship between microbial numbers and quality, sometimes there is a tendency for organoleptic acceptability to improve with increased total counts (Hill et al., 1976). Certain microorganisms found on meat are deliberately cultured to impact desirable organoloptic characteristics to the meat, for example, some lactic acid bacteria and yeasts.

In spite of considerable work, no single method or criterion has found general acceptance as an index of quality or potential shelf-life. Assembled evidence suggests that reproducible relationships between quality and microbial numbers only exist for products handled, packed and stored under carefully defined conditions (WHO, 1979). The biochemical, physical and chemical methods available for quality assessment have been reviewed by Pearson (1968), Jay (1978) and Baumgart (1980). Because present methods rely on detecting gross changes in the chemical composition or physical properties of meat they are only sensitive to changes caused by microbial numbers in excess of 10^6/g. The changes caused by fewer microbes may be smaller than the variation in the chemical composition of the meat, unless the specific chemical markers of microbial activity can be found. Many tests for evaluating meat quality share a common lower detection level (The changes caused by 10^5 organisms/g) and so provide only a crude quality assessment. In fact many tests are only

marginally more sensitive than touch to detect slime, smell or color observation, although they have the potential to produce more objective results than sensory measurements. Various chemical and physical markers have been recommended for demonstrating incipient spoilage in meats. A relationship has been shown between the extract release volume or water holding capacity of meat homogenate and its microbial quality (Jay, 1966). Beef of poor microbiological quality releases a smaller volume of liquid than beef of good quality when it is filtered for a set period of time. Other authors have suggested that a fall (to below 5) followed by a rise in pH (from 6 to 8) resulting from deamination of amino acids may indicate incipient spoilage (Pearson, 1968). This pH shift may have a direct effect on the water holding capacity. Changes in the concentrations of a number of chemicals, e.g., low-molecular-weight, nitrogen-containing compounds, amino sugars, nucleotides and lactic acid, may be used as markers of quality, but in general the concentrations associated with spoilage are so variable that any test based on them cannot be used routinely except under carefully specified conditions. Fat degradation has also been used as an indicator of spoilage but is less satisfactory than the others because the changes observed occur more slowly than the degradation of high- or low-molecular-weight, nitrogen-containing compounds (Jay and Kontou, 1971; Ingram and Dainty, 1967). It is difficult to identify the reasons for changes in fats, for

example, to separate chemical from microbial oxidation. In addition, some of the products formed during either protein or fat degradation (e.g., carboxyl-containing compounds) may be further metabolized by certain species of microbes (Alford and Pierce, 1961). The significance of various biochemical changes have been reviewed (Ingram and Dainty, 1971). They suggested that unless meat has been stored for very long periods, the structural macromolecules will not be degraded by microbes. Changes caused by microbes will be limited to the direct effect on low-molecular-weight substances or to indirect effects on the environment surrounding large molecules and incidently causing changes to their tertiary or quaternary structure.

The assessment of meat quality by the measurement of products of microbial metabolism or of microbial enzyme levels has particular appeal as it is possible to accurately and rapidly determine minute amounts of specific enzymes and metabolites (Bascomb, 1981). Some progress has been made in using gas liquid chromatography and mass spectroscopy to identify microbial induced changes that can be related to the quality of meats, but such techniques are not currently suitable for routine use. Other methods proposed include the determination of the degree of hydration of the meat, the rate at which the redox indicator is reduced and also the measurement of titratable acidity and presence and concentration of specific compounds (Shelef and

Jay, 1969; Baumgart and Niermann, 1974; Holley et al., 1977; Dodsworth and Kempton, 1977). Further consideration should be given to such methods, as they may provide the tools for quality inspectors to distinguish between meats that contain the same total number of microbes but have a different potential for spoilage. Using conventional microbiological techniques it is impossible to distinguish between meats which have been contaminated during processing and those which have been badly stored under warm conditions. Identification of meats with such different histories is essential for control of quality and safety and may be possible by the use of markers.

Rapid methods in food microbiology

In the last few years the development of rapid microbiological tests for food products have been increasing due to the limitations of traditional techniques. Conventional microbiological techniques, and, some of the more rapid alternatives, rely on the amplification of microbial numbers by growth and division to provide a result. This dependence on growth ensures sensitivity, since a few microbial cells can be easily distinguished from food particles by their ability to divide; however, the time taken to obtain a result is limited by the generation time of the organism. Faster detection of microorganisms is possible if the cells are considered as a collection of compounds that can be assayed in minutes using a

biochemical test. Several cell components have been used as the basis for such assays, for example, Gram-negative lipopolysaccharide (Jay, 1977), haematin (Mason et al., 1978), and adenosine triphosphate (ATP). The lack of amplification by growth in this type of test means that they are more prone to interference, eg., from non-microbial ATP in foods. In order for these assays to be useful, interfering compounds must be removed.

The molecule of ATP is found in all forms of life, and is extremely important for energy-transfer reactions in the cell. One reaction that utilizes ATP is the production of light by fireflies. The enzyme that catalyses the reaction, luciferase, and its substrate luciferin, are present in the tail of the firefly. Strehler and Totter (1952) were the first to realize the potential of the firefly luciferin-luciferase system for the accurate biochemical assay for ATP. The ATP assay is simpler to use when the sample contains only microbial cells. The assay has proven useful in fermentation control, where progress of fermentation in terms of biomass production can be easily determined by measurement of ATP. Viable yeast for wort pitching during the brewing process can be quantified by ATP assay (Miller et al., 1978), permitting more consistent pitching. ATP assay has also been used as a biomass measurement for *Trichoderma reesei* during fermentation (Gaunt et al., 1985), correlating well with cellulose consumption, absorbance and dry weight.

With all the success of the ATP assay for samples containing microbial cell only scientists then tried the same assay for the same purpose in foods. However, early workers were hindered by interference from large quantities of non-microbial ATP in foods, which in many cases masked the ATP from bacteria (Hysert et al., 1979). This problem delayed further efforts to utilize the ATP assay for enumeration of microorganisms in foods by almost a decade. In order to solve this problem for foodstuffs, a means of differentiating between microbial and non-microbial ATP was necessary. The only two possibilities were to extract and destroy non-microbial ATP or to separate microorganisms from interfering food components before extraction and assay of their ATP. The first approach is dependent on the differential resistance of somatic cells and microbial cells to extractants. A mild extraction procedure is used first with a surfactant preparation (Kennedy and Oblinger, 1985), although ultrasonics have also been used (Catsaras, 1982). This releases ATP from the somatic cells.

The second approach to the problem of interference from non-microbial ATP is to separate the organism from the food before extraction and then assay for their ATP. Liquids pose no problem in separation since they can be filtered. Organisms can be collected on a filter and interfering compounds like non-microbial ATP can be removed. This method has been used for

carbonated beverages, where filtration was used to isolate yeasts in the samples for ATP assay (Littel and LaRocco, 1986).

In the food industry there are several uses for ATP assays other than merely enumerating and detecting microbes. The rapidity of the assay means that corrective action can be taken immediately, which has importance in the monitoring of cleanliness and hygiene in food-processing areas. The assay has been used to determine microbial numbers on swabs of work surfaces (Shaw, 1981). ATP assays can also be used for shelf-life determinations. It is likely that ATP assay, which is closely linked to the cell' s metabolism will be more appropriate to shelf-life studies than counting colonies, since the organism that grow on the agar are not necessarily those that will grow in the food. For pasteurized milk and cream, the incubation and selective extraction/destruction techniques have been studied as a means of predicting shelf-life (Waes and Bossuyt, 1982).

Some of the new instruments for ATP analysis can detect as little as 10^2-10^3 organisms/g. The amount of ATP in one colony-forming unit has been reported as 0.47 ng with a range of 0.22-1.03 ng. Yeast cells have about 100 times more ATP than bacterial cells. Using this principle many researchers have tested the efficacy of using ATP to estimate microbial cells in foods and beverages. Littel et al. (1986) indicated that the ATP procedure was able to predict bacterial levels within 0.5 $\log_{10}$ of the actual

count for beef and chicken samples. Minimum sensitivity is 5×10^4 colony forming units/g of meat sample. Ward et al. (1986) also found positive correlation between the ATP method and the conventional method in evaluating fish samples.

Lumac (Landgraaf, the Netherlands) markets several models of ATP instruments and provide customers with test kits with all necessary reagents such as a fruit juice kit, hygiene monitor kit, etc. The reagents are injected into the instrument automatically and readout is reported as relative light units (RLUs). By knowing the number of organisms responsible for generating known RLUs, one can estimate the number of microorganisms in the food sample. In some food systems, such as wine, the occurrence of any living matter is undesirable; thus; monitoring of ATP can be a useful tool for quality assurance in the winery.

ATP assay is a technique that has great potential in microbiology for providing rapid results. At present, the technique is fairly labor-intensive, and provides only limited information. Various developments are necessary to increase the acceptance of the method, in particular automation and the provision of more detailed information.

Another well known rapid microbiological detection method is the direct epifluorescent technique (DEFT). DEFT uses membrane filtration and epifluorescence microscopy, takes less than 30 minutes to complete and does not suffer from many of the

disadvantages of other microscopic methods. Microscopic techniques can be improved by using membrane filtration to concentrate microorganisms prior to enumeration. Filtration is usually done by either applying vacuum below the filter or pressure above the filter. Particles in the sample being filtered which exceed the mesh or pore size are retained in or on the filter, smaller particles pass through in the filtrate. The size of particles retained can be selected by using a filter with the appropriate mesh or pore size. To retain bacteria a mesh or pore size of 0.2-0.6 um is generally used.

Fluorescence is essentially an optical phenomenon in which light energy is absorbed by a substance, known as a fluorophore, and almost instantaneously re-emmited as light of a longer wavelength. Fluorescence is distinguished from phosphorescence by the duration of luminescence after the excited light is switched off. When fluorophore molecules absorb energy from the exiting light their electronic state changes to an exciting state in which the energy of the molecule is increased above its normal or ground state. Exited molecules are unstable and they quickly return to the ground state, emitting surplus energy as heat, fluorescence or in a photochemical reaction. The fact that the emitted light is of a longer wavelength than the exited light is the basis for the optical system of the fluorescence microscope.

One of the big applications of DEFT has been in the dairy industry. Treatment with trypsin and Triton X-100 is essential for efficient filtration and clear preparations for microscope analysis (Pettipher and Rodriguez, 1981). The DEFT count is not sufficient sensitive to determine accurately bacterial numbers in pasteurized products with counts below 10^3/ml, but it would detect grossly contaminated products immediately after production. The microflora of pasteurized milk consist of thermoduric organisms that survive the heat treatment and post-pasteurization contaminants. Post-pasteurization contamination by psychrotrophic Gram-negative bacteria is the most significant factor in reducing the shelflife of refrigerated pasteurized milk (Schroder, 1982). In poorly refrigerated milk, however, the thermoduric flora may also cause spoilage (Cox, 1975).

Rapid microbiological methods for pasteurized products are needed, preferably one that predicts the quality of the product. Pre-incubation of milks containing inhibitors and subsequent estimation of post-pasteurization contamination levels by measurements of bacterial ATP has been used to predict the shelf life of pasteurized milk within 24 hours (Waes and Bossuyt, 1982).

Depending on the food, 4 to 15 ml of stomachered food suspensions (10 g food plus 90 ml diluent) could be filtered through a single nylon pre-filter and 3 to 10 ml of these pre-filtered suspensions could be filtered in the DEFT (Pettipher and

Rodriguez, 1982). For fresh meat and fish, the pre-filtered DEFT count agrees well with plate colony counts over the range of 10^4 to 10^{10}/g. For counts on frozen meat and frozen fish the prefiltered DEFT count also agrees well with plate colony counts over the range of 5×10^4 to 5×10^7 g. For cooked meats, cream doughnut and spices good agreement was found between the pre-filtered DEFT and plate colony counts (Pettipher and Rodriguez, 1982).

Bacterial counts in natural waters obtained by membrane filtration epifluorescence microscopy techniques generally agree with counts obtained using the scanning electron microscope (Bowden, 1977). The DEFT could be used to count bacteria in rinses of milking equipment, this enables the source of high numbers of bacteria to be traced within one working day permitting speedy corrective action. Pre-filtration through a 5.0 um pore size nylon filter increased the sensitivity of the rinse DEFT by five times giving a sensitivity of 200 bacteria per ml of rinse. Hunter and McCorquodale (1983) suggested that DEFT is a useful and rapid means of counting bacteria in rinses of equipment where disinfection is primarily achieved by chlorination but, in the absence of a stain which can differentiate more accurately between dead and living organisms, its use is limited where disinfection is carried out solely by heat.

For the microbiologist the acceptability and use of DEFT is increasing and the method is becoming widely used in the dairy,

food and beverage industries. Automation of the technique is likely to speed its introduction but it should not be forgotten that no automation is required if only small numbers of samples are analyzed daily. The DEFT has the advantage that, as a manual technique, it can be used for the rapid assessment of small numbers of samples of different foods in less than 30 minutes, whereas a fully automated DEFT instrument has the potential to become the future method of choice for the bacteriological analysis of milk.

Another very important way of analyzing bacterial loads is by using immunological techniques. These methods by definition, depend on the highly specific binding reaction between an antibody and the antigen to which it is directed. Immunogens, which can vary from a pentasaccharide to a whole cell are composed of two parts: the site/s to which antibody molecule/s attach and the carrier which is also needed to generate the immune response. The site of antibody attachment on an immunogenic molecule is called the antigen or epitope, as distinct from a hapten which is a small molecule capable of binding to antibody but not capable of generating an immune response. In order to generate antobodies for use in an immunoassay, the antigen must first be isolated and purified before it can be used to stimulate antibody production. The degree of purity of the substance used for immunization is of critical importance to any

subsequent assay, since impurities may also elicit antibody production.

In recent years many developments in the field of immunoassays indicate the continuing activity in this complex area. Amplification of the signal generated in an assay serves as a useful means of improving its sensitivity and can also increase the speed of reaction. Several methods have been developed and utilized. The use of cycling enzyme-substrate reaction mixtures, using high turnover enzymes which generate large amounts of measurable end product, is one approach that has been developed. In a system developed by Stanley et al. (1985), alkaline phosphatase (the label used in enzyme-linked immunosorbent assay ELISA) dephosphorilases nicotinamid adenine dinucleotide phosphate (NADP) to generate the substrate for a NAD^+/NADH redox cycle. In this cycle which uses the enzyme alcohol dehydrogenase and diaphorase and their substrates, a colorless dye is converted to a colored end product which is measured. This system has been employed by Shone et al. (1985) to produce 40 fold amplification of their ELISA to detect *Clostridium botulinum* neurotoxin B.

In recent years immunological techniques have been use in the detection of pathogens and their metabolites. Campylobacters are now recognized as one of the major causes of gastrointestinal disturbances, with *Campylobacter jejuni* being the species most commonly involved (Stern, 1986). Several coagglutination assays

have been developed using protein A on *Staphylococcus aureus* cells to bind antibody which detects *Campylobacter* antigens and these have been used successfully for initial screening of strains. Lauwers et al. (1985) developed a serotyping method utilizing complement mediated lysis of strains selected by antibody. Fricker and Park (1986) have developed a competitive microtitration plate ELISA for specific *Campylobacter* strains.These same authors also investigated the use of monoclonal antibodies in ELISA and agglutination tests (Fricker and Park, 1987).

Another area of great advances in the area of immunology have been in the development of new techniques for the detection of *Clostridium botulinum* toxins. ELISA techniques have been most widely developed and applied for detecting toxins in foods, however, a variety of assay systems have been exploiting monoclonal and polyclonal antibodies in sandwich assays and primarily directed against toxin A, B and E. Sensitivities of 0.5-1.0 ng/ml are generally achieved (Tranter et al., 1987). Modi et al. (1988) have developed a sandwich ELISA employing a monoclonal capture antibody and polyclonal antibody conjugate which, when coupled to an amplification step, enabled toxins A and B to be detected in food extracts at a level of about 50 pg/ml. This assay can be completed within about 5h, but shows low levels of cross reactivity with other clostridial toxins and *Vibrio cholerae* toxin. The availability of more highly purified toxins of all types and

the production of monoclonal antibodies should enable assays of high specificity to be produced, thereby limiting problems of non-specificity. On the other hand the production of antibodies capable of detecting all toxins types simultaneously could be of use in screening applications. Evidence has been presented showing that unpurified toxin is more toxic that purified toxin, and that in these types of assay it is the immunological activity only that is being detected (Ohishi and Sakaguchi, 1980).

Several forms of foodborne diarrhea caused by enteropathogenic strains of *E. coli* have been identified: classical, facultative, enteroinvasive and enterotoxigenic. Two types of enterotoxin, produced in the small intestine of infected individuals, have been isolated which are distinguished immunologically and by their ability to withstand heating at 60°C for 30 minutes; the heat stable (ST) and the heat labile (LT) toxins. Enterotoxigenic strains produce one or both toxins but ST production is most commonly found. LT is antigenically similar to *Vibrio cholerae* enterotoxin (Karnacki and Marth (1982). The availability of purified ST has similarly led to the development of several ELISAs for this toxin. Some of the ELISAs were developed by the use of sandwich techniques, which show considerable improvement in sensitivity over the suckling-mouse assay (Klipstein et al., 1984). Monoclonal antibodies to ST have now been produced and utilized in a competitive ganglioside ELISA (Svennerholm, et al., 1986).

Methods have been applied to the detection of toxins in both culture preparations and feces, but the latter enables more rapid determination of the cause of diarrhoeas by avoiding the necessity for isolating and culturing *E. coli*. Shah and Rhea (1986) developed an enzyme immunoassay for LT which is carried out on a nitrocellulose membrane. The membrane is inoculated with food/culture and incubated overnight on a growth medium. Colonies on the membrane are then lysed and assayed for the presence of LT by a direct double antibody technique.

One of the pathogens with growing concern in the food industry is *Listeria monocytogenes* which has caused some major outbreaks of food poisoning in the last nine to ten years. Faber and Spiers (1987) have produced antibodies specific against flagella of *Listeria* spp. which did not react with any of the 30 non-*Listeria* cultures examined. Cheese and milk samples naturally contaminated with *L.monocytogenes* were found to be positive for *Listeria* within two working days using the monoclonal antibodies in an ELISA. A series of 15 monoclonal antibodies which reacted specifically with a protein antigen found in all species of *Listeria* were developed by Butman et al. (1988). Those antibodies were tested by an ELISA method and western blot analysis for cross-reaction with non-*Listeria* organisms. From this work, a rapid diagnostic kit was developed for the detection of *Listeria* in food products (Mattingly et al., 1988). This is a sandwich ELISA method

in which the monoclonal antibodies to *Listeria* are used to coat the wells of a microtitred plate. Donnelly and Baigent (1986) investigated the feasibility of automating fluorescent-antibody techniques through the use of flow cytometry. Problems of cross reactivity were overcome by selective enrichment. This approach enables the rapid characterization of a cell population based on a number of parameters such as morphology, nucleic acid content and surface antigeneity.

One of the major causes of foodborne illness in the United States and worldwide is due to *Salmonella*. *Salmonella* is one of the genera of the *Enterobacteriaceae* and identification is traditionally based on serological typing where serotypes are distinguished from each other by their O (somatic), H (flagellar) and Vi (capsular) antigens. Based on these antigens the salmonellae can be classified according to the Kauffman-White scheme and serotypes are grouped according to their common O antigens. The identification of *Salmonella* and the diagnostic uses of monoclonal antibodies have been extensively reviewed by Lim (1987). The development of specific and sensitive rapid methods for the detection of *Salmonella* require up to 7 days to produce and confirm positive results, thus there is an urgent requirement for methods which are cheaper, simpler and more rapid than cultural procedures. A number of alternative methods use labelled antibodies which are specific to *Salmonella*, including ELISA.

Among the latest rapid methods developed for *Salmonella* are the enzyme immunoassay membrane filter method, the combined electrical immunological method and the Tecra method. In the enzyme immunoassay membrane filter method an ELISA technique using a horseradish peroxidase-protein A Spicer Edward antiserum complex is use for the detection of *Salmonella* on membrane filters (Farber, 1985). In the combined electrical and immunological method *Salmonella* is captured utilizing antibody coated beads which can then be transferred to a selective enrichment broth and a specific electrical signal monitored (Eastern, 1988). Jay and Comar (1988) developed the Tecra method in which a sandwich ELISA uses antibodies absorbed onto wells of microtiter plate and visual endpoints are obtained by the use of a color comparator card.

Another organism that causes food poisoning is *Staphylococcus aureus* which remains a common analysis in the quality control of foods, even though the occurrence of the organism itself does not necessarily correlate with the ability of a food to cause poisoning. Foods may retain toxicity despite the loss of viable organisms and not all strains of *S. aureus* produce enterotoxin (Minor and Marth, 1976). Several immunological methods using enterotoxins as analytes have been developed as kits. An ELISA for the detection of staphylococcal enterotoxins (SEs) A-D using polystyrene beads and microtiter plates as the

solid phase have made commercially available (Notermans, 1983). These assays can detect as little as 0.1 ng SE/g in foods within two days. Patel and Gibbs (1984) produced a novel rapid (30h) assay for SE-B in food which exploited phase separation by use of antibody attached to a magnetic gel. Currently SE types are detected separately resulting in the necessity for parallel assay of food/culture samples. The development of an assay capable of simultaneous detection of all SEs would be an advantage in routing screening and may be possible to develop monoclonal antibodies capable of cross-reacting with all SE types.

Microorganisms and their toxins are continually being identified as causes of foodborne illness. In some cases the role of the microbial species itself in foodborne disease is not clearly defined and the role of toxic substances produced by these organisms is even less clearly identified. These organisms and toxins are likely to be the subject of much study in the next few years and immunological methods are likely to be an important feature of these studies. The species of interest include *Yersinia enterocolitica, Vibrio* sp., *Klebsiella pneumonia, Edwardsiella tarda, Citrobacter* sp., *C. freundii* and *Aeromonas hydrophila.* In addition to food poisoning, spoilage of foods can and does have considerable commercial implications to food manufacturing, and yet to date immunological assay of spoilage organisms have receive little attention. These organisms offer a vast potential for

commercial immunoassays, particularly since many analytical techniques currently available rely on microbiological culture with its attendant problems of low throughput and long assay time. In a recent publication fimbriae (CFA/I) were isolated after detachment from an enterotoxigenic *Echerichia coli* (ETEC), purified by successive isoelectric point precipitation at pH 3.5 and injected into rabbits (Singleton et al., 1991). The antisera containing the polyclonal antibodies against the fimbral proteins (antigens) were then used to detect the presence of CFA/I on other *E. coli* cultures using an indirect ELISA system. The ELISA procedure for the CFA/I assay was as sensitive as the DNA hybridization for detecting the ETEC.

Another rapid method developed a few years ago is the hydrophobic grid-membrane filter. The filter itself is composed of 1600 grid cells. The black hydrophobic print divides the substrate into discrete growth compartments. These compartment are inoculated by filtration. During incubation, nutrients and selective agents diffuse through the membrane filter, and microbial growth form within the grid cell. The usual appearance after incubation is of a set of square colonies distributed over the hydrophobic grid-membrane filter. The lateral restraint isolating growths to particular grid-cells results entirely from the hydrophobicity of the grid lines.

Microorganisms stressed by dry conditions, heat, or freezing etc, may be able to survive and multiply in foods, yet may escape detection is suspension are inoculated directly onto selective agar media. The conventional broth resuscitation procedures (e.g., pre-enrichment of dried foods in *Salmonella* analyses) permit the analysis to be used only as a "presence-absence" type of test. It is, of course, possible to carry out an MPN-type procedure by using a plurality of pre-enrichment vessels if an estimate of the contamination level is needed. Such procedures are usually too expensive and time-consuming for routine work. Solid repair procedures which involve overlays of non-selective with selective agar media allow enumeration but are more tedious and the conditions must be carefully defined for each group of organisms being enumerated (Hartman, 1979). Membrane filters may be used to advantage in enumerative and diagnostic analyses since they may be transfered from one growth medium to another without masking the identity or changing the number of individual growth units. Thus, stressed organisms may, after inoculation onto membrane filters, be permitted a period of recovery on a relative good growth medium before being transfered to selective medium. The quantitative nature of the analysis is not lost. A hydrophobic grid-membrane filter method which includes a 4 hour resuscitation period at 35°C on a non-selective medium gave significantly higher counts of *Vibrio parahaemolyticus* which had

been stressed by freezing, heating or chilling (Entis and Boleszczuk, 1983). The use of up to 4 hours of incubation on non-selective medium prior to transfer to tryptone bile agar at 44°C was used by Brodsky (1982) for enumeration of *E. coli*. A similar resuscitation period has been prescribed for enumeration of E. coli from dried, frozen or acidified in both the Anderson and Baird-Parker direct plate (Rayman, 1979) and hydrophobic grid-membrane filter methods (Sharpe, 1983).

Detection of a species through its biochemical profile, on transferring a single hydrophobic grid-membrane filter across several media has been done for *A. hydrophila*, but this approach has limited applications (Sharpe, 1986). However, with the development of an easy to use replicator the hydrophobic grid-membrane filter holds much potential for biochemical profiling, identification, screening, and culture maintenance. The replicator permits a master hydrophobic grid-membrane filter to be reprinted in seconds, and a virtually unlimited number of copies to be made. These may be incubated on different substrates, or stored for future use. It has been used by both Peterkin (1987) and Lo and Cameron (1986) in the preparation of genomic libraries.

Lin et al. (1984) developed a trypan blue agar for use in conjunction with the ISOGRID system for effective enumeration of yeasts and molds from foods. A comprehensive review on all aspects of membrane filter technology for food microbiology has

been made by Sharpe and Peterkin (1988). Topics in the book include basic principles of membrane filter technology, mathematics and statistics involved in using membrane filtration for enumeration of microorganisms, applications of membrane filtration for general microbiology as well as for specific organisms such as *Salmonella, E. coli, Listeria monocytogenes, Clostridium perfringens, Staphylococcus aureus, Yersinia enterocolitica*, etc., and the use of DNA probes and antibodies in conjunction with the membrane filter system. In a recent study, enumeration of bacteria in different foods was compared by using two different methods (Hart et al., 1991). The Trypan blue and the Congo red methods were compared using the HGMF technique of the ISO-GRID system. Results from this experiment indicated that the Trypan blue agar observed in incandescent light is more convenient to use for enumerating yeast and mold in food samples.

Another viable cell count system is the Petrifilm (3M Co., St. Paul, Minnesota). In this system, rehydratable nutrients are imbedded into a series of films. When needed, the first layer of the protective cover is lifted and 1 ml of liquid sample is introduced to the center of the unit, and the cover is replaced. A plastic template is placed on the cover to make a round mold. The rehydrated medium will support the growth of microorganisms after suitable incubation time and temperature. The colonies can be counted

directly in the unit. The unit is about the size and thickness of a plastic credit card; thus, providing great savings of space in storage and incubation. Petrifilm units have been developed for total bacterial counts, coliform count, and fecal coliform count. Petrifilm has been successfully used to count organisms from milk and meat (Ginn et al., 1986; Smith et al., 1986). Fung et al. (1987) obtained a 0.99 correlation coefficient comparing Petrifilm SM with the conventional viable cell count method for seafood analysis of mesophiles. Bishop and Juan (1988) found that the petrifilm technique was not statistically different from the agar pour plate method for enumeration of psychrotrophs in raw milk samples. The advantages of the Petrifilm system include its ease of operation, savings of storage and incubation space, its long shelflife because of the use of dehydrated medium in the film, and independence from heat treatment compared with the conventional agar pour plate method.

One of the newest systems in the market is the Redigel system (RCR Scientific, Inc., Goshen, Indiana). This system consist of sterile nutrients with a pectin gel in a tube. A 1-ml sample is first pipetted into the tube. After mixing, the sample is poured into a special Petri dish previously coated with a gelation material. When liquid comes in contact with the gelation material it forms a complex that swells to resemble conventional agar. After an appropriate incubation time and temperature, the colonies can be

counted. Chain and Fung (1987) compared 17 different foods and obtained a correlation of 0.964. Later Roth (1988) reported a comparative analysis of nine different nondairy and dairy food products and found that the temperature-independent pectin gel method (Redigel) provided statistically significant results compared with the conventional method. Also Roth and Bontrager (1989) evaluated the coliform count of cream, cheddar cheese, cottage cheese, homogenized milk, raw milk, sour milk, and yogurt by the Redigel method and the conventional pour plate method and again found statistically significant results.

One of the big advances in the area of rapid microbiological methods has been the rapid growth of gene probes. Gene probes are small segments of single stranded nucleic acid that can be used to detect specific genetic sequences in test samples. Probes can be developed against DNA or RNA segments and gene probe is a generic name to describe both types of systems. Currently probes have been developed for the detection of a range of viruses and bacteria causing infectious diseases such as herpes and cytomegalovirus. The specific characteristics of any organism depend on the particular sequences of nucleic acid contained in its genome. It is possible to find sequences that are common to a large group of organisms and code for some elementary function needed by them all. It is also possible to find more specific sequences that are present in a much smaller number of

organisms and code for a specific biological function such as the production of a toxin. The possession of the nucleic acid sequence defining toxin production would allow the discrimination of toxin-producing strains from all other strains.

Nucleic acids are made up of just four nucleotides in a double helical structure. Pairing occurs between the bases on each strand (adenine with thymine and guanine with cytosine). Two strands of DNA in which the base pairs match are complementary and form double stranded structures known as hybrids. So, a probe sequence can be selected to match the DNA sequence of a target organism and the target organism can be treated to make single strand of its DNA sequence available for hybridisation with the complementary prove sequence. The attraction of gene probes to the problem of microbial detection is that a probe consisting of 20+ nucleotide sequences can be shown to be statistically unique and, if it hybridises with part of the microbial genome, then this can be used to generate an identification of unparalleled accuracy (Klausner and Wilson, 1983). Methods for producing gene probes have been reviewed by Wilson (1983). Probes can be constructed by biochemical synthesis using enzymes, by cloning in appropriate vectors or by chemical synthesis in automated gene synthesisers. It is fair to say that the methodology for the production of gene probes is now part of the routine of molecular biology. Taking the development of reliable and sensitive systems for the detection of

hybridization and it is with this aspect that current developments are concerned. The detection of hybridization depends upon the attachment of a label or signal to the gene probe. It is important that this label should not interfere with the hybridization process. Early labels were radioisotopes, in particular P^{32}, which were detected by autoradiograph or scintillation counting. Food laboratories are usually reluctant to use radioisotic methods and the widespread adoption of gene probes for food testing requires the development of other detection systems based on, fluorescent antibodies, enzyme induced color reactions or chemiluminescence. The biotin-avidin link has been used to attach labels to DNA sequences. The biotin is chemically bound to nucleotides and the avidin is linked to antibodies or enzymes that can trigger a detection system. Extra nucleotides ca be attached to the prove sequence to act as carriers for signal molecules and increase the sensitivity of the prove. The use of different ligands to carry different signal systems might allow testing for a number of organisms to be carried out simultaneously.

A number of DNA probes have now been developed for the detection of microorganisms of interest to the food microbiologist. Hill (1981) has reported the use of DNA hybridization for the detection of enterotoxiganic *E. coli.* Hill et al. (1983) used DNA colony hybridization for the detection of *Yersinia enterocolitica* and Fitts et al, (1983) have applied the methodology to the detection of

Salmonella in foods. This gene probe has been released commercially by Gene Track Systems (Portland, OR) and a collaborative study using it has been reported by Flowers et al. (1987). The gene probe in this system is labelled using P^{32} and detected by a dedicated counter on a scintillation counter. The assay requires that the sample under test go through a conventional pre-enrichment and selective enrichment procedure before filtration to capture the microbial population on a membrane. The bacteria are lysed and exposed to DNA denatured to single strands which are fixed to the membrane. The filters are incubated in hybridization solution containing the radiolabelled probe. If salmonellae are present in the enrichment culture then the labelled prove will bind to the *Salmonella* DNA. Unbound probe is washed away and the filters are dried before the radioactivity is measured. Positive and negative controls cultures are used as part of the routine of the test. Other companies also market a non-isotopic gene probe for *Campylobacter* which uses an enzyme catalysed color change as the detection principle. This probe targets ribosomal RNA rather than genomic DNA. The greater abundance of ribosomal RNA improves the sensitivity of the test and this is likely to be exploited in a wider range of gene probes in the future.

Some of the problems of gene probes are lack of reliable non-isotopic detection methods and poor sensitivity. The Gene

Trak *Salmonella* system will only detect a number above 10^5 per ml; hence the need to amplify numbers through enrichment. However, the theoretical sensitivity of gene probes is a single cell. Loss of sensitivity occurs because of the practical problems associated with manipulation small quantities of DNA. In the future we can expect to see the development of reliable detection and amplification systems and the evolution of gene probe instruments which can carry out the manipulations automatically and can probe for several target organisms simultaneously.

Methods involving electrical measurements have been gaining a lot of recognition as a tried and tested means of replacing traditional bench techniques in microbiological laboratories. Assays systems generally consist of a dilute suspension of cells in buffer, sometime partially solidified with agar which does not obstruct the passage of small molecules. Two metal electrodes of the same material are placed in the solution and the circuit is completed. If the circuit is completed with a source of direct current (DC) electricity, then ions migrate in the solution to their oppositely charged electrode, and electrolysis occurs. Thus, the composition of the medium would change. If the source is an alternating current (AC), then by definition, no ions actually move. The charge is passed to and from at the frequency of the AC and the medium would not change its composition. However, the measurement being made, be it current, resistance, or potential is affected by

polarization of the electrodes. This arises at the surface or boundary between the electrode and the solution. In DC circuits polarization of the electrodes eventually blocks flow of current, but in AC circuits, it can be a help, but more generally gives erroneous values. As the AC cycle varies, so the boundary potential also changes. This gives rise to a polarization impedance which has been found to have two components, capacitance and resistance/reactance. It decreases with increasing frequency of the AC. Different metals have different polarization impedances. Thus besides any electrical parameters that may be changing in the solution due to microbiological effects, other reactions are occuring.

The application of electrical methods to the determination of the total mesophilic bacterial population has been examined for almost all types of foodstuffs including fresh, frozen and preserved meat, fish, milk and dairy products, fruit and vegetables and dehydrated products (Martins ang Shelby, 1980; Fryer and Ford, 1988; Sorrells, 1981). The measurements performed are either enumeration (from comparison with a calibration curve) or a presence or absence test to assess sterility by the lack of change of conductance or impedance. Psychrophilic bacterial populations have been less well investigated but some studies have been carried out on products susceptible to low temperature spoilage and fish (Martins, 1984; Ogden, 1986).

Electrical methods, like conventional microbiology, can be made specific for certain groups of organisms by adding inhibitory or selective agents to the culture medium, and thereby can detect organisms of public health significance. The detection and enumeration of coliform bacteria in food stuffs has long been used to indicate the hygienic practices applied during their growth, harvest and processing of food products. Coliforms and Enterobacteriaceae are easily and specifically detected by electrical methods and this test has been applied to a wide variety of foods. *Salmonella* spp. have received considerable attention recently and two protocols have been developed. The first one developed by Easter and Gibson (1985) and the second one developed by Arnott et al. (1988). Both methods rely on a non-selective pre-enrichment followed by a period of selective enrichment during which the electrical detection is made. The medium developed by Easter and Gibson is based on lysine decarboxylase and dulcitol fermentation in the presence and absence of bacteriophage specific for *Salmonella* ssp. The TMAO which is used in this procedure was modified by the substitution of dulcitol by mannitol and by the substitution of TMAO with dimethylsulfoxide in the pre-enrichment broth only (Gibson, 1987; Ogden and Cann, 1987). These modifications facilitate the detection of dulcitol negative strains of *Salmonella* and reduce the cost per test. The TMAO medium and method of Easter and

Gibson have been compared with traditional methodology in investigations of milk powder with 8 different serotypes of *Salmonella*, and no significant difference was found (Prentice et al., 1988). Prentice and Neaves (1987) have developed a medium for the detection of *Staphylococcus aureus* in milk powders using conductance measurements. The medium uses traditional selective agents in addition to glycerol which is present to reduce the water activity of the medium, since incorporating high concentrations of sodium chloride for this purpose have an adverse effect on the conductance responses. Neaves et al. (1988) have also developed a conductance method for the detection of fecal streptococci in milk and milk powders. Scombrotoxin food poisoning is associated with the consumption of fish. Klausen and Huss (1987) have utilized this characteristic reaction in the development of a conductance method for the detection of these organisms.

Improvements in the performance of electrical methods can be achieved by a short pre-treatment step to separate and concentrate the target organism(s). Techniques such as centrifugation, filtration and non-specific adsorption onto magnetic or electro positive supports have been successfully applied to the removal of bacteria from aqueous suspensions, but relatively little work has been carried out in combination with electrical methods Kroll, 1985). Easter et al. (1988) described a 24 hour test for

Salmonella involving a combination of three techniques - an immunological recognition on magnetic beads for selective removal from pre-enrichment broths, detection by measurement of conductance and rapid confirmation using colored latex agglutination test.

The Bactometer is an instrument designed to measure impedance changes in foods. Samples are placed in the wells of a 16-well module. After the module is completely or partially filled, it is plugged into the incubator unit to start the monitoring sequence. As the microorganisms metabolize the substrates, change in impedance increases sharply, an the monitor screen shows a slope similar to the log phase of a growth curve. The point at which the point of impedance begins is the "detection time" and this is measured in hours from the start of the experiment. The detection time is inversely proportional to the number of microorganisms in the sample. By knowing the number of microorganisms per milliliter in a series of liquid samples and the detection time of each sample, one can establish a scattergram, similar to a standard curve. One can also establish cutoff points to monitor certain specifications of the food products. For example, if one finds that meat with 10^6 organisms/g will result in a detection time of 5 hr, then one can use 5 hr as a cutoff point for an indicator that meat samples have fewer that 10^6 organisms/g. In newer models of the Bactometer, the screen displays bars with one of three colors

instead of an impedance curve. A red bar signals that the food being analyzed is "out of spec." These new developments are designed to be "user friendly." Hardware and software are provided for users to conveniently monitor their food products as far as impedance microbiology is concerned.

Another instrument similar in principle and operation is the Malthus system (Malthus Instruments, Crawley, England). The Malthus method works by measuring the conductance of the fluid as the organisms grow in the system. It also generates a conductance curve similar to the impedance curve of the Bactometer, and it also uses detection time in monitoring the density of the microorganisms in the food. The major difference between the two systems, besides the scientific principle (impedance versus conductance), is the incubation units. In the Bactometer system, the size of the well (about 2 ml in capacity) in the 16-well module is fixed. No modification is possible because the module is designed to fit into the incubator chamber. The Malthus system, however, allows analysts to choose three size ranging from 2- to 100-ml samples, depending on the material involved. Another important difference is that the modules of the Bactometer are disposable, whereas the jars, tubes, and electrodes of the Malthus system are autoclavable and reusable. In terms of performance, the systems are equivalent in sensitivity and detection time. The Malthus system has been used for

microbial monitoring of brewing liquids (Day, 1983; Evans, 1985), fish and seafoods (Gibson and Hobbs, 1987), and hygiene monitoring (McMurdo and Whyward, 1984).

Manufacturers of microbiological products are continuously upgrading the software for data analysis in response to customer requests and improvements in computational facilities and skills. Software modifications are generally concerned with the display, interpretation and presentation of the captured data. Much more useful information could be obtained by the analysis of things like mean generation time, lag time and inoculum size (Henshke and Thomas, 1988).

Another method which is widely used in the limulus amoebocyte test (LAL). It is a sensitive method to detect endotoxin levels of lipopolysaccharides (LPS) of Gram-negative cell walls. The method was first used by scientists working with horse shoe crab (Limulus) to detect the presence of endotoxins (Levin and Bang, 1964). The classical LAL test is one in which gel formation occurs upon the mixing of LAL reagent with LPS followed by a suitable incubation period. Gelation occurs as a result of a series of cascading reactions that are triggered by LPS. According to Nakamura et al. (1986), LPS induces the activation of factor C in the reagent and its active form activates factor B, which in turn activates the proclotting enzyme. The latter, is a serine protease that transform coagulogen to coagulin gel. While factor C is LPS

sensitive, the LAL reagent contains another substance designated factor G. Obayashi et al. (1985) obtained partial purification Of LAL and reconstituted the test material without factor G and found that the new reagent could detect 1 pg/ml of LPS from *E. coli.* Another LAL automated method that employs turbidity is the one developed by Oishi et al. (1985) which employs a Toxinometer apparatus. The instrument could monitor 64 samples simultaneously at 12 sec increments, and quantified endotoxin content. The instrument responds to 0/0005 unit (EU)/ml (1 ng is about 5 EU).

One of the areas in which scientists are working is in the development of a rapid method to detect *Listeria monocytogenes.* One of the methods which was developed for the detection of *Listeria* in meat samples uses a U-shaped glass apparatus (Fung-Yu tube). This system utilizes unique biochemical and physical properties of *Listeria* for selective enrichment. Using this system, isolation of *Listeria* in the presence of mixed bacterial flora was successful in 24-48 hr (Yu and Fung, 1991). In another study the effect of oxyrase enzyme on *Listeria* and other facultative anaerobes was studied (Yu and Fung, 1991). FDA-approved *Listeria* Enrichment Broth containing oxyrase was found to be substantially superior for facilitating the growth of *L. monocytogenes* than commonly used but nonspecific reducing agents such as L-cystine HCL and sodium thioglycolate. Another

area in which the Fung's tube method was used together with the oxyrase enzyme system and the Gas Pack Anaerobic system was in the area of *Clostridium perfringens* detection (Mohammad and Fung, 1991). By using the Fung's double tube method the number of *C. perfringens* increased from 44/g up to 1.3 x 10^9/g.

The area of rapid method in food microbiology is exciting. It shall continue to fluorish in the years to come because applied microbiologists will always want to find more sensitive, efficient and cheaper methods to enumerate, isolate and identify microbes from food to ensure the safety of our food supplies, and therefore the health of consumers. Mancini (1992), published an excellent review of all the rapid methods that are currently used in the food industry. Her article provides basic technical information on how each test works, the time frame for ech test and the total cost per test.

Structure of bacteria

With the exception of *Mollicutes (Mycoplasmas, Spiroplasma*) the cell envelopes of prokaryotic microorganisms are characterized by the presence of two distinct components: an inner cytoplasmic membrane, which controls the substrate and electron transport of the cell and which is the site of biosynthesis of extracellular macrocomponents; and the strong, outer cell wall, which maintains the shape of the cells and protects the

mechanically fragile cytoplasmic membrane from rupture owing to the high osmotic pressure exerted on it by the cell cytoplasm. These components can be separated in vivo and are seen as two distinct layers by electron microscopy. In gluteraldehyde-fixed specimens the cell wall can be seen to make intimate contact with the cytoplasmic membrane, however, at sites of cell wall synthesis, where nascent wall polymers may form transient covalent bridges between synthetic complexes in the membrane and sites of incorporation into the wall. These localized interactions have proven strong enough to permit the isolation of specific contact regions "Bayer juctions" in some Gram-negative bacteria. There is evidence for localized adhesion zones on either side of the nascent division septum, that effectively compartmentalize the periplasm at the division site to form 'periseptal' compartments (Eisenberg and McLaughlin, 1976).

The cell wall of the typical Gram-positive and Gram-negative eubacteria, where strength and shape are provided by peptidoglycan which is cross-linked by way of its peptide components to form a single, cell-shaped macromolecule. This material, sometimes described as 'murein', finds an analogue in pseudomurein, the principal wall component of the archaebacterial genus *Methanobacterium* (Pollack and Richmond, 1965). Both polymers contain a glycan backbone consisting of a disaccharide repeating unit of two different N-acetylated amino sugars, to one of

which is attached a short peptide chain of between four and nine amino acid residues. Peptide bonds between the peptide chain of different glycan strands create a covalent network that has great mechanical strength as well as considerable elasticity.

The structure of the peptidoglycan is best considered in three parts; the glycan backbone, the linear muramyl tetrapeptide and the peptide cross-link, each of which is subject to a variation from strain to strain and may vary with growth conditions or during differentiation processes within a given strain (Brock, 1979). The glycan backbone usually consists of the disaccharide repeating unit (N-acetylglucosamine B1-4 N-acetylmuramic acid) polymerized by B1-4 glycosidic linkages. Variations occur in which the muramic acid residues are partially O-acelylated at C6 or partially de-N-acetilated. In *Mycobacterium* the amino group of the muramyl residue carries a glycolyl substituent instead of acetyl (Adam et al., 1969), while in the spore cortex peptidoglycan of *Bacillus* the O-lactyl groups of some muramic acid lack peptide substituents and instead form internal lactams with the amino groups of the sugars (Warth and Strominger, 1969). The in vivo chain length of the glycan strands has proven difficult to determine owing to the presence in cell walls of endogenous endo-glycosidases that may catalyze chain degradation during the isolation of peptidoglycan. Chain lengths of approximately 45 disaccharides units in *Bacillus licheniformis* and 79 units in a lytic-

deficient stain of the same species were measured after taking precautions against autolysis (Ward, 1973). An average length of 35 units was determined for *Echerichia coli*, though this may vary from 20 to 200 for individual chains. In some Gram-negative bacteria, glycan chains may terminate in 1, 6-anhydromuramic acid owing to the action of a murein transglycosylase (Holtje et al., 1975).

The structure of the tetrapeptide attached to the lactyl group of the muramic acid residues is well conserved. In most types of peptidoglycan (types A) it consist of the sequence L-ala-D-glu-amino acid(3)-D-ala, in which the glutamic acid residue is usually linked through its side-chain carboxyl to the amino acid at position 3. amino acid(3) is a species-specific diamino acid, most commonly L-lysine, an isomer of diaminopimelic acid (DAP), or ornithine, though several other variants occur (Schleifer and Kandler, 1972). Inter-species variety may also be introduced by the partial amidation of the side- chain carboxyl groups of D-glutamic acid and diaminopimelic acid and by the replacement of L-alanine at position 1 by glycine or serine in *Corynebacteria* (type B peptidoglycan; Schleifer and Kandler, 1972). Important differences occur between the muramyl peptides of peptidoglycan from vegetative cells and from the cortex of endospores in *Bacillus*. In *B. subtilis* spores, about 20 per cent of muramyl residues carry only L-alanine (Warth and Strominger, 1972) instead of

tetrapeptide, while in *B. sphaericus* spores the diaminopimelic acid of vegetative cells is replaced by L-lysine (Tipper and Gauthier, 1972).

The nature of the cross-linkage (-X- in Figure 1) is the most variable feature of the peptidoglycan structure and is a valuable taxonomic character (Schleifer and Stackebrandt, 1983). In its simplest form the cross-link consists of a direct peptide bond between the side chain amino group of the diamino acid at position 3 of one tetrapeptide and the carboxyl of the terminal D-alanine of a tetrapeptide on another peptidoglycan strand. This type occurs in *B. subtilis* and in all Gram-negative eubacteria with the exception of spirochaetes and *Fusobacterium* (Kato et al., 1979). Recent developments in the analysis of peptidoglycan structure have revealed an alternative cross-linkage in Gram-negative bacteria, between the diaminopimelic acid residues of two tetrapeptides. Gram-positive species exhibit a wide range of cross-bridges consisting of peptide chains of up to five amino acids. These may be chains of a single amino acid or more complex heteropeptides. An interesting type found in a number of strains of *Micrococcus* consist of a tetrapeptide identical to that linked to muramic acid, and there is good evidence that this cross-bridge originates as a muramic acid-linked tetrapeptide that is subsequently transferred to its new position in a transpeptidation reaction.

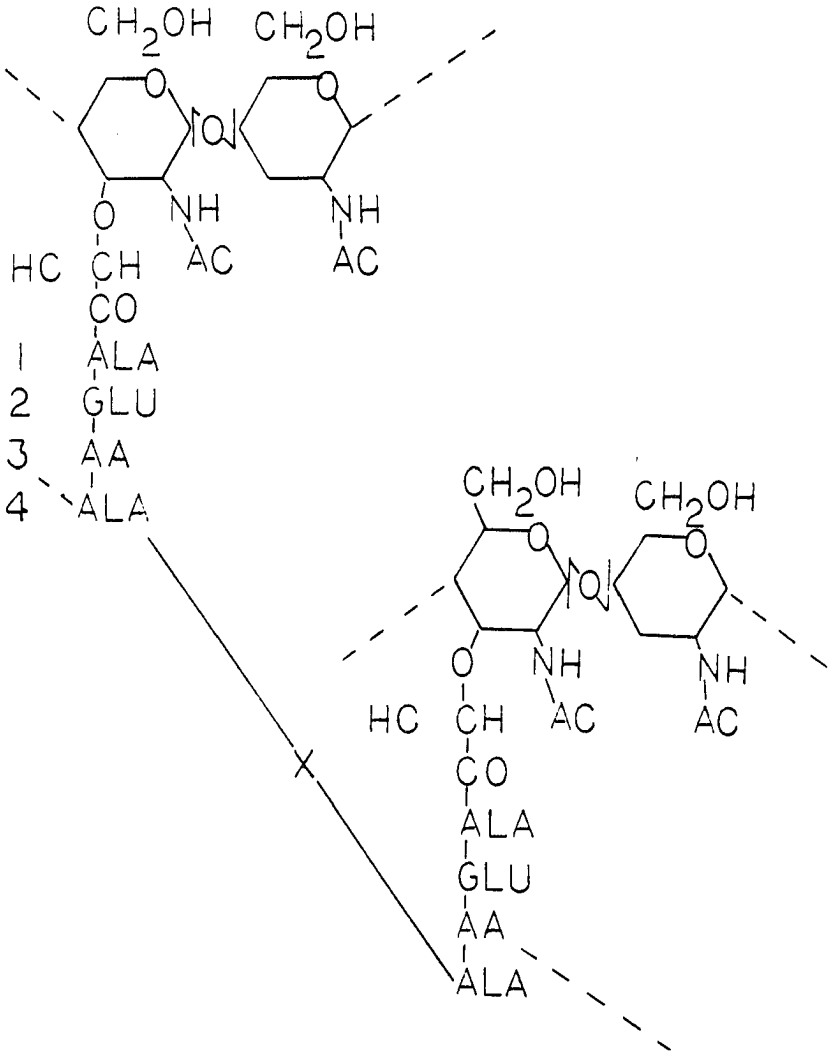

Figure 1. Peptidoglycan structure

The presence in these strains of muramic acid residues carrying no peptide chain is consistent with such a process. In type B peptidoglycans found in some plant-pathogenic corynebacteria, the cross-bridge is formed between the side-chain carboxyl of glutamic acid at position 2 of the tetrapeptide and the terminal D-alanine of another tetrapeptide (Stanier et al., 1976).

The chemical nature and the extent of cross-linking in peptidoglycan are both subject to phenotypic variation. The effects of growth conditions and the description of the interferences with normal cross-link formation of high concentrations of certain amino acids, particularly alanine, serine and glycine, in the culture medium was very well reviewed (Schleiffer et al., 1976). A high concentration ratio of serine to glycine in the medium results in the replacement of some of the glycine residues in the cross-bridge by serine. For a given strain of bacterium the extent of cross-linking of the peptidoglycan depends very much on growth conditions. It was found that *S.aureus* growing at a constant rate in a chemostat on fully defined medium, the proportion of muramyl tetrapeptide lysine residues whose side-chain amino groups were involved in peptide linkages varied between 62 and 90 per cent depending on the limiting nutrient in the medium.

Although peptidoglycan determines the shape and strength of walls of Gram-positive and Gram-negative eubacteria, electron microscopy and chemical analysis reveal differences both in the

architecture of the peptidoglycan layer and in accessory wall structures between the two groups. In Gram-positive bacteria the entire wall is seen as a single, amorphous layer with no internal features, that can be isolated, almost intact, as a covalently linked complex of peptidoglycan and accessory polymers. In its hydrated form it represents of the order of 10 per cent of the total cell volume and is between 20 and 50 nm thick. In Gram-negative bacteria the peptidoglycan forms only a thin layer; quantitative measurements of peptidoglycan and assumptions about its space-filling properties suggest that it may be only one molecule thick. Most of the Gram-negative wall consists of an outer membrane, seen in electron microscopy as a double-track membrane but chemically very different from the bacterial cytoplasmic membrane. Both groups of bacteria often display a regularly arrayed layer of protein units (S-layer) on the outer surface of the wall, and the cells may be surrounded by a loosely-attached capsule of polysaccharide or protein (Klein, 1982).

Muramic acid

Elucidation of the structural and functional relationships in microflora communities has long been a goal of microbial ecologists. Toward this end, some means of biomass characterization is obviously necessary. Existing methods of biomass determination, however, are not wholly satisfactory.

Classical plate counting methods reportedly understate the numbers of organisms by factors of 6 to as much as 700,000 when compared with counts made by direct microscopic examination (Butkevich, 1932; Collins and Kipling, 1957; Jannasch and Jones, 1959; Perfil'ev and Gabe, 1969). The viability of a great majority of these organisms has been shown in studies of vital stain incorporation (Alfimov, 1954; Kusnetsov, 1958; Strugger, 1949). Unfortunately, direct counting techniques are tedious as well as difficult to carry out on sediments and organic detritus.

Another measure of biomass in wide use is the determination of adenosine triphosphate (ATP) levels in water columns and sediments (Ausmus, 1973). It has been shown that ATP levels in the water column correspond to approximately 0.04% of the cellular organic carbon content (Holm-Hansen, 1973). ATP levels, which are rapidly dissipated after metabolic death, indicate 50 to 2000 times more organisms than can be determined by plate counts (Holm-Hansen and Booth, 1966). On the other hand, ATP levels depend on the physiological state of the organisms. Thus, rapid changes in ATP levels in a constant number of cells are readily induced (Goldenbaum, 1975). ATP is also found in the microeucaryotes.

Clearly, other measures of biomass are necessary for the better description of complex environmental assemblages of microflora. Further differentiation is possible with an assay of

muramic acid (3-O-carboxyethyl-D-glucosamine). To date, this compound has been detected only in the muramyl peptide of cell walls in prokaryotic bacteria and blue-green algae. This test was first used with the analysis of muramic acid in soils (Millard and Casida, 1970). Assays of laboratory-grown cultures by these workers showed a muramic acid content of 3.44 ug/mg (dry weight) for seven species of Gram-positive organisms and 9.6 ug/mg for five species of Gram-negative organisms. Based on these assays, an estimation of the average bacterial muramic acid content of 6.4 ug/mg can be calculated. Variability by a factor of two in muramic acid levels has been reported in various bacilli under nutrient-limiting conditions in a chemostat (Ellwood and Tempest, 1972). In other experiments conducted in marine environments they found good separation of muramic acid from other interfering compounds with satisfactory reproducibility. They also found that the muramic acid content of natural marine water decreased with increasing depth (Mimura and Romano, 1985).

Some methods for the detection of bacterial cell wall in tissues such as external labeling with 125 I, fluoroscein, or rhodamine, present several problems (Doble et al., 1975; Ginsburg and Trost, 1971; Vernon-Roberts et al., 1976). These include uncertainty of the precise component which is labeled, possible alteration of biological properties by the conjugation, and uncertainly that the label remains associated with the cell wall

structures *in vivo*. Detection of cell wall antigens by immunofluorescent or radioimmunoassay can be applied to human tissue specimens, but the specificity of the antigen being sought must be known before testing. In addition, host antibody could block the antigenic site, or the host may modify immunodeterminants on cell wall antigens (Wheat et al., 1978). A chemical assay of muramic acid does not suffer from these constraints. The obvious advantage of the detection of this natural internal label is that the presence of cell wall debris from any bacterial species can be detected and quantitated in human as well as animal tissues.

Amines in foods and their analysis

Amines are basic nitrogenous compounds in which one, two or three atoms of hydrogen in ammonia are replaced by alkyl or aryl groups. Aryl-substituted amines are uncommon in nature, and the carcinogenic property of many synthetic aryl amines may explain their rarity. The simple aliphatic monoamines are widespread. These amines are usually formed by decarboxylation of amino acids in bacteria, but in plants aldehyde amination is a common mode of formation. Methylamine and ethanolamine are probably ubiquitous, but together with di- and trimethylamine they may also be easily formed as artifacts by degradative processes in

the course of isolation (Lerch and Stegemann, 1966; Hartmann et al., 1972).

The diamine putrescine and the polyamines spermidine and spermine probably occur universally in animals and plants, and at least putrescine and spermidine are found in most bacteria. These amines are important in the regulation of nucleic acid function and protein synthesis, and probably also in the stabilization of membranes. In all organisms spermidine and spermine are formed from putrescine by successive donation of one or two aminopropyl groups, respectively, from decarboxylated S-adenosylmethionine (Janne et al., 1978; Smith, 1977). Certain classes of amines, the cathecolamines, indoleamines and histamine, fulfil important metabolic functions in man, especially in the nervous system and in the control of blood pressure. These amines occur widely in animals, plants and bacteria, and they are frequently found in high concentrations in foods, especially in that which has been subjected to deliberate or accidental bacterial contamination (Stockley, 1973).

Amino acid decarboxylation is the most common mode of synthesis of most of the amines. Phenethylamine and the cathecolamines, like tyramine, causes a rise in blood pressure by constricting the vascular system and increasing the heart rate and force of contraction of the heart. These are known as pressor amines. By contrast, histamine reduces the blood pressure by

causing vasodialation. Phenethylamine, tyramine and histamine are found in animals, plants and bacteria. Adrenaline, also known as epinephrine, is formed only by animals, but noradrenaline is formed by animals and plants. Bacteria do not appear to produce noradrenaline and adrenaline. Under normal circumstances in man exogenous amines aborted from food are rapidly detoxified by amine oxidases or by conjugation. Histamine is oxidized by a diamine oxidase while the cathecolamines are oxidized by a different multiple enzyme system known as monoamine oxidase (MAO). For instance after oxidation by MAO, tyramine is excreted as p-hydroxyphenylacetic acid.

When MAO inhibitors were first used in the treatment of tuberculosis and subsequently for depressive illness, a number of patients developed severe hypertension and these attacks were traced to the accumulation in the body of high concentrations of pressor amines like tyramine derived from foods, notably cheese. The parent amino acid, tyrosine, occurs at high concentrations in cheese and it was from this source that tyrosine was first isolated. Some pressor amines, like noradrenaline, may increase blood pressure directly by constricting the vascular system and stimulating the heart muscle. However, tyramine does not indirectly by causing the release of noradrenaline from the sympathetic nervous system (Stockley, 1973). The increase in blood pressure, now known as the 'cheese reaction', can cause

severe headache and may induce a brain hemorrhage or heart failure. One of the functions of MAO in the intestine and liver is apparently to destroy potentially harmful amines derived from food before they reach the blood. The rapidity of the reaction, in some cases within five minutes, suggested that absorption of at least part of the amine may take place through the oral mucosa, bypassing the intestinal and hepatic MAO. Inhibition of the neuronal MAO would therefore render the subject very sensitive to amines entering by this route. The absorption of amines in this way is dependent on alkaline pH and on the length of time the food is retained in the buccal cavity (Price and Smith, 1971).

It is well known that fish in the family *Scombridae*, which includes tuna and mackarel, are liable to cause a characteristic food poisoning. Tuna fish may be unsuitable for consumption for this reason even before spoilage can be detected by organoleptic examination (Lenistea, 1972). Recent literature on this subject has been reviewed by Arnold and Brown (1978), and Sinnel (1978). Vomiting, abdominal pain, facial flushing and headache are the most notable effects of scombroid fish poisoning. Onset is rapid, but recovery is usually complete within 8 h (Cruickshank and Williams, 1978). Some of these symptom closely resemble those of a histamine reaction, and it is not surprising to find high concentrations of histamine in suspect foods. Unlike tyramine, histamine is a powerful vasodialator, and this accounts for the

facial flushing in histamine toxicity. Fish with a histamine content above 1 mg/g is usually toxic (Cruickshank and Williams, 1978). Early reports of a separate toxin named 'saurine' have proven to be attributable to histamine (Foo, 1976). The histamine is formed by bacterial decarboxylation of the amino acid histidine, which is a major component in muscle in the fish causing this reaction. Histidine decarboxylase is an inducible enzyme, and it occurs in many species of bacteria. The apparent high incidence of scombroid fish poisoning in hospitals could be related to the availability of the means for diagnosis and may indicate that such symptoms are fairly common in the general population (Popovich et al., 1960). One interesting fact is that some seawater cultures are able to degrade puterscine and cadaverine. In this experiment, it was shown that bacteria show a potential to mineralize putrescine, indicating a general degradation potential for this compound by marine bacteria even if the compound was not present during growth (Hofle, 1984).

In order to explain the histamine reaction found when the histamine in the food does not achieve high concentration, it has been suggested that other substances found in the food may be synergistic, enabling absorption of amounts of histamine larger that could be achieved in the absence of food. In man, histamine is normally detoxified by a diamine oxidase found in the intestinal wall, and the high concentrations of the diamines, putrescine and

cadaverine, in spoiled fish may depress histamine oxidation and act as synergistic factors (Karmas and Mietz, 1978; Parrot and Nicot, 1966). However, the possible presence of an alternative toxin cannot be eliminated. The relative proportions of putrescine, cadaverine, spermidine, spermine and histamine may be useful as quality indicators of fish, lobster and shrimp (Karmas and Mietz, 1978).

Considerable interest has been generated in recent years regarding biogenic amines in foodstuffs. Microbial spoilage in foods leads to the production of decarboxylases which under appropriate conditions can convert amino acids into their corresponding amines. Thus, the diamines cadaverine and putrescine, which are formed from the amino acids lysine and ornithine, are products of bacterial decomposition of animal tissue, and histamine is formed from the amino acid histidine in some foods by a similar mechanism.

The separation and analysis of these nitrogenous compounds is generally accomplished by gas and liquid chromatography or by High-Performance Liquid Chromatography (HPLC) but the sample preparation steps are very tedious (Straruszkiewicz and Bond, 1981; Henion et al., 1981). Fluorimetry is a sensitive method but it is limited in its specificity to a few amines. Ion-exchange column chromatography (IEC) with automated instruments is the most favored method for routine

analysis of mono-, di- and polyamines in biological material. However, most of the ion exchange methods only involve separations of putrescine, cadaverine, spermidine, spermine, histamine and a few other related compounds such as tyramine and tryptamine (Adler et al., 1977).

Physiologically active amines may accumulate in meat products due to bacterial activity. Usually only low concentrations of histamine are found, but tyramine can occur at over 300 ug/g. Several meat products would provide sufficient tyramine in normal servings to induce a pressor response in patients taking MAO inhibitors. Chicken liver which caused a hypertensive crisis contained about 100ug/g, though normally such liver would contain only 0.5 ug/g (Hedberg et al., 1966). Histamine of physiological origin occurs in quite high concentrations (140 ug/g) in the skin of birds (Henry, 1960). In a study of the formation of volatile amines in uncured pork during storage at -20°C to +20°C, amine formation was independent of bacterial growth until the count reached $10^9/cm^2$. The meat was spoiled before dimethylamine was formed in amounts sufficient for the detection of N-nitrosodimethylamine (Patterson and Edwards, 1975).

Meat freshness can be evaluated by organoleptic assessment, bacterial count and chemical determination of bacterial metabolites. Since bacterial analysis is time-consuming, requiring 1 to 3 days, and organoleptic assessment still contains

elements of subjectivity, chemical methods offer an alternative to conventional bacteriologic and organoleptic techniques.

For decades, chemical evaluation of meat freshness has been the target of many investigations in the search of rapid, objective tests for determining meat quality (Pearson, 1968; Turner, 1960). Gilka et al. (1980) surveyed the recent chemical methods. All these tests have drawbacks such as the limited specificity for certain bacteria like the reductase-positive types for the resazurin test, or they do not correlate with organoleptic assessment, or compounds that are measured appear after the meat is spoiled. These disadvantages stem from the fact that bacterial metabolites used for chemical assays are not formed by the dominant spoilage microflora (Fields et al., 1968). There are several considerations when studying bacterial metabolites as chemical indicators of freshness, among them the relationship of each compound to specific spoilage microorganisms and whether or not their formation occurs during the initial stages of spoilage and in quantities sufficient for detection (Slerm ,1981). Also, the generally low carbohydrate content of beef (1% or less) places spoilage organisms in the position of utilizing free amino acids as sources of energy. Spoilage is generally caused by *Pseudomonas* sp., psychrotrophs and enterobacteria (Jay, 1978).

Many of the published methods for the analysis of amines in food products use High Performance Liquid Chromatography

(HPLC) techniques as well as Gas Chromatography (GC). Lin and Chen (1980) developed a high performance liquid chromatography method for the analysis of ammonia, and other amines in fish and shrimp. The aliphatic amines were modified by pre-column derivatization with dabsyl chloride which stabilizes the molecules, facilitates extraction and improves detection at nanogram amount. The complete assay procedure takes about 3 hours. Another method uses a single column amino acid auto-analyzer (Sayem-El-Daher, 1983). Using a Technicon (Tarrytown, NY) C_4 ion-exange resin column adapted to a Technocon TSM amino acid analyzer 15 different amines were separated and quantified. The method was assayed on ground beef, cheese and wine. One of the of the most rapid, effective and sensitive ways to analyze amines in by the use of HPLC in conjunction with paired-ion chromatography. In a survey of 61 cheeses, 13 sausages and 10 chocolates samples for amines, tyramine was found to be the most prevalent and with higher concentrations (Koehler and Eitenmiller, 1983). In the analysis of chocolate and other derivatives spectrofluorometric and thin layer chromatography techniques have also been used (Santos-Buelga et al., 1983). In their study they found that the amounts of tyramine ranged between 0.1 and 2.8 ug/g. A flow-injection procedure utilizing fluorescence detection has been developed for the determination of primary, secondary and tertiary amines in nonaqueous media.

Primary amines are derivatized with o-phthaladehyde and 2-mercaptoethanol and secondary amines with 7-choro-4-nitrobenzo-2-oxa-1,3-diazole. In the secondary amine determination, interfering primary amines are masked by reaction with o-phthalaldehyde and 2-mercaptoethanol. Tertiary amines were determined by observing their catalytic effect on the reaction between acetic anhydride and malonic acid (Whiteside et al., 1988; Whiteside et al., 1988). An on-column derivatization of primary and secondary amines with trifluoroacetic anhydride or heptafluorobutyryl chloride is the basis of their determination by gas chromatography. Detection of their derivatives can then be accomplished by mass spectroscopy (Mikaya et al., 1989). The HPLC separation of dansyl derivatives of amines is the basis of several methods for amine analysis. Among the types of amines analyzed in this way are mono-, di-, and triamines, aniline derivatives in surface water, and morpholine, benzylamine, cyclohexylamine, ammonia, and hydrazine in water samples (Geerdink, 1988; Gaetani et al., 1989). In another study it was found that ethylenediamines give bisdansyl derivatives which can be detected by their fluorescence spectra. Derivatization, however, cannot be carried out in alcohol solutions because the dansyl esters are also highly fluorescent (Kallmayer, 1989). Various derivatization procedures have been reported for the analysis of amines by reverse-phase HPLC. In one method, 2, 4

dinitrofluorobenzene derivatives of amino alcohols were used (Chang et al., 1987), while in another salicylaldehyde-diphenyl-boron chelates of ethylenediamine and triethylenetetramine were employed (Winkler, 1988). In the method described by Chang et al., the 2,4 dinitrofluorophenyl derivatives are separated using reverse-phase liquid chromatography and are detected using the hybrid photolysis-electrochemical detector in tandem with UV absorbance detection. Following optimization of reaction, chromatographic, and detection variables, the derivatization detection approach provides limits of detection in the low parts per billion range, with a linearity of three orders of magnitude. A method for the determination of the enantiomeric composition of tertiary amines is based on selective N-demethylation using vinyl chloroformate, followed by an acid-catalyzed cleavage of the vinyl carbamate. The enantiomeric secondary amines are converted into the diastereomeric urea derivatives with (R)-(+)-1-phenyllethyl isocyanate which can then be analyzed by HPLC (Mailbaum, 1988).

The evolution of putrescine, cadaverine, histamine, spermine and spermidine concentrations in spoiling pork and beef has been studied by Slerm (1981). Sterile meat was inoculated with *Pseudomonas, Enterobacteriaceae* species separately and with a mixture of several bacterial strains. She obtained a strong correlation attributing putrescine formation to *Pseudomonas* and

cadaverine to *Enterobacteria,* while no correlation was found with histamine, spermine and spermidine during spoilage of pork. On the other hand, the increases in putrescine and cadaverine was slightly lower in beef than in pork. Few studies report on the relationship of microbial quality of ground beef and its content of putrefactive amines (Sayem-El-Daher, 1983). Spinelli et al. (1974) studied the effects of processing on the amine content of pork bellies. They found that the concentration per 100 grams of tissue ranged from 0.03 mg for cadaverine to 8.1 mg for spermine. They also found that processing does not significantly alter the levels of the free amines.

Bacterial sources of putrescine and cadaverine have been studied by Dainty (1986). Of the meat strains of Leuconostocts *Enterobacteriaceae and Brochothrix thermosphacta* tested, only *Hafnia alvei* and *Serratia liquefaciens* showed diamine-producing potential during growth in pure culture on beef stored in vacuum packs at 1°C. Putrescine concentrations produced by the two organisms were an order of magnitude lower. During the growth on beef of either *H. alvei* or *S. liquefaciens* in a mixed culture with arginine-utilizing strains of Streptobacteria, putrescine as well as cadaverine concentrations were similar to those detected in naturally contaminated samples.

In another study, the possible production of putrescine and cadaverine in meat by single strains of bacteria was studied (Slerm

and Ritter, 1984). Beef samples were inoculated with one of the following cultures, *Lactobacillus sake, L. curvatus, L. plantarum, Staphylococcus xylosus, Staph. simulans, Streptococcus faecalis* and *Strep. faecium*. All samples were vacuum-packaged and stored at 4°C. Concentrations of putrescine and cadaverine remained throughout in the ranges found in fresh meats, i.e. traces-0.28 and traces-0.35 mg/100 g respectively. None of the strains studied increased diamine content during storage of vacuum-packaged beef. Yano et al. (1990) studied the potential of using diamines as indices for freshness of both aerobically packed and vacuum-packed meat. They found that at advanced stages of putrefaction, the major spoilage bacteria were Gram-negative psychrophiles in meat stored under modified atmosphere of 25% CO_2/75%N_2, and lactic acid bacteria in meat stored under vacuum-packaging conditions. Levels of spermine and spermidine were fairly constant during storage. Putrescine and cadaverine were not detected in fresh samples, becoming detectable after 8 and 7 days, respectively, in samples stored under aerobic conditions. In another study conducted in beef stored under vacuum-packaging conditions, the development of a natural spoilage flora during storage led to increases of concentrations of both putrescine and cadaverine and production of a 6th amine, tyramine, in addition to spermine, spermidine and histamine. Pure culture meat inoculation experiments showed tyramine formation

to be restricted to lactobacilli and to stains of *Lactobacillus divergens* and *L. carnis* in particular (Edwards et al., 1987).

In a study conducted on beef, pork and lamb during aerobic chill storage, putrescine concentrations increased consistently with 'total' aerobic viable count but cadaverine concentration increased only when high numbers of presumptive *Enterobactericeae* were present (Edwards et al., 1983). In another study conducted in inoculated pork spoilage induced by Pseudomonas increased the putrescine concentration greater than ten fold, while enterobacteria raised the cadaverine concentration greater than 100 fold (Slerm, 1981). Concentrations of histamine, spermine and spermidine did not change considerably in the initial stage of spoilage. Natural spoilage was simulated by a mixture of the tested strains. The bacterial mixture caused a significant increase of putrescine and cadaverine concentrations. The concentration increase was well correlated with the sensory assessment and surface bacterial counts. In beef the bacterial mixture caused somewhat lower increases in amine concentration. In a publication by Lin and Chen (1982) a fast and sensitive method for the analysis of amines by HPLC and TLC with dabsyl chloride was developed. In this method the amines were derivatized with dabsyl choride and then separated by a normal-phase chromatographic column with 2% acetone in chloroform as mobile phase. In addition they were also able to separate the dabsyl derivatives of

putrescine, spermidine, spermine, ammonia and ornithine on TLC. In this dissertation the extraction and derivatization processes described in this publication were modified in order to analyze cadaverine in beef, pork and poultry. In addition all the quantification process was performed directly on the TLC by the use of a densitometer. The use of these two techniques (TLC followed by densitometry) would help the bacteriological laboratory in any industry since both techniques require instruments which are not as expensive and technically complicated as an HPLC or a GC. In addition the time require to run this test is only 2 hr which would be a great advantage over the traditional bacteriological techniques which are used in the meat industry.

PART 1. STUDY OF THE POSSIBLE CORRELATION BETWEEN MURAMIC ACID AND TOTAL BACTERIAL NUMBERS IN MEATS

INTRODUCTION

Interpretation of the structural and functional relationships in microfloral communities has long been a goal of microbiologists. Toward this end, some method of biomass characterization is obviously necessary. Existing methods of biomass determination, however, are not wholly satisfactory. Classical plate counting methods many times understate the number of microorganisms when compared with counts made by direct microscopic examination (Collins and Kipling, 1957; Jannasch and Jones, 1959; Perfil'ev and Gabe, 1969).

Another measure to characterize biomass that is widely used is the determination of adenosine triphosphate (ATP) levels in water columns and sediments (Ausmus, 1973). Clearly, other measures to characterize biomass are necessary for a better description of complex environmental assemblages of microflora. One possibility is the analysis of muramic acid, which is a component of the muramyl peptide found only in the cell wall of bacteria and blue-green algae. King and White (1977) worked on a method to measure microbial biomass in estuarine and marine samples. They were able to find muramic acid levels of 100-700 ug/g. Muramic acid is an interesting compound because of its unique occurrence in the peptidoglycan polymer of bacterial cell walls. Millar and Cassida (1970) demonstrated a method for

muramic acid analysis that involved the extraction of soil, deionization, hydrolysis of lactic acid from the muramic acid, and, finally, analysis for lactic acid. In addition, Zelles (1988) investigated muramic acid and glucosamine in soil by HPLC with the use of fluorescence derivatization.

The present study was undertaken to recover muramic acid, quantitate it, and determine if there is a possible correlation of the amounts of muramic acid and total bacterial counts in meat samples.

This part of the dissertation is arranged by experiments with materials and methods followed by results and discussion of each experiment.

MATERIALS AND METHODS (EXPERIMENT I)

Fresh, boneless pork loins and eye of the beef round obtained from the Iowa State University Meats Laboratory were cut into 2.5 cm thick pork chops or beef steaks. Groups of pork chops and beef steaks were arranged randomly and assigned also at random to any one of two treatments (air permeable storage or vacuum-packaging storage). Vacuum-packaging bags were used in the second treatment (O_2 permeability < 1 ml/645 cm^2/24 hr at 22.8°C and 0% RH) of Nylon/Saran Surlyn laminate (Curlon 892, Curwood, Inc., New London, WI). The packaged meat was vacuum sealed (1 KPa) in a multivac MG-2 (Sepp-Haggenmuller KG, West Germany). All samples were stored at 2-4°C until the day of the examination.

Samples stored under air permeable conditions were tested on day 0 and then every other day until the meat reached bacterial counts of logCFU (log of colony forming units) 6 or above. In the case of meat samples stored under vacuum-packaging conditions all samples were tested every week for a period of five weeks until the samples reached bacterial counts of logCFU 6 or above. On each sampling day one pork chop or one steak from each treatment was taken from storage for microbiological determinations as well as for the analysis of muramic acid. Each package was opened aseptically, and sterile tongs were used to

remove the piece of meat. Using a 2 cm^2 sterile aluminum template, five different 2 cm^2 areas of meat surface (for a total of 10 cm^2) were swabbed with two sterile cotton swabs. Swabs were aseptically broken into 100-ml 0.1% sterile peptone water blanks and shaken according to recommended procedures. Appropriate serial dilutions were made for plating. Total psychrotrophic counts were enumerated using APT agar (30°C, 48 hr). Lactobacilli were enumerated by using lactobacilli selective (LBS, BBL) agar acidified with acetic acid (30°C, 71 hr). Experiments with pork chops under aerobic conditions were replicated nine times. Pork under anaerobic conditions were replicated six times. Both beef treatments were replicated five times.

For the analysis of muramic acid the method described by Hadzija (1974) was used. Areas of meat measuring 10 cm2 were swabbed and the swabs were placed in screw-cap tubes. Five ml of 4 N HCl were added to each tube to destroy the cell wall of any bacteria present. The tubes were held at room temperature. After 4 hr, 5 ml of 4 N NaOH were added to each tube to neutralize the contents of the tubes. At the same time, standards of muramic acid containing .90 to 9.00 ug of lactic acid from the muramic acid stock solution were prepared as well as a blank. .01 or .05 ml samples were transfered to other tubes and the volume adjusted to 1 ml. Then, .5 ml of 1.0 N NaOH was added to each tube and incubated for 30 minutes at 37°C. After this step 10 ml of

concentrated sulfuric acid were added to each tube to separate the muramic acid molecule N-acetyl-glucosamine. The tubes were well stoppered and placed for 10 minutes in boiling water. This step helps to release the muramic acid molecule. After heating, the tubes were cooled in running water and 0.1 ml $CuSO_4$ solution and 0.2 ml of p-hydroxydiphenyl solution were added. These two reagents provide the color needed for the actual quantification of the muramic acid. The tubes were stoppered again, shaken, and kept at 30°C for 30 minutes for the color reaction to develop. The absorbance was measured at 560 nm in a Baush and Lomb Spectronic 20 spectrophotometer. A reagent blank was used to adjust the spectrophotometer to zero absorbance.

RESULTS AND DISCUSSION (EXPERIMENT I)

Both the quantity of muramic acid and total numbers of psychrotrophic bacteria in beef and pork increased with time in storage. In pork samples stored under aerobic conditions, the muramic acid content ranged from 10 ug/cm^2 to about 120 ug/cm^2 and total psychrotrophic bacteria numbers ranged from 10^1 to 10^5 (Figure 1). The correlation between bacterial numbers and muramic acid levels was only 0.22 (Figure 1). Since the points on this figure do not remove the variation due to replications, bacterial numbers and muramic acid levels were averaged over replications and a correlation among these averages on each day was made (Figure 2). Using replication averages, a correlation of 0.98 was obtained. This would indicate a very strong relationship between bacterial numbers and muramic acid levels. However, nine replications would be needed to reach this correlation level.

In pork samples stored under anaerobic conditions, the muramic acid content ranged from zero to over 200 ug/cm^2 and bacterial numbers from 10^2 to 10^7 (Figure 3). The correlation between bacterial numbers and muramic acid levels was only 0.28 (Figure 3). Averaging both bacterial numbers and muramic acid values over replications on each day improved the correlation to 0.84 (Figure 4). This correlation was based on averages of six replications. If more replications were used then higher

correlations could be expected; similar to pork stored in aerobic conditions.

The numbers of lactic acid bacteria and muramic acid levels were averaged over replication for each day and a correlation between the averages was determined (Figure 5). However with lactic acid bacteria a low correlation (0.33) was found even after averaging. This indicates that muramic acid is not a good indicator of numbers of lactobacilli.

In beef samples stored under aerobic conditions, the muramic acid content ranged from 20 ug/cm^2 to about 110 ug/cm^2 and total psychrotrophic bacteria numbers ranged from 10^1 to 10^5 (Figure 6). The correlation between bacterial numbers and muramic acid levels was only 0.24 (Figure 6). Since the points in this figure do not remove the variation due to replication, bacterial numbers and muramic acid levels were averaged over replications and a correlation among these averages on each day was made (Figure 7). Using replication averages, a correlation of 0.37 was obtained. This would indicate a weak relationship between bacterial numbers and muramic acid.

In beef samples stored under anaerobic conditions, the muramic acid content ranged from near zero to nearly 300 ug/cm^2 and bacterial numbers form 10^1 to over 10^7 (Figure 8). The correlation between bacterial numbers and muramic acid levels was only 0.44 (Figure 8). Averaging both bacterial numbers and

muramic acid values over replications on each day a correlation of 0.46 was obtained.

Lactobacilli numbers and muramic acid levels were averaged over replication for each day and a correlation between the averages was determined (Figure 10). However, with lactic acid bacteria a low correlation (0.36) was found even after averaging. This again indicates that muramic acid is not a good indicator of lactic acid bacterial numbers.

In both pork and beef stored under vacuum-packaging, larger amounts of muramic acid were obtained that those found in both pork and beef stored under total aerobic conditions. This perhaps can be explained by the fact that when meat is stored under vacuum-packaging conditions, a different bacterial flora will predominate. *Lactobacillus, Bacillus, and Enterobacter* are good examples of the type of flora which predominates in vacuum-packaged meats (Allen and Foster, 1960). These are all Gram positive bacteria, which means that larger amounts of muramic acid are expected because of the thicker peptidoglycan network which is present in all Gram positive bacteria. However muramic acid was not a good indicator of lactic acid bacteria (Figure 5). Under aerobic conditions, a Gram negative bacteria load is expected. Bacteria such as *Pseudomonas, Moraxella*, and *Acinetobacter* are the predominant species here. In Gram positive bacteria, as much as 90% of the cell wall consist of the

peptidoglycan, although another kind of constituent, teichoic acid, is present in small amounts. In Gram negative bacteria, only 5 to 20% of the cell wall is peptidoglycan, the rest of the wall consists of lipid, polysaccharide, and protein, usually present in a layer outside the peptidoglycan layer.

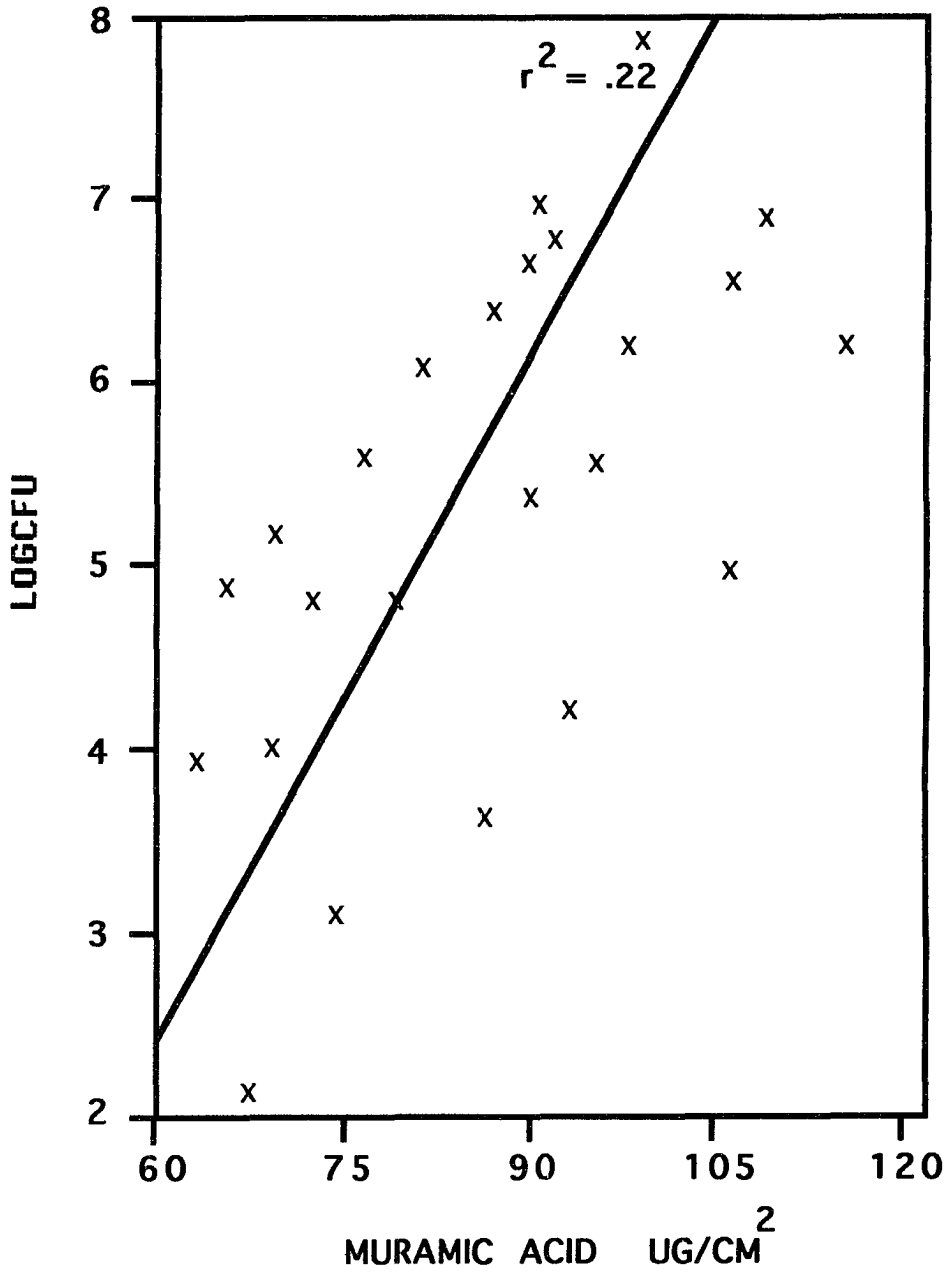

Figure 1. Correlation analysis between muramic acid and LogCFU in pork samples stored under aerobic conditions

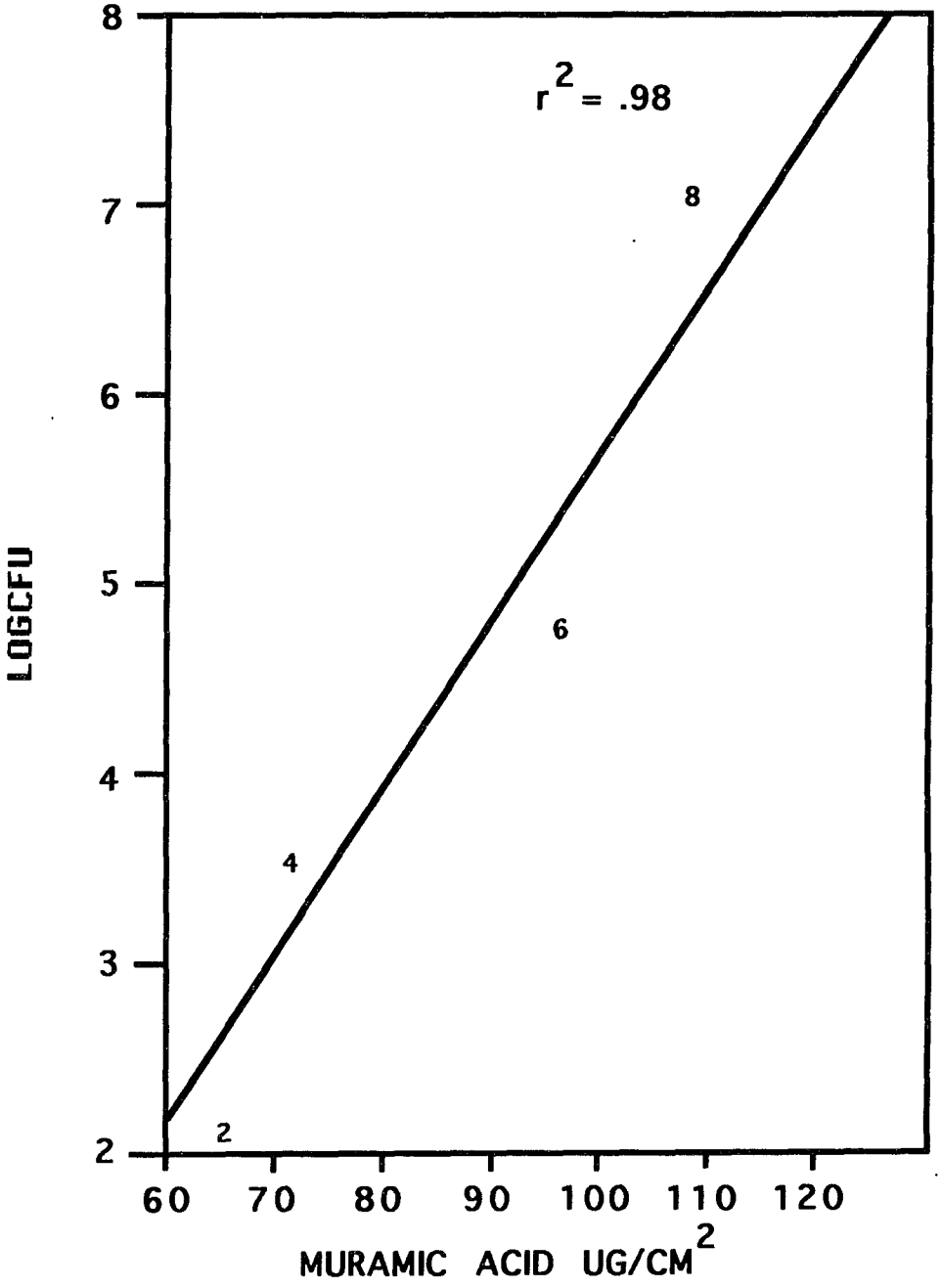

Figure 2. Correlation analysis between muramic acid and LogCFU in pork samples stored under aerobic conditions averaged over replications on days 2, 4, 6 and 8

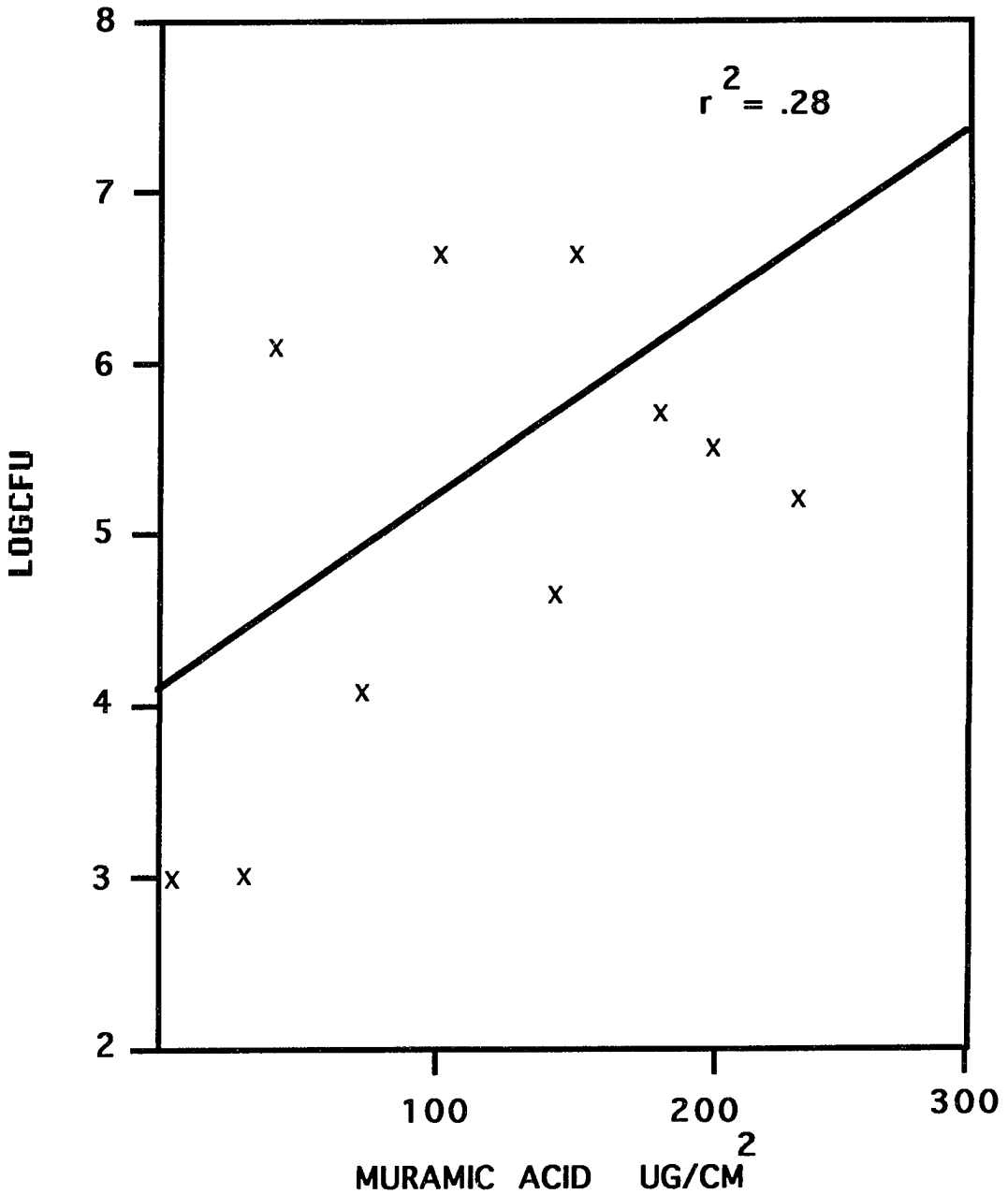

Figure 3. Correlation analysis between muramic acid and LogCFU in pork samples stored under anaerobic conditions

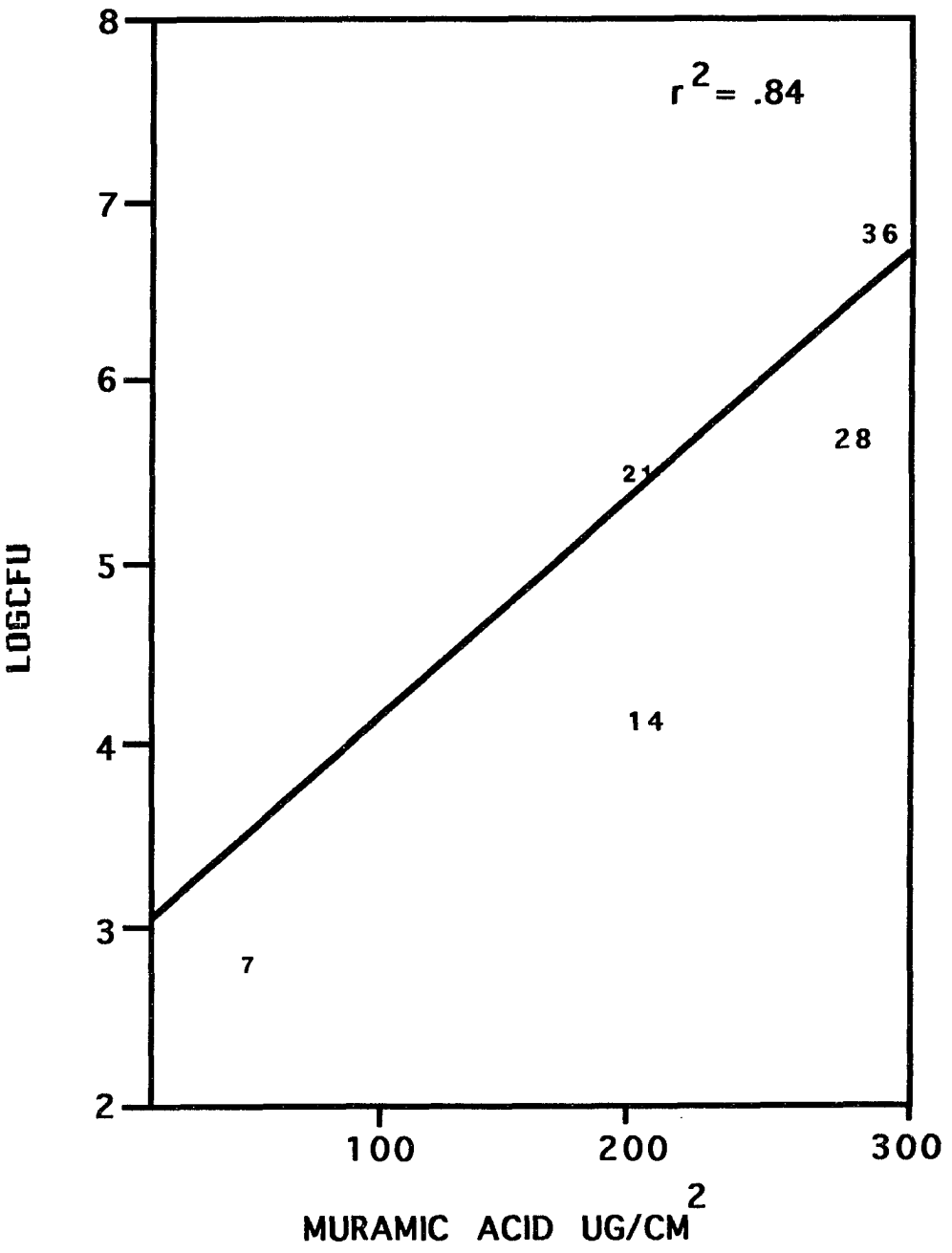

Figure 4. Correlation analysis between muramic acid and LogCFU of psychrotrophs in pork stored under anaerobic conditions averaged over replications on days 7, 14, 21, 28 and 36

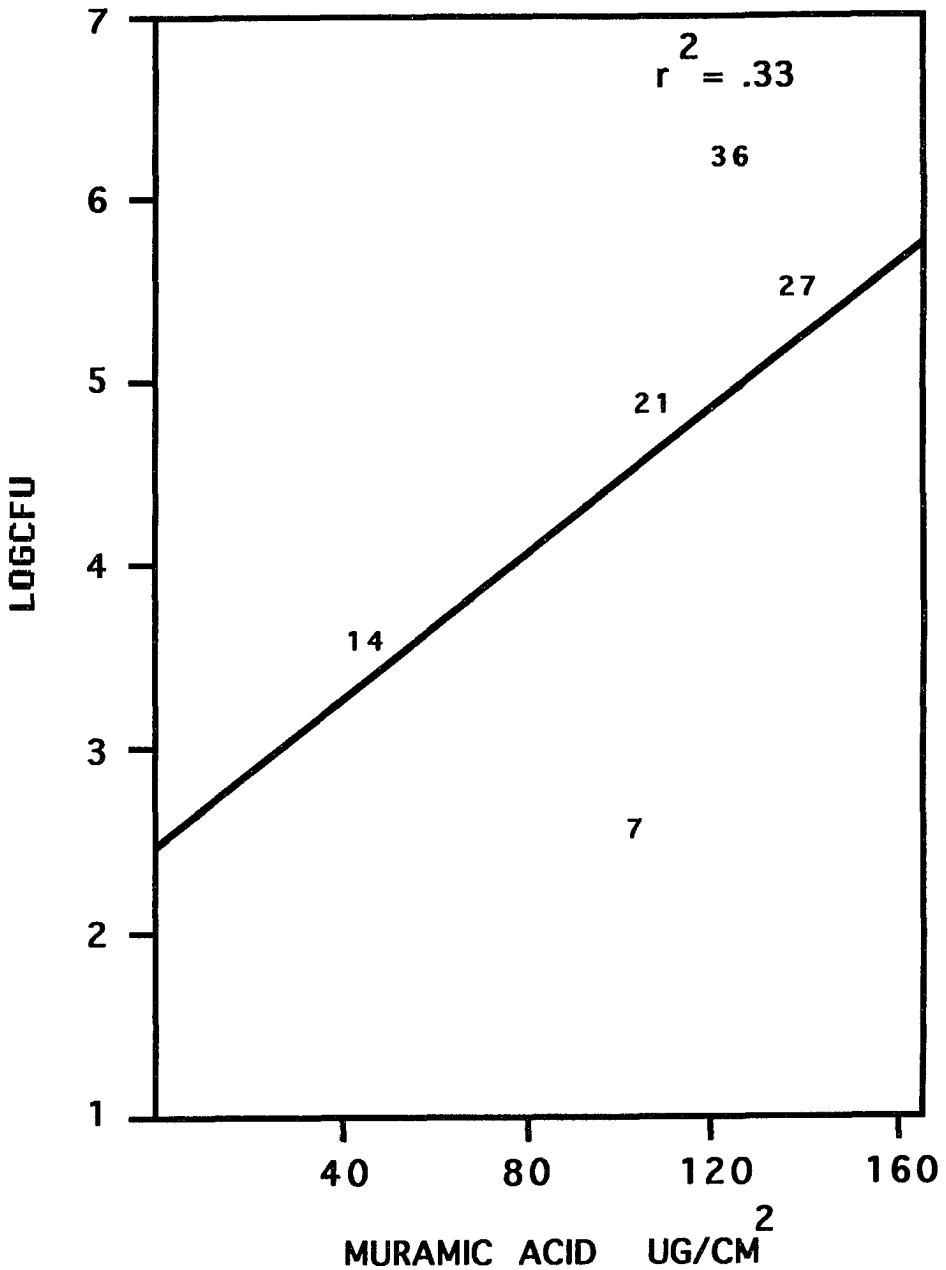

Figure 5. Correlation analysis between muramic acid and LogCFU of lactic acid bacteria in pork samples stored under anaerobic conditions averaged over replications on days 7, 14, 21, 28 and 36

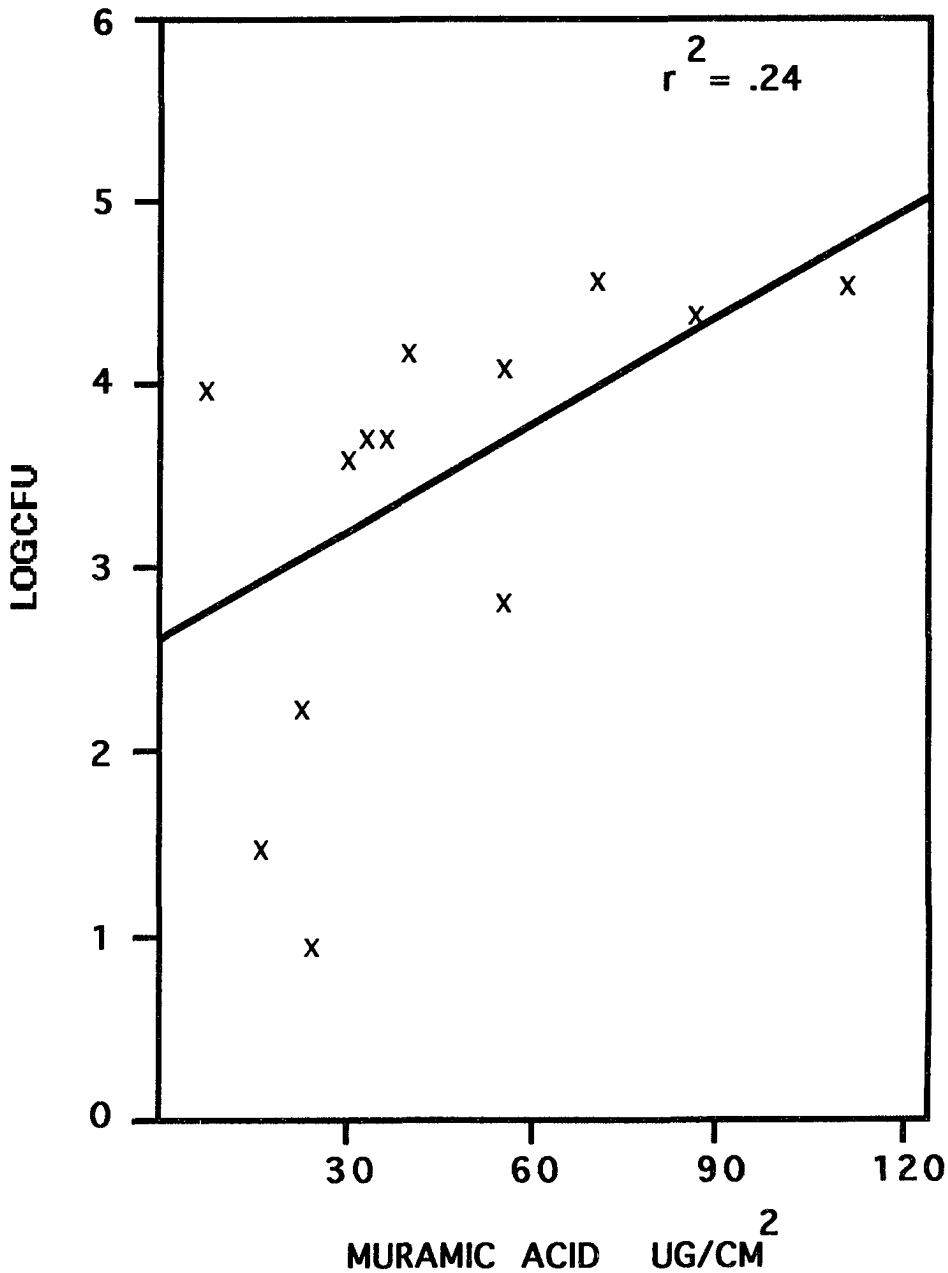

Figure 6. Correlation analysis between muramic acid and LogCFU in beef samples stored under aerobic conditions

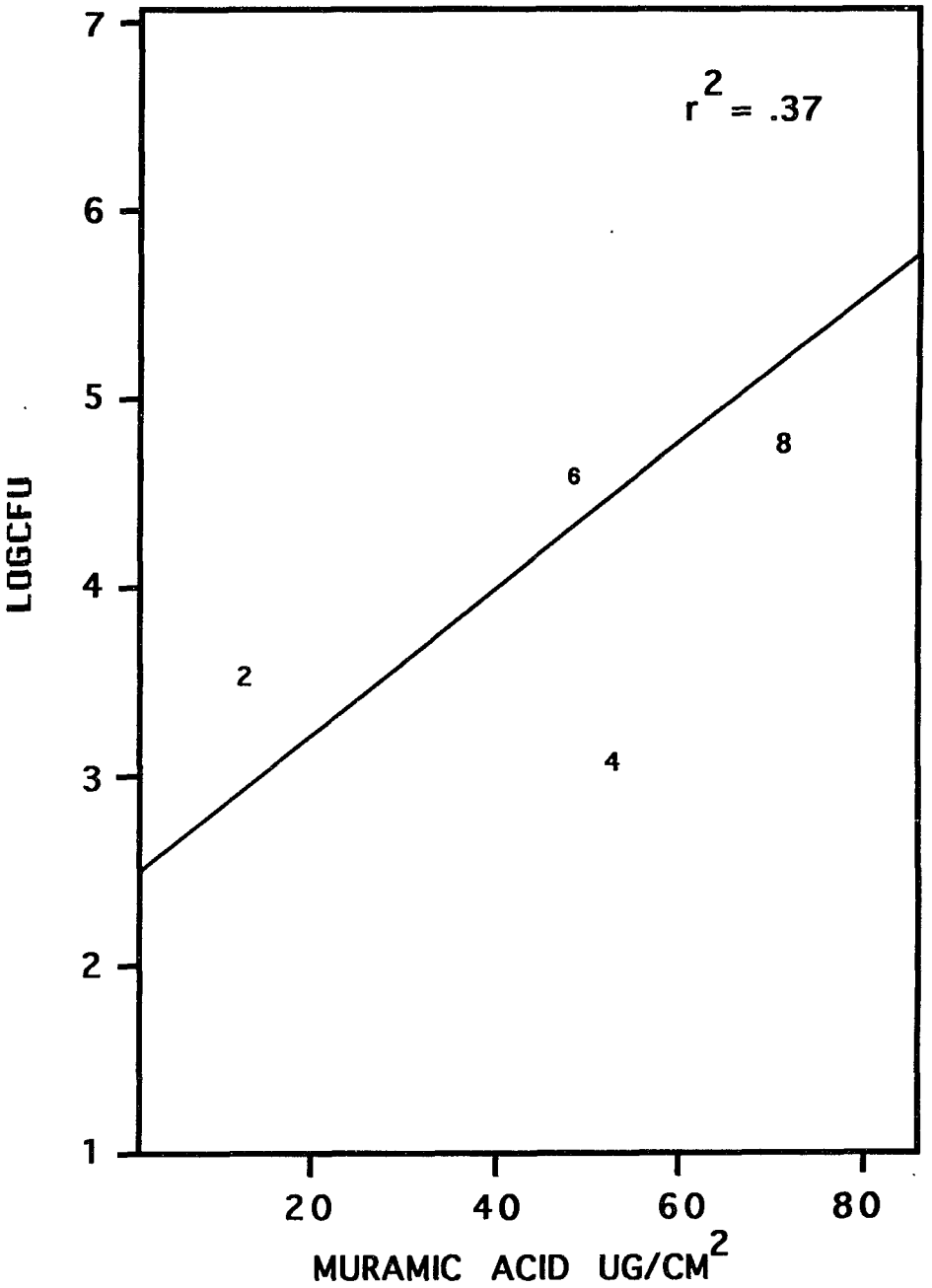

Figure 7. Correlation analysis between muramic acid and LogCFU in beef samples stored in aerobic conditions averaged over replications on days 2, 4, 6 and 8

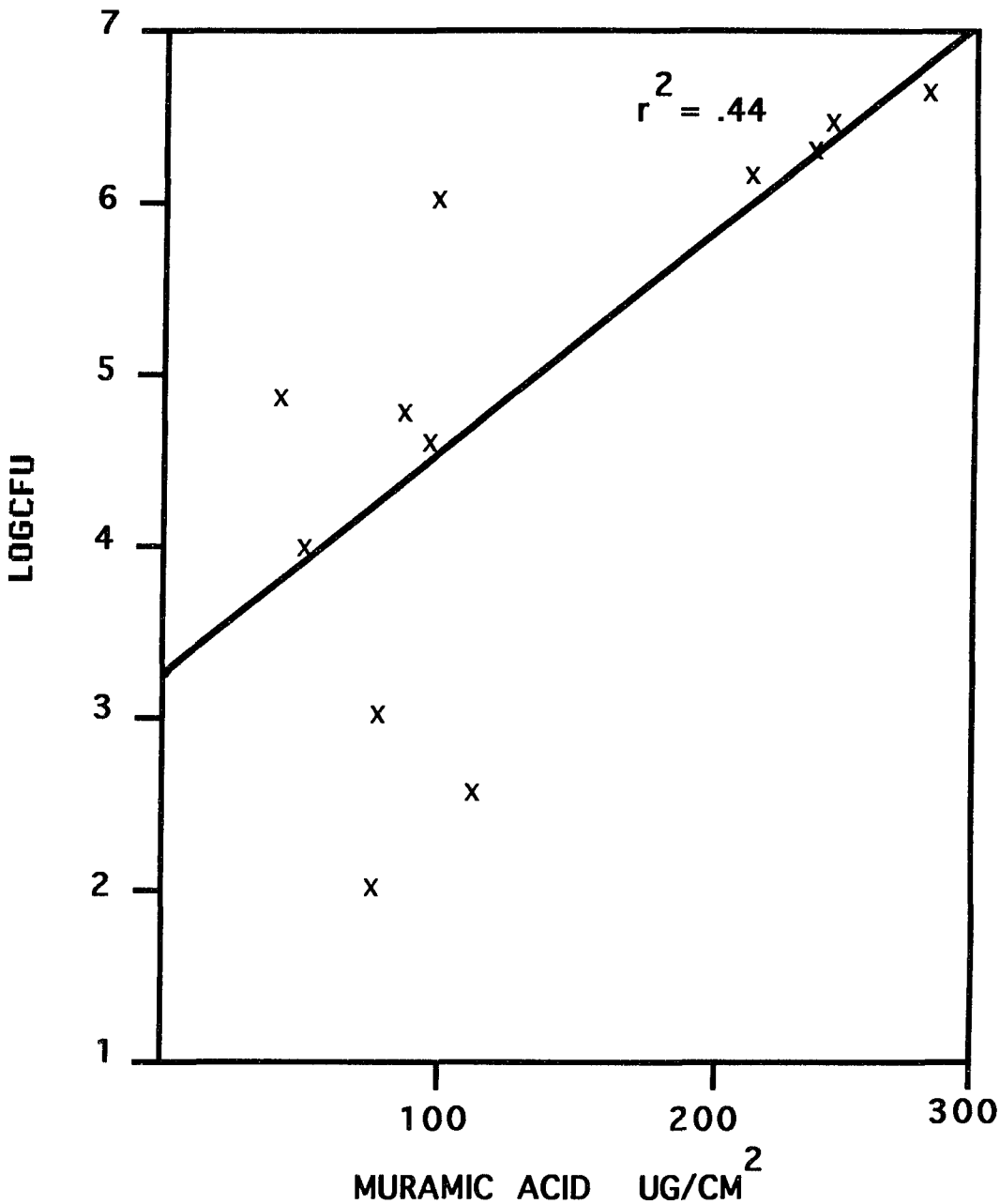

Figure 8. Correlation analysis between muramic acid and LogCFU in beef samples stored under anaerobic conditions

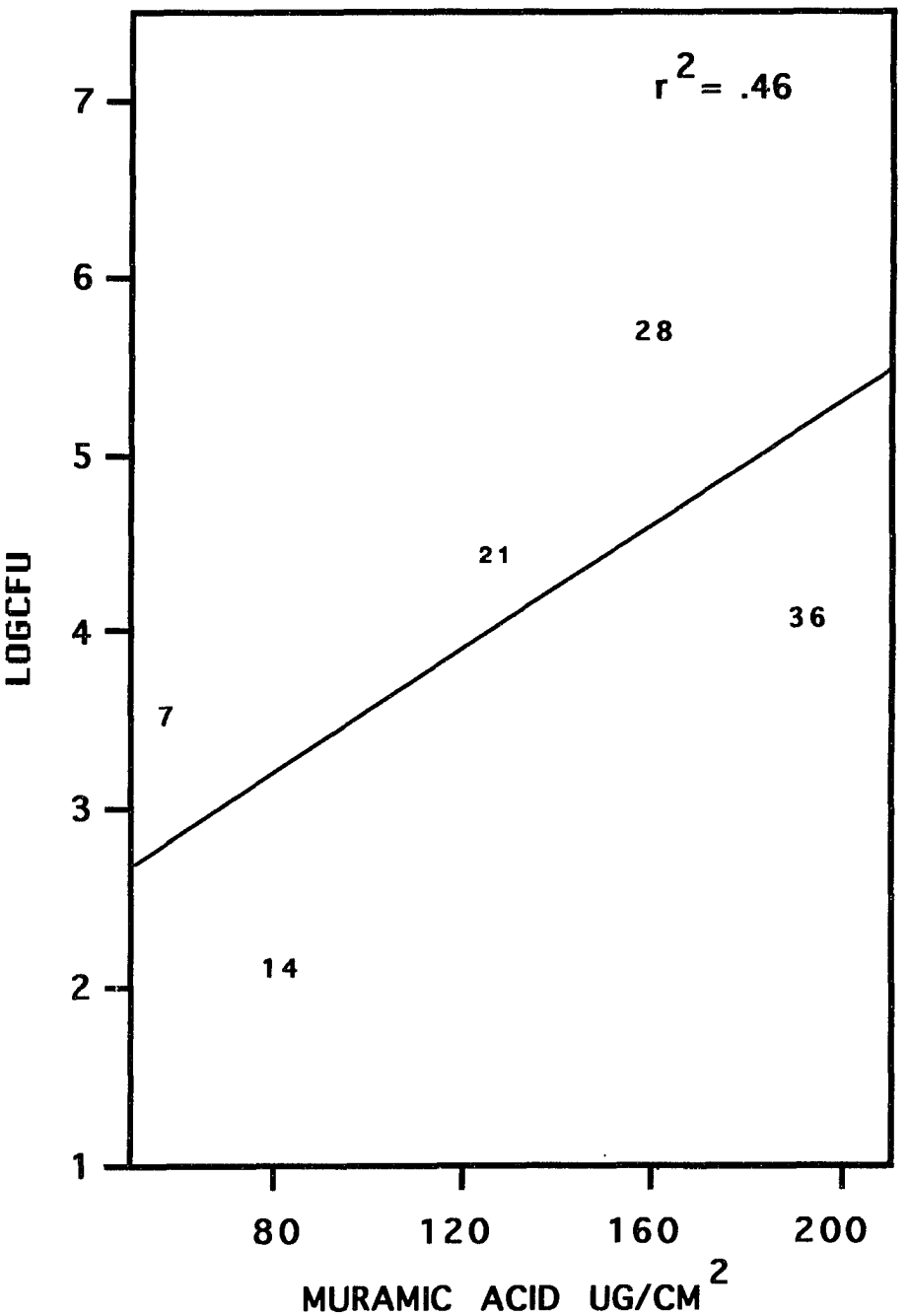

Figure 9. Correlation analysis between muramic acid and LogCFU in beef stored under anaerobic conditions averaged over replications on days 7, 14, 21, 28 and 36

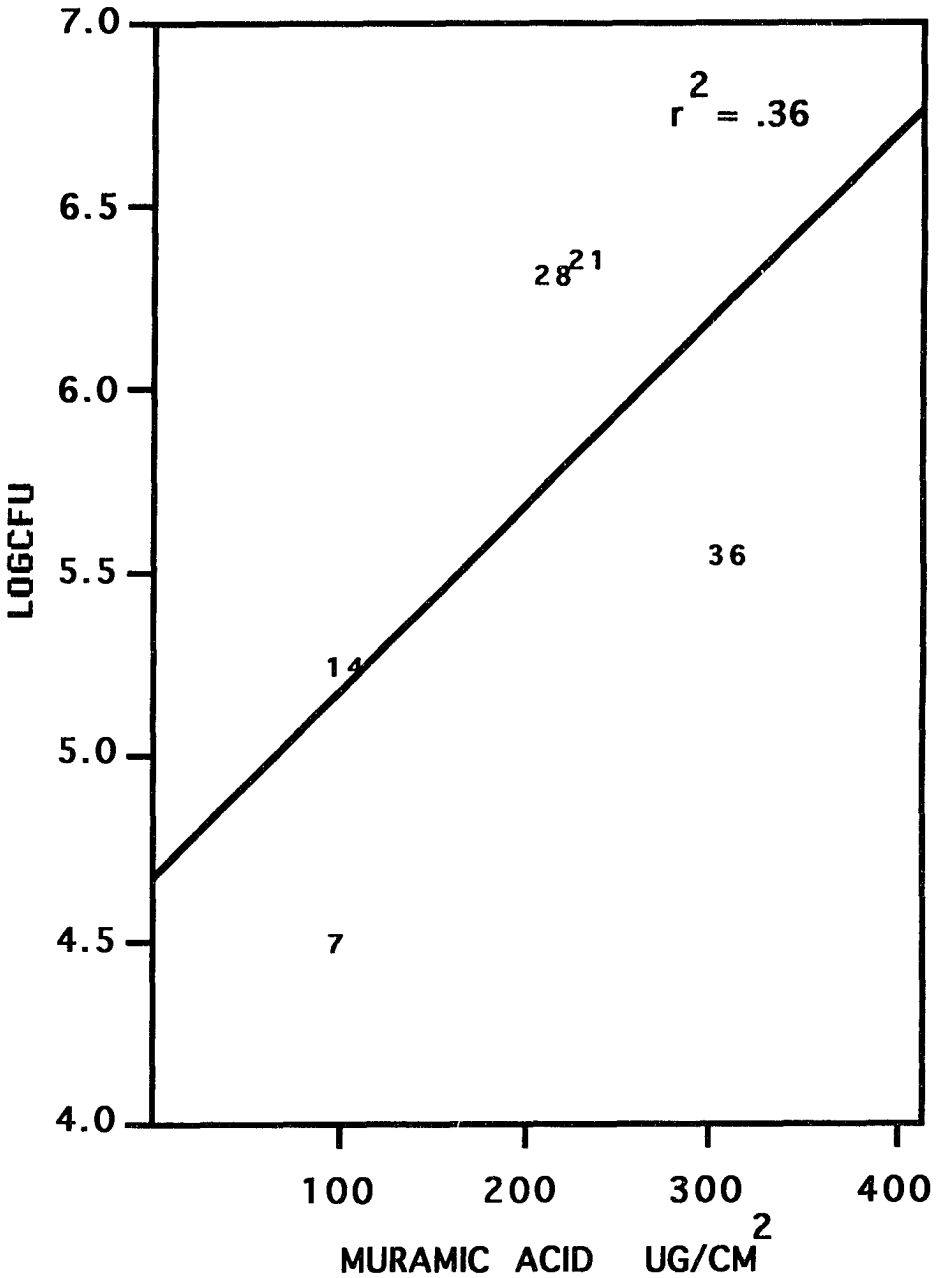

Figure 10. Correlation analysis between muramic acid and LogCFU of lactic acid bacteria in beef samples stored under anaerobic conditions averaged over replications on days 7, 14, 21, 28 and 36

MATERIALS AND METHODS (EXPERIMENT II)

In order to determine if the amounts of muramic acid were similar in comparable meat samples, a chemical analysis as well as a microbiological analysis was performed on three different samples which were stored together in the same package. All three pieces of meat within the same package should have about the same amounts of muramic acid as well as the same bacterial loads. Muramic acid was analyzed by the method described by Hadzija (1974).

For pork chops, all three samples within the same package came from the same loin and they were all packaged the same day, and in the case of the beef, all samples came from the same round. The tests were performed on each sample within the same package. This was repeated three times. Boneless pork loins and beef eye of the round were cut into 2.5 cm thick pork chops and beef steaks. All samples were stored under either vacuum packaging or under air permeable conditions. In the case of the air permeable samples, the tests were performed after 2 days and again after 4 days and on the samples stored under vacuum-packaging conditions the samples were tested after 7 days in storage and again after 14 days in storage. On sampling days, (days 2 and 4 for air permeable samples and days 7 and 14

for vacuum-packaging samples) all three pork chops and beef steaks were tested for muramic acid and for total counts.

In addition another experiment was conducted to determine if swabbing effectively recovers most of the muramic acid and bacteria. The same amount of pork chops and steaks were used as in experiment II. All chops and steaks were stored under air permeable conditions and under vacuum-packaging conditions. On sampling days (days 2 and 4 for the air permeable samples and days 7 and 14 for the samples stored under vacuum-packaging conditions) all three pork chops and steaks were tested for muramic acid as well as for microbiological content. However instead of using a swabbing technique for collecting the sample an actual piece of meat was used. An area of 12.5 cm^2 was taken out of the piece of meat by using a core. The core was made of stainless steel and measured 12.5 cm^2. The same area was taken out of the center of each piece of meat. Only the top 3 mm of the piece of meat was used. The pieces of meat were homogenized with 4 N HCl in a Stomacher (Techmar, Co. Cincinnati, Ohio). From here on the same method for the analysis of muramic acid was used. This was repeated three times.

RESULTS AND DISCUSSION (EXPERIMENT II)

Three pork chops and beef steaks were place and stored in the same package (aerobic and anaerobic conditions) to determine the source of variance of muramic acid values. The variance was larger due to replication than it was for chop or steak within the same package for aerobic-packaged pork (Table 1), aerobic-packaged beef (Table 2), anaerobic-packaged pork (Table 3) and anaerobic-packaged beef (Table 4). There was greater variation due to replication in the anaerobic packaged samples than the aerobic packaged samples. This explains why better correlation coefficients were found when values were averaged over replications in Experiment I.

Another potential source of variation in determining muramic acid levels is that swabbing the surface of meat may not recover a high enough portion of the muramic acid present. Hence this experiment used the top thin layer (3 mm) of a meat sample to recover all of the muramic acid. Results for aerobic-packaged pork (Table 5), aerobic-packaged beef (Table 6), anaerobic-packaged pork (Table 7), and anaerobic-packaged beef (Table 8) show that the replication contributed to the greatest variance as shown in Experiment I and II.

In conclusion, because there is a high variance between replications in quantifying the muramic acid content, this test does

not lend itself to a rapid chemical test for bacterial numbers. While it is unknown why replication gave such varying results, it may be due differences of muramic acid content among bacteria within the sample or to operator or procedural influences that vary with time. Hence, it was prudent to proceed with identifying other chemical measures to indicate bacterial loads.

Table 1. Muramic acid results obtained when performing the analysis on the same pork chops by using a swabbing technique

	Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1	DAY 2		
	1	3.21	63
	2	3.35	60
	3	3.02	70
	DAY 4		
	1	5.31	97
	2	5.12	109
	3	5.42	119
REP 2	DAY 2		
	1	3.51	74
	2	4.01	82
	3	3.91	91
	DAY 4		
	1	4.02	98
	2	4.21	102
	3	4.09	89
REP 3	DAY 2		
	1	3.90	112
	2	4.02	98
	3	4.05	91
	DAY 4		
	1	4.31	131
	2	4.22	108
	3	4.36	112

Table 2. Muramic acid results obtained when performing the analysis on the same beef steaks by using a swabbing technique

	Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1	DAY 2		
	1	1.90	61
	2	2.01	67
	3	1.71	79
	DAY 4		
	1	2.36	97
	2	2.41	102
	3	2.46	113
REP 2	DAY 2		
	1	2.41	91
	2	2.36	86
	3	2.51	101
	DAY 4		
	1	3.30	152
	2	3.61	149
	3	3.46	152
REP 3	DAY 2		
	1	2.01	91
	2	2.16	106
	3	2.36	121
	DAY 4		
	1	4.01	138
	2	3.96	152
	3	3.89	145

Table 3. Muramic acid results obtained when performing the analysis on the same pork chop stored under vacuum-packaging conditions and using a swabbing technique

	Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1	DAY 2		
	1	2.11	65
	2	2.50	72
	3	2.25	57
	DAY 4		
	1	3.90	101
	2	3.25	95
	3	3.52	82
REP 2	DAY 2		
	1	1.92	41
	2	1.61	31
	3	1.66	36
	DAY 4		
	1	2.90	86
	2	3.06	91
	3	3.18	97
REP 3	DAY 2		
	1	2.40	121
	2	2.36	131
	3	2.41	112
	DAY 4		
	1	4.06	184
	2	3.21	191
	3	3.36	151

Table 4. Muramic acid results obtained when performing the analysis on the same beef steaks stored under vacuum-packaging conditions and using a swabbing technique

	Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1	DAY 2		
	1	1.90	50
	2	2.25	69
	3	2.60	58
	DAY 4		
	1	3.01	96
	2	3.20	115
	3	3.71	125
REP 2	DAY 2		
	1	2.50	79
	2	2.31	86
	3	2.26	72
	DAY 4		
	1	3.42	118
	2	3.19	126
	3	3.36	131
REP 3	DAY 2		
	1	1.31	101
	2	1.16	96
	3	1.21	112
	DAY 4		
	1	2.28	136
	2	2.31	151
	3	2.06	132

Table 5. Muramic acid results obtained when performing the analysis on the same pork chop by using 12.5 cm^2 of sample

	Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1	DAY 2		
	1	1.50	42
	2	1.32	39
	3	1.46	56
	DAY 4		
	1	2.45	82
	2	2.80	94
	3	2.91	101
REP 2	DAY 2		
	1	1.71	46
	2	1.81	51
	3	1.68	47
	DAY 4		
	1	2.52	87
	2	2.38	97
	3	2.71	109
REP 3	DAY 2		
	1	1.80	53
	2	1.91	48
	3	1.96	61
	DAY 4		
	1	2.61	92
	2	2.71	101
	3	2.62	90

Table 6. Muramic acid results obtained when performing the analysis on the same beef steaks by using 12.5 cm^2 of sample

Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1 DAY 2		
1	1.96	71
2	2.06	86
3	2.11	91
DAY 4		
1	1.36	136
2	1.28	146
3	1.35	132
REP 2 DAY 2		
1	2.02	89
2	1.96	96
3	1.99	102
DAY 4		
1	1.89	102
2	2.03	99
3	2.26	126
REP 3 DAY 2		
1	1.86	102
2	1.76	112
3	1.80	121
DAY 4		
1	3.25	152
2	3.40	176
3	3.56	181

Table 7. Muramic acid results obtained when performing the analysis on the same beef steaks stored under vacuum-packaging conditions and using 12.5 cm^2 of sample

Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1 DAY 2		
1	1.97	30
2	1.50	41
3	1.37	24
DAY 4		
1	2.90	84
2	3.01	93
3	3.15	89
REP 2 DAY 2		
1	2.51	76
2	2.46	86
3	2.52	91
DAY 4		
1	4.15	116
2	4.06	122
3	4.15	116
REP 3 DAY 2		
1	2.51	76
2	2.46	86
3	2.52	91
DAY 4		
1	4.15	116
2	4.06	122
3	4.15	116

Table 8. Muramic acid results obtained when performing the analysis on the same beef steaks stored under vacuum-packaging conditions and using 12.5 cm^2 of sample

Chop	LOGCFU	Muramic acid (ug/cm^2)
REP 1 DAY 2		
1	2.30	71
2	2.53	52
3	2.80	61
DAY 4		
1	3.35	125
2	3.18	142
3	3.91	118
REP 2 DAY 2		
1	2.10	67
2	2.31	73
3	2.16	61
DAY 4		
1	3.81	96
2	3.92	101
3	4.03	116
REP 3 DAY 2		
1	2.31	71
2	2.41	82
3	2.32	71
DAY 4		
1	3.40	126
2	3.51	136
3	3.42	141

PART 2. STUDY OF THE POSSIBLE CORRELATION BETWEEN TOTAL BACTERIAL NUMBERS AND CADAVERINE ON REFRIGERATED PORK, BEEF AND TURKEY

INTRODUCTION

Meat freshness can be evaluated by organoleptic assessment, bacterial count and chemical determination of bacterial metabolites. Chemical tests for assessment of the degree of freshness in meats has long been of interest. Microbiological methods which require at least 48 hours of incubation time are too lengthy for meat processing firms. Therefore, a chemical test should eliminate the disadvantages of the currently used methods for spoilage assessment, such as the measurement of bacterial numbers or sensory judgment. For many years, chemical evaluation of meat freshness has been the target of many investigators in the search of rapid, objective tests for determination of meat quality (Pearson, 1968a,b; Turner, 1960). Chemical tests reflect the biochemical changes which occur in meat during storage. Some consideration must be taken into account when studying bacterial metabolites as chemical indicators of freshness, among them the relationship of each compound to specific spoilage microorganisms and whether or not their formation occurs during the initial stages of spoilage and in quantities sufficient for detection (Slerm, 1981). Karmas (1978) developed a biogenic amine index to classify seafood as acceptable, borderline, or unacceptable. The chemical quality index, which includes measurement of levels of putrescine,

cadaverine, histamine, spermine, and spermidine concentrations, was reported to be in satisfactory agreement with sensory evaluations.

Though the biochemistry of amines was first investigated more than a hundred years ago, the enormous Interest in these substances during the last two decades was primarily induced by the discovery of biologically very active amines, such as epinephrine, norepinephrine, histamine and serotonin (Seiler, and Wiechmann, 1970). Parallel to the still growing interest in the biochemistry of the biogenic amines was the increasing demand for sensitive and simple analytical methods for their detection and quantitative determination. This demand resulted because the old methods of isolating amines were only achievable in special cases, these methods being far too insensitive, complicated, and troublesome, and for quantitative purposes too inaccurate, for the quatitative analysis of small tissue samples (Siler and Wiechmann, 1970).

Baugmart (1979) studied the correlation between biogenic amines such as histamine and tyramine and bacterial flora in meat salads during spoilage, and observed that *Lactobacillus brevis* produced histamine and tyramine while *Pediococcus cerevisiae* produced only histamine. In another study, a simple method using HPLC was developed for the analysis of amines in canned fish. In this study scientists found low levels of putrescine, cadaverine,

tryptamine, 2-phenylethylamine, spermidine, spermine, tyramine, and agmatine (Yen and Hsieh, 1991). In the present study, meat freshness is compared to the content of cadaverine and total bacterial numbers throughout aerobic chilled storage of commercial fresh pork, beef, and turkey.

MATERIALS AND METHODS (EXPERIMENT I, ANALYSIS OF CADAVERINE STANDARD BY TLC)

Cadaverine standard was obtained form Sigma Co. (St. Louis, MO). A standard solution containing 50 ug/ul was prepared using water as the solvent. The standard solution was stored at 5-7°C until the time of use. To 1.0 ml of standard solution 1.6 mg of dabsyl chloride in 1.0 ml of acetone was added. The mixture was thoroughly mixed and maintained at room temperature (25-26°C) for one hour. Then additional dabsyl chloride (0.8 mg in 0.5 ml of acetone) was added and kept at the same temperature for 30 minutes to ensure the completeness of the dabsylation. The excess dabsyl chloride was consumed by the reaction with 5 mg of glycine in 0.5 ml of water for 30 minutes. The resulting mixture was extracted with 5 ml of benzene and the organic layer was washed with 5 ml of water twice and dehydrated with anhydrous sodium sulfate (.3-.5 g). From here an aliquot of the clear benzene extract was taken for Thin Layer Chromatography (TLC) analyses (Lin and Chen, 1982).

Two kind of TLC plates were used in this part of the experiment. The plates were .2mm N-HR (High Resolution) E. Merck (Emsience, N. J.) plates and the second type were polyamide plates. High Resolution plates are widely used for the

analysis and separation of many different compounds. In addition many different solvent systems could be used with this type of TLC plate. Polyamide plates can also be used for a wide variety of compounds and different solvent systems. One advantage of polyamide plates is that samples could be run in shorter periods of time compared to the High Resolution plates.

Cadaverine standards were applied to the plates using Hamilton (1-10 ul and 10-100 ul) syringes and with the help of a soft current of dryed air to evaporate the moisture of the sample rapidly. When using Hamilton syringes successive round spots are applied to the plate. The spotting process is time-consuming and tedious. The irregularities that necessarily are a part of this method of sample application are magnified in the separation process and often can make isolation of developed material very difficult. Of course, if the separations are widely spaced, the hand "spot streaking" method is perfectly satisfactory. With experience, it is possible to streak a plate by hand satisfactorily using a syringe. The syringe is held nearly vertically and not quite touching the coating surface. Then, using the end of a bench or some such guide to help steady the hands, the syringe is moved across the plate while the plunger is slowly pushed into the barrel. Several solvent systems were used for the separation of the different compounds present in the extracted sample. Solvents were selected on the basis of previous information on the separation of

other amines in urine samples (Fleisher and Russell, 1975). Other solvents were selected from a publication by Lin and Chen (1982) and (Stahr, 1991). There are many variables when working with TLC, like temperature in the chamber, amount of sample plated, concentration of the sample and uniformity of the silica on the plate; therefore, it was necessary to experiment with each different system before going on to the next one.

Solvent systems used were:

1. Chloroform-dichloromethane-acetone-95% ethanol (5:2:.5:1)
2. Chloroform-dichloromethane-acetone-abs.ethanol (5:2:.5:1)
3. Chloroform-dichloromethane-abs.ethanol (5:2:1)
4. Chloroform-trimethy. .ıe (5:1)
5. Chloroform-dichloromethane-absolute ethanol-n-hexane (5:2:1:.5:3)
6. Chloroform-acetone-2-propanol (85:10:5)
7. Dichloromethane-acetone (90:10)
8. Methanol-water-ammonium hydroxide (85:15:1)
9. Methanol-water-ammonium hydroxide (95:5:1)
10. Ethanol-water-acetic acid (65:35:1)

Solvent 10 was used together with reverse phase Whatman C18 plates (Whippany, N. J.). Polyamide plates took approximately 15 minutes to develop and the N-HR plates took around 90 minutes. The C18 reverse phase plates took 35 minutes to develop.

After the TLC plates were dried they were read on a Kontes Scanner 800 densitometer used with a 425 nm filter to measure visible yellow spots. A chart recorder was used for the recording of all results. Peak areas were calculated by multiplying peak height by peak width at half-height.

After obtaining the results from solvent #1, the polarity of the system was increased by the use of absolute ethanol (solvent #2). Poor to no separation was obtained. With both solvent systems the concentration and the amounts plated were varied in order to obtain some separation; however, concentrations or amounts plated proved to be independent from the separation factors. A third attempt to increase polarity was made by the use of solvent #3 in which the amount of absolute ethanol was increased once more. With this third solvent some separation was obtained; however, poor reproducibility was obtained.

Other polar chemicals were tried such as trimethylamine (solvent #4) and n-hexane (solvent #5), however with these two other solvent systems again poor to no separation was observed. With solvent #6 the ethanol in the first three systems was replaced with 2-propanol which is slightly less polar than ethanol. Again no separation was obtained. The last four solvents as well as the reverse plate technique (using C18 TLC plates) used with solvent #10 were chosen from Stahr (1991).

RESULTS AND DISCUSSION (EXPERIMENT I)

The results of the thin layer chromatography on N-HR plates and polyamide plates with 9 solvent systems are listed on tables 1 and 2, respectively. When no separation was obtained it is expressed in the tables as 'none' and when partial separation was obtained it is expressed as 'partial'. When using silica N-HR plates only partial separation was obtained when using solvent 2 (chloroform-dichlomethane-acetone-absolute. ethanol 5:2:5:1) and with solvent 3 (chloroform-dichloromethane-absolute. ethanol 5:2:1). Their Rf_S were .70 and .60 respectively but mixed bands were obtained. From the results obtained from the first three systems it can be concluded that that the polarity of the solvent mixture exerted some effect on the mobility of the dabsyl-cadaverine. From the minimal results obtained it was observed that the migration rate of the dabsylated amine increased as the concentration of ethanol was increased. All the other solvent systems failed to separate cadaverine on the N-HR TLC plates. When using the polyamide plates no single solvent system proves to be an effective way to analyze amines. The only system that proves to be an efficient method, providing both good separation and simple application was by the use of reverse phase C18 TLC plates and using ethanol-water-acetic acid (65:35:1) as a developing solvent. The Rf obtained here for cadaverine was .55.

From all the results obtained in this experiment it can be concluded that reverse phase C18 plates using the mentioned solvent is not only an efficient way to analyze cadaverine but the plate only takes 30 minutes to develop, compared to 90 minutes when using N-HR plates.

Chemical bonding onto the surface of silica allows for the ability to vary chain length, to alter selectivity and to enhance retention by chain variation. Understanding of these principles allows for the creation of an optimized structure for the greatest possible resolution capabilities. This effect has been investigated by high performance liquid chromatography (HPLC), and planar TLC.

The complex surface of silica, handled correctly, can be a source of a host of products, all with different identities. The results are that carbon chain length is not the only criterion for retention, but molecular functional groups, size and shape all contribute to migration rates (Felton, 1982).

The reverse phase TLC plate C18 shows wide applicability to the separation of various classes of both nonpolar and polar compounds which opened new opportunities for TLC. Care must be exercised in spotting reverse phase plates, taking into account the spotting volume and concentration. It is better to have small spots containing high concentrations of the compounds to be examined. C18 plates provided the highest speed of analysis over

the broadest range of mobile-phase compositions used. Unique separations can be obtained employing selected solvent additions or absorbed species.

Linearity and sensitivity

Linear calibration curves passing through the origin were obtained with known amounts of polyamines ranging from 1 to 4 ug. The equation for the linear curve obtained with cadaverine was: $y = -0.14506 + 0.15403x$ ($r = .97$) shown in Figure 1. The minimum detectable concentration of cadaverine was about 0.5 ug. The volume spotted on the plate was 50 ul. of solvent.

The next step was to actually determine if cadaverine is present in meat samples followed by experimentation to decide how much sample would be needed to obtain measurable amounts of cadaverine.

Table 1. TLC analysis of dabsyl polyamines on H-HR plates

Solvent system	Rf	Separation
1	—	none
2	.70	partial
3	.60	partial
4	—	none
5	—	none
6	—	none
7	—	none
8	—	none
9	—	none

1 = chloroform-dichloromethane-acetone-95%ethanol (5:2:5:1)
2 = chloroform-dichloromethane-acetone-absolute ethanol (5:2:5:1)
3 = chloroform-dichloromethane-absolute ethanol (5:2:1)
4 = chloroform-trimethylamine (5:1)
5 = chloroform-dichloromethane-abs. ethanol n-hexane (5:2:1:5:3)
6 = chloroform-acetone-2 propanol (85:10:5)
7 = methylene chloride-acetone (90:10)
8 = methanol-water-ammonium hydroxide (85:15:1)
9 = methanol-water-ammonium hydroxide (95:5:1)

Table 2. TLC analysis of dabsyl polyamines on polyamide plates

Solvent system	Rf	Separation
1	—	none
2	—	none
3	—	none
4	—	none
5	—	none
6	—	none
7	—	none
8	—	none
9	—	none

1 = chloroform-dichloromethane-acetone-95%ethanol (5:2:5:1)
2 = chloroform-dichloromethane-acetone-absolute ethanol (5:2:5:1)
3 = chloroform-dichloromethane-absolute ethanol (5:2:1)
4 = chloroform-trimethylamine (5:1)
5 = chloroform-dichloromethane-abs. ethanol n-hexane (5:2:1:5:3)
6 = chloroform-acetone-2 propanol (85:10:5)
7 = methylene chloride-acetone (90:10)
8 = methanol-water-ammonium hydroxide (85:15:1)
9 = methanol-water-ammonium hydroxide (95:5:1)

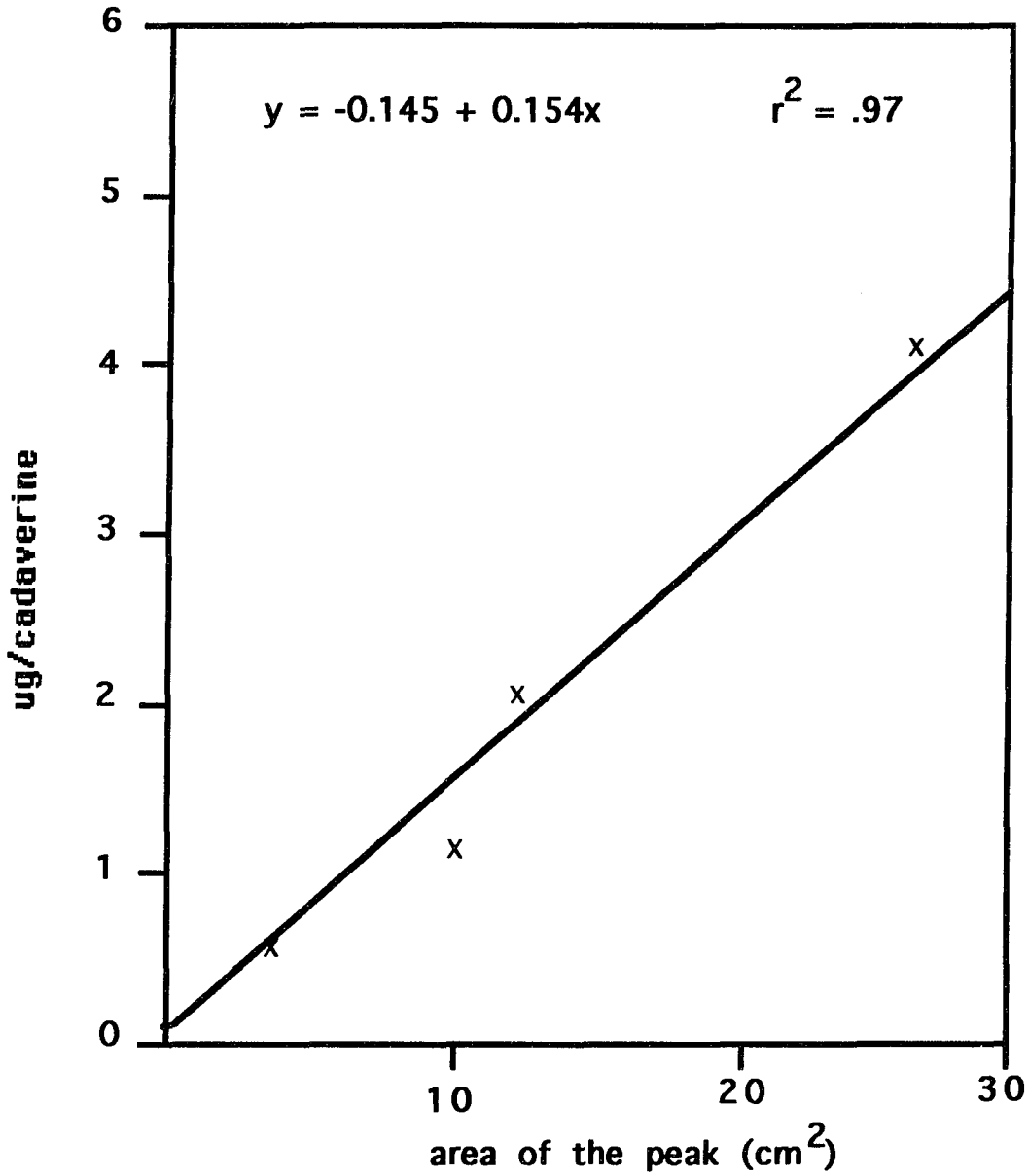

Figure 1. Standard curve for cadaverine

MATERIALS AND METHODS
(EXPERIMENT II, MEAT SAMPLE ANALYSIS)

This experiment was performed to find how much meat sample was necessary to obtain measurable amine quantities when bacterial loads are low. Since low bacterial loads would be expected to produce low levels of cadaverine, larger sample sizes would be needed to get sufficient quantity of cadaverine to fit on a standard curve developed in Experiment I.

Fresh, boneless pork loins were obtained from the Iowa State University Meats Laboratory. Loins were cut into large chunks and wrapped into oxygen permeable film and then stored at 4 to 6°C until the day of examination. On sampling days (0, 2, 4, 6 and 8), one piece of loin was taken from storage for microbiological and chemical analysis on cadaverine. Each package was opened aseptically, and sterile tongs were used to remove the piece of meat. Using a stainless steel borer that measures 10 cm^2 two samples were taken, one for microbiological testing and the other for the analysis of cadaverine. After the amine quantification results were obtained by using 10 cm^2, it was decided to use more meat sample. The same chemical analysis was then performed by using 25 cm^2 followed by 50 cm^2 and finally by using 75 cm^2. The reason for doing this was to obtain measurable amounts of cadaverine in the samples. An area of 25

cm^2 was taken for microbiological analysis. A stainless steel borer that, measures 25 cm^2 was used. Only the top 3-4 mm was used for both, chemical and microbiological examinations. For the microbiological analysis the meat was placed into a sterile bag (Tekmar Company, OH) with 99 ml of sterile 0.1% peptone water and vigorously macerated in a stomacher (STO 400, Tekmar Company, OH) for two minutes. Appropriate serial dilutions were made for plating. All Purpose Tween (APT) agar (BBL) was used for the enumeration of total mesophilic (30°C, 48 hr) and psychrotrophic (5°C, 7-10 days) bacteria.

For the cadaverine evaluation the 10, 25, 50 and 75 cm^2 samples were placed into sterile bag with 30 ml of 0.38N trichloro acetic acid (TCA) and vigorously macerated in a stomacher for 5 minutes. The sample was then placed into a tube and centrifuged at 8000 g for 15 minutes. Two ml of the supernatant were taken and mixed with 2 ml of acetone. The resulting mixture was centrifuged again at 8000 g for 15 minutes; 1 ml aliquots of the clear supernatant were used for the determination of cadaverine. 1.6 mg of dabsyl chloride in 1.0 ml of acetone was added to 1 ml of sample and then the pH adjusted to 9-10 with 0.1N NaOH. The mixture was thoroughly mixed and allowed to stand at ambient temperature (25-26°C) for half an hour. Then additional dabsyl chloride (0.8 mg in 0.5 ml of acetone) was added and kept at the same temperature for 30 minutes to ensure the completeness of

dabsylation. The excess dabsyl chloride was consumed by reaction with 5 mg of glycine in 0.5 ml of water for 30 minutes. The resulting mixture was extracted with 5 ml of benzene and the organic layer was washed with 5 ml of water twice and dehydrated with anhydrous sodium sulfate (0.3-0.5 g). An aliquot of the clear benzene extract was taken for TLC analyses (Lin and Chen, 1982). Whatman C18 TLC plates were used. 65:35:1 (V/V/V) (ethanol:water:acetic acid) was used as the developing solvent. The plates were dryed and then read on a Kontes scanner 800 densitometer equipped with a 425nm filter to measure visible yellow spots. Each experiment was replicated three times with each different amount of sample and analyzed by using correlation coefficients.

A standard curve using cadaverine was included on each plate along with the samples. The amount of sample was divided by the amount of TCA in which it was homogenized followed by subsequent dilutions with the idea of knowing the amount of sample spotted which in our case it represented a specific area of the initial sample (area). The standard curve was used to calculate the amount of cadaverine present in the sample.

RESULTS AND DISCUSSION (EXPERIMENT II)

The main purpose of doing this experiment was to find a sufficient amount of meat sample that would be necessary to obtain quantitative cadaverine results when bacterial loads are still at 10^4 CFU/cm^2 or lower. Every pork loin used in this experiment had bacterial loads of less than 10^3 CFU/cm^2. This is typical of meat purchased at the Iowa State Meats Laboratory. These pork loins provide excellent experimental conditions since the bacterial history could be followed from the beginning. Figure 2 shows the quantitative results when the cadaverine analysis was performed in pork loins using an area of 10 cm^2. Bacterial counts were performed on 25 cm^2 meat samples. No cadaverine was detected on the first three testing days (0, 2, and 4), however cadaverine was detected on the fourth and fifth day when bacterial counts were at almost 10^6 and 10^7 CFU/cm^2. A correlation factor of 0.91 was found for the three point shown in the graph. Only three points are shown since no cadaverine was detected on days 0, 2 and 4.

When performing the same analysis on 25 cm^2 (Figure 3) cadaverine was detected on the third testing day when bacterial counts were at 10^5 CFU/cm^2 which corresponded to about 100 ug/cm^2 of cadaverine. A correlation factor of 0.88 was found when using 25 cm^2. These results still would not be appropriate since

the main purpose of the experiment was to detect measurable quantities of cadaverine when low bacterial loads are present.

Figure 4 shows the results obtained when 50 cm^2 were used. Here cadaverine was detected when bacterial loads were at about 10^4 CFU/cm^2 which corresponded to about 90 ug/cm^2. Here a correlation of 0.87 was obtained. 50 cm^2 would be the least amount of meat that would be useful to measure cadaverine in commercial meat samples since most meat obtained from commercial sources very rarely contain less than 10^4 bacteria/cm^2 (Nortje et al., 1990).

However, by using 75 cm^2 (Figure 5) cadaverine was detected when bacterial counts were at about 10^3 CFU/cm^2 and a correlation of 0.89 was obtained. Therefore, using 75 cm^2 samples not only provides excellent cadaverine quantifications at low levels of bacteria, but, is also an area that could easily be extracted from any cut specially if they come from commercial sources. However, the procedures to determine cadaverine are still too long for any direct commercial application, so refinement of the system is needed.

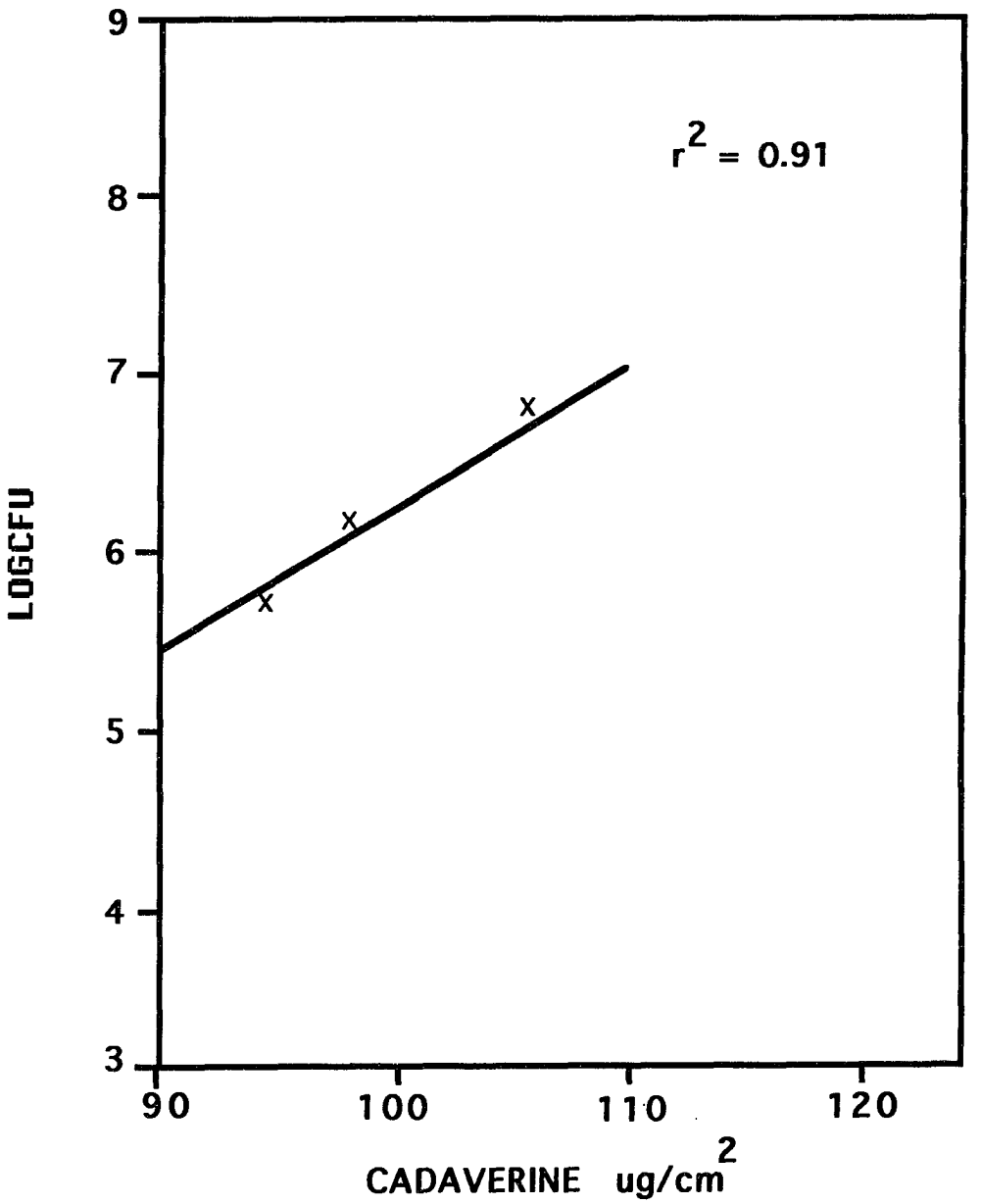

Figure 2. Analysis of cadaverine using 10 cm ^{2} pork loin sample

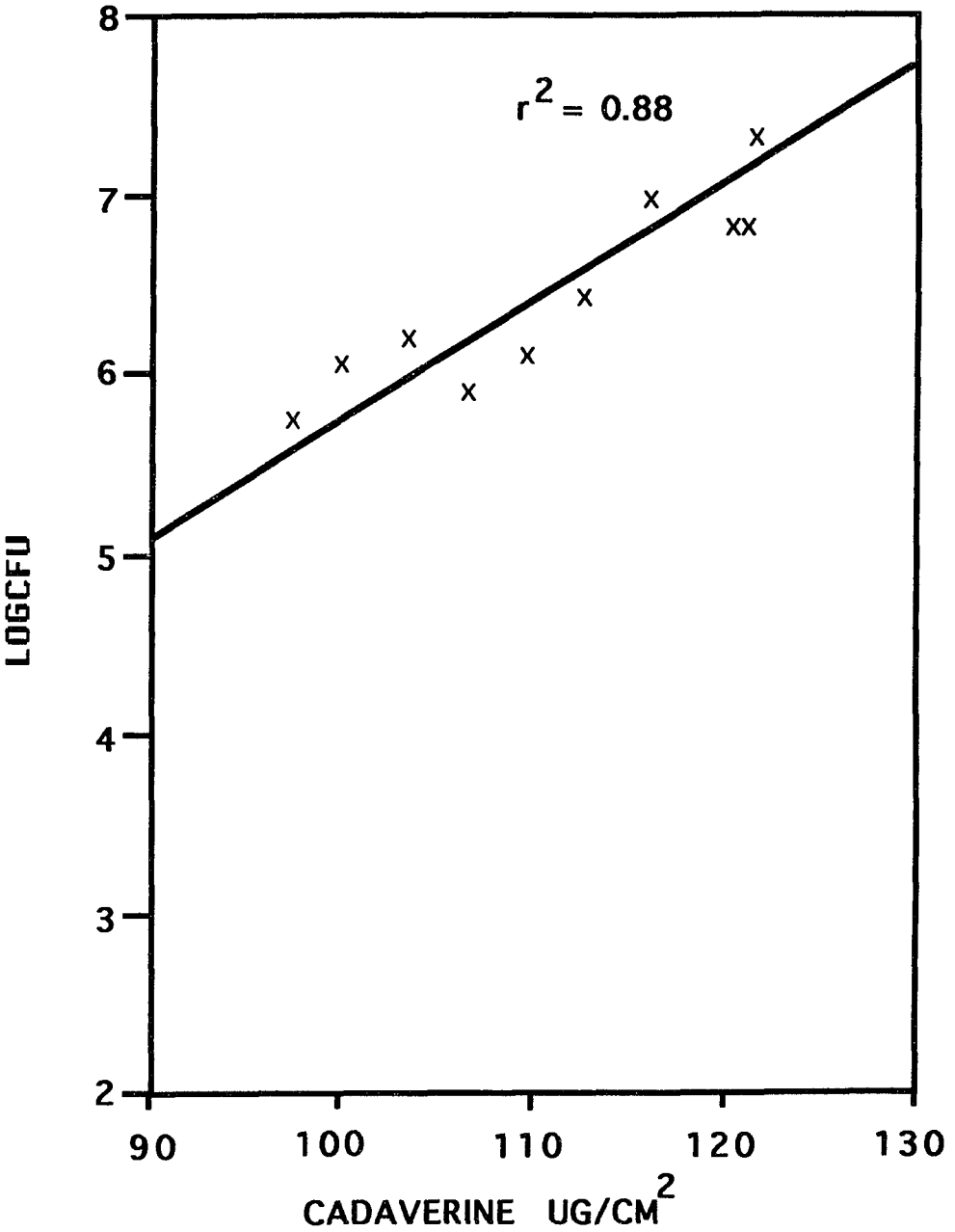

Figure 3. Analysis of cadaverine using 25 cm^2 pork loin samples

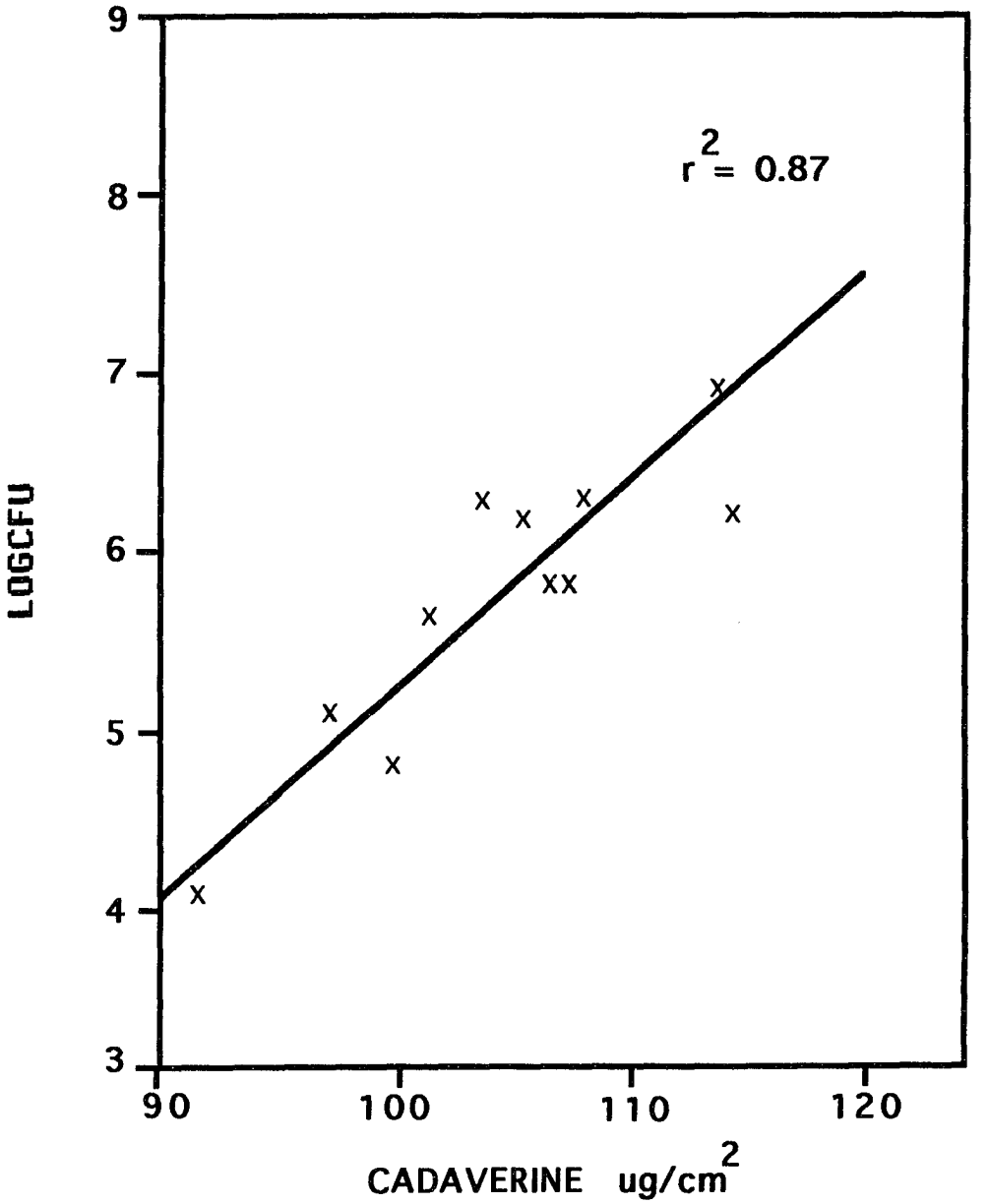

Figure 4. Analysis of cadaverine using 50 cm^2 pork loin sample

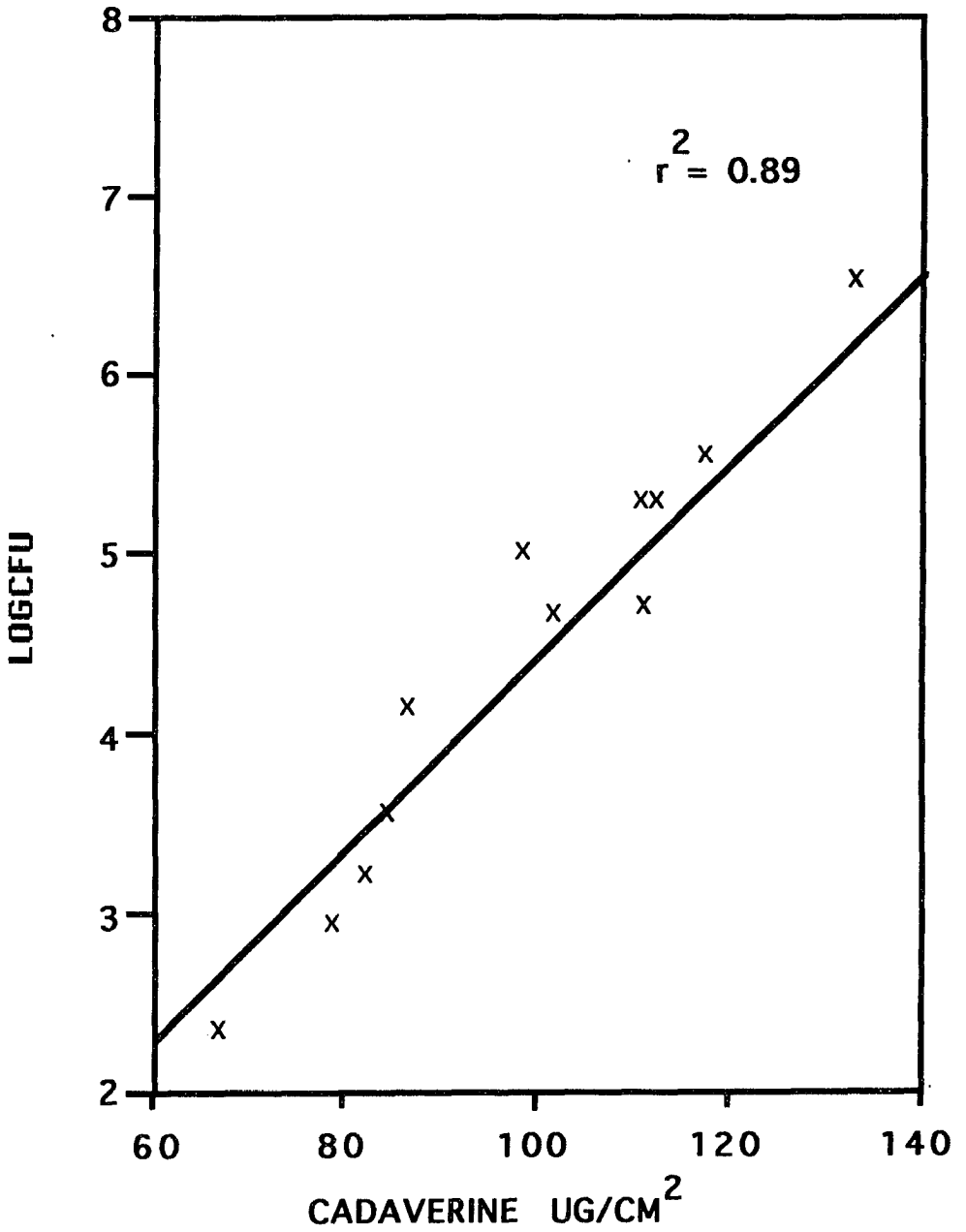

Figure 5. Analysis of cadaverine using 75 cm^2 pork loin samples

MATERIALS AND METHODS
(EXPERIMENT III, EFFECT OF INCREASING DABSYL CHLORIDE AND TEMPERATURE)

To refine the procedure for measuring cadaverine, levels of dabsyl chloride and reaction temperature were investigated. The effect on increasing the amounts of dabsyl chloride from 1.6 mg/ml to 3.2 mg/ml was studied. In this first experiment two pork loins were stored at 5°C under aerobic conditions and they were tested for cadaverine on days 2, 4, 6, and 8 by using both dabsylation solutions on the same sample.

In addition the dabsylation process was compared when the reaction temperature was changed from 25°C to 35°C. Three different pork loins were tested. The purpose of increasing the temperature was to examine if the dabsylation reaction time could be reduced from one hour to at least half an hour.

RESULTS AND DISCUSSION (EXPERIMENT III)

When the amount of dabsyl chloride was increased from 1.6 mg/ml to 3.2 mg/ml, essentially the same quantitative results were obtained (Figure 6). Again, the purpose of this experiment was to find if by increasing the amount of dabsyl chloride would affect the final cadaverine content. This experiment clearly shows that 1.6 mg/ml of dabsyl chloride is enough for all cadaverine molecules to react with dabsyl chloride and therefore accurate amine quatification could be obtained.

When the temperature of the dabsylation reaction was increased from 25°C to 35°C it was observed that the amounts of cadaverine remained constant after 30 minutes in all three loins (Figure 7). In figure 7 each set of lines represent a different loins in which measurements were taken after 30, 60 and 120 minutes. Each set of lines represent a different loin all of them containing different bacterial loads. Loin 1 had bacterial counts of 4.5 LogCFU, loin 2 had 5.1 and loin 3 had 5.4. From this experiment it can be concluded that by using a temperature of 35°C the dabsylation reaction rate can be shortened from 1 hour to 30 minutes and still obtain the same quantitative results. This is very significant since 30 minutes could be saved form the entire cadaverine analysis.

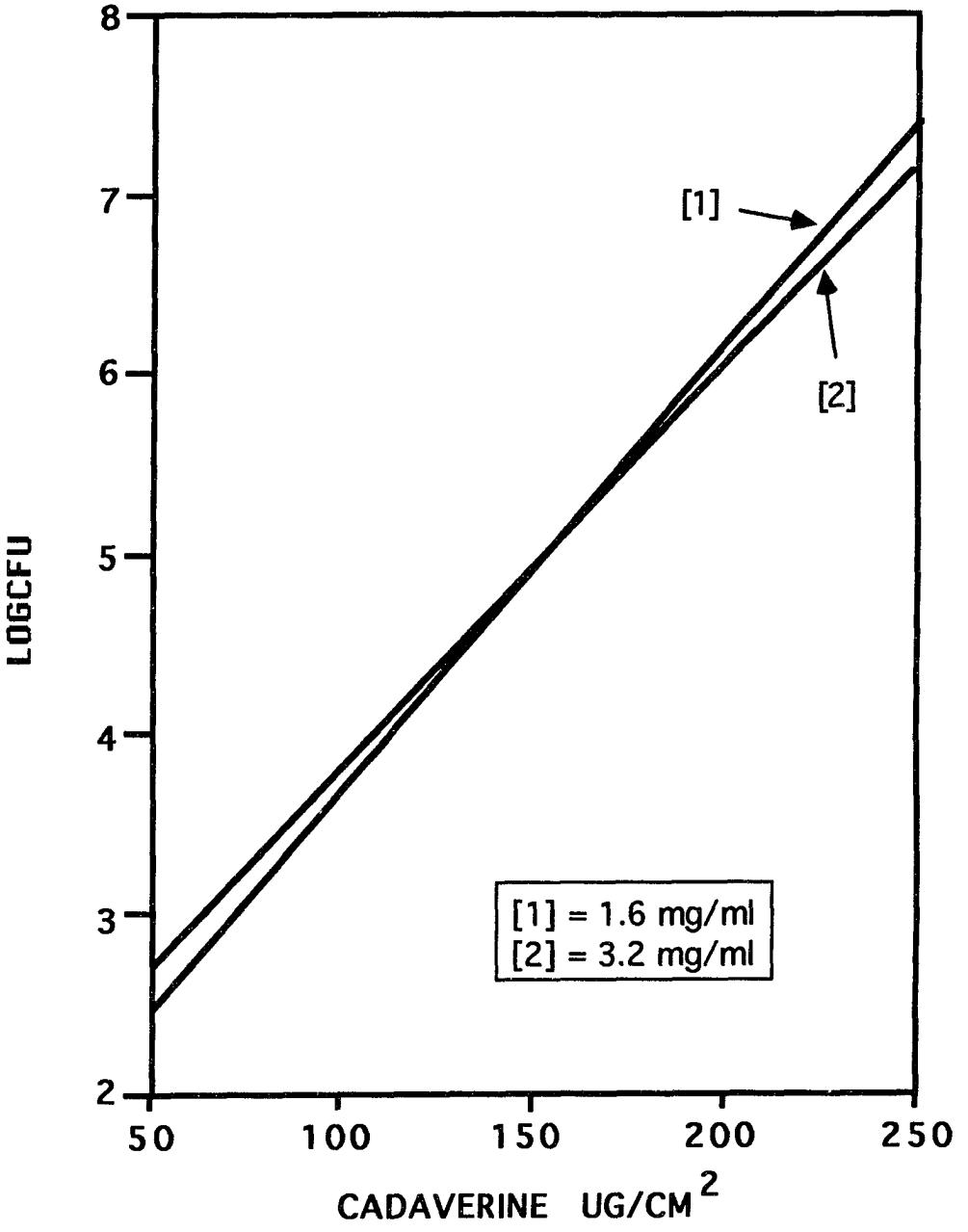

Figure 6. Analysis of cadaverine using two different concentrations of dabsyl chloride

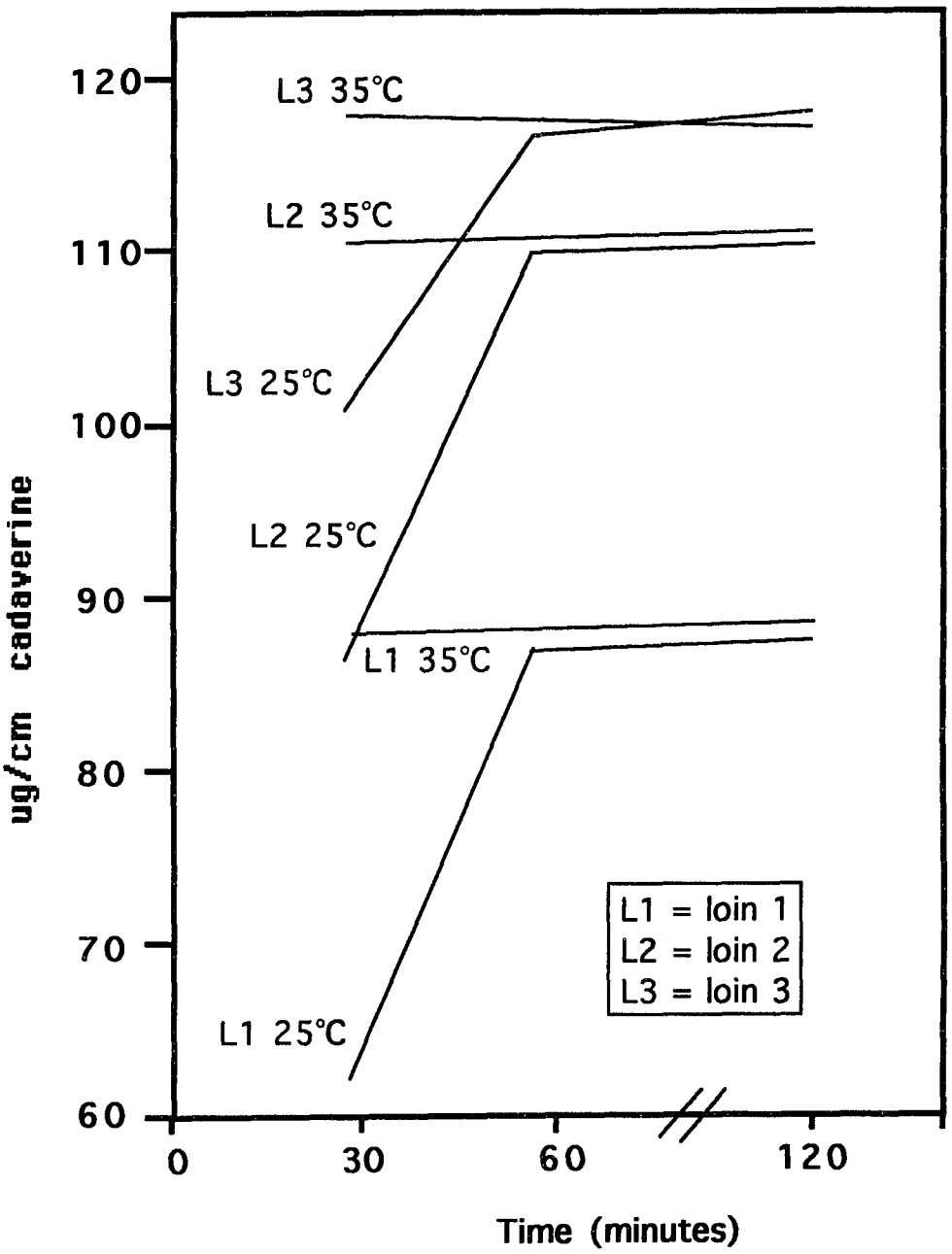

Figure 7. Analysis of cadaverine using two different temperatures

MATERIALS AND METHODS (EXPERIMENT IV, CORRELATION STUDY BETWEEN CADAVERINE AND BACTERIAL NUMBERS IN PORK, BEEF AND TURKEY)

Fresh, boneless pork loins were obtained at the Iowa State University Meats Laboratory. In addition beef loins, beef rounds and turkey breast samples were obtained from local supermarkets. Pork loins were cut into large chunks and wrapped into oxygen permeable film and then stored at 4 to 6°C until the day of examination. On sampling days (2, 4, 6 and 8) one piece of loin was taken from storage for microbiological and chemical analysis of cadaverine. Beef loins, beef rounds and turkey samples were tested the same day they were purchased. Each package was opened aseptically, and sterile tongs were used to remove the piece of meat. Using a stainless steel borer that measured 25 cm^2, a total area of 75 cm^2 were removed for chemical analysis of cadaverine and an area of 25 cm^2 was used for microbiological analysis. Only the top 3-4 mm was used for both examinations. The microbiological analysis was performed as explained in experiment II. For the amine determination the derivatization and extraction method used by Lin and Chen (1982) was used.

A sample measuring 75 cm^2 was placed into a sterile bag (Tekmar Company, OH) with 30 ml of 0.38N trichloro acetic acid

(TCA) and vigorously macerated in a stomacher (STO 400, Tekmar Company, OH) for 5 minutes. The sample was then placed into a tube and centrifuged at 8000 g for 15 minutes. Two ml. of the supernatant were taken and mixed with 2 ml of acetone. The resulting mixture was centrifuged again at 8000 g for 15 minutes; 1 ml aliquots of the clear supernatant were used for the determination of cadaverine. 1.6 mg of dabsyl chloride in 1.0 ml of acetone was added to 1 ml of sample and the pH adjusted to 9 with 0.1N NaOH. The mixture was thoroughly mixed and placed in a water bath at 35°C for half an hour. Then additional dabsyl chloride (0.8 mg in 0.5 ml of acetone) was added and kept at the same temperature for 30 minutes to ensure the completeness of dabsylation. The excess dabsyl chloride was consumed by the reaction with 5 mg of glycine in 0.5 ml of water for 15 minutes. The resulting mixture was extracted with 5 ml of benzene and the organic layer was washed with 5 ml of water twice and dehydrated with anhydrous sodium sulfate (0.3-0.5 g). An aliquot of the clear benzene extract was taken for TLC analyses. Whatman C18 TLC plates were used. 65:35:1 (V/V/V) (ethanol:water:acetic acid) was used as the developing solvent. The plates were dryed and then read on a Kontes scanner 200 densitometer equipped with a 425nm filter to measure visible yellow spots. The sensitivity and scan rate were optimized for each individual plate in order to obtain the best reading.

Data derived from plate counts were transformed into logarithms and correlated with the amounts of cadaverine found in the samples.

RESULTS AND DISCUSSION (EXPERIMENT IV)

In this study, the relation of cadaverine release and bacterial growth in pork, beef, and turkey meat was determined. The range of the bacterial counts was from 10^3 to 10^9 bacteria/cm^2 for both mesophilic and psychrotrophic bacteria. In the first part of the experiment the formation of cadaverine in pork loins and total bacterial counts were measured. With pork loins, a correlation of 0.95 for psychrotrophic organisms (Figure 8) and 0.89 for mesophiles (Figure 12) was found for cadaverine and total counts. Therefore, cadaverine appears to have a potential as an indicator of the microbial load (freshness) in pork. Cadaverine levels were about 100 ug/cm^2 when counts were at about log^5 and to about 280 ug/cm^2 for log^8 for psychrotrophic organisms. For mesophilic organisms cadaverine levels were about 60 ug/cm^2 when counts were at about log^4 and about 285 ug/cm^2 when counts were at about log^9. The study was based on the appearance of cadaverine on the surface of the meat, however; in most studies amine values are presented in mg of amine per 100 grams of meat. The method used in this study would simplify the preparation of the sample for the actual analysis as well as providing a good measure for surface contamination.

In previous studies, scientists have demonstrated that the amine, putrescine, in ground beef purchased in retail stores has

potential as a bacterial index, while 1, 3 diamino-propane was present as an intermediary compound in some samples only and was not significantly correlated with total bacterial counts (Sayem-El-Daher, 1983). This suggests that factors influencing the growth and metabolism of spoilage flora as well as errors associated with the determination of the diamine level and the bacterial count are relatively large. In meats held under aerobic conditions, increasing levels of cadaverine have been detected (Nakamura et al., 1979; Edward et al., 1983). Cadaverine as well as other amines have been shown to be produced by metabolic activities of *Pseudomonas* and *Enterobacteriaceae* (Stern, 1981). These are species generally known as dominant elements of chilled meats flora (Gill C. O, 1983).

On the samples obtained from local supermarkets, a correlation between cadaverine and total counts on both beef loins and rounds (Figures 9) was 0.94 and 0.95 for beef loins alone (Figure 10) for psychrotrophic organisms and 0.87 and 0.91 for mesophilic organisms (Figures 13 and 14). This again shows that cadaverine appears to have an excellent potential as quality indicator not only in pork but also in beef. In all beef samples bacterial loads (psychrotrophic) were between LogCFU 4 and 5.5, and cadaverine amounts ranged from about 130 ug/cm^2 to about 180 ug/cm^2. For mesophilic organisms bacterial loads were between LogCFU 3 and 8.6, and cadaverine amounts ranged

between 110 ug/cm^2 and 250 ug/cm^2. The lowest correlation coefficients were found on turkey breast meat, 0.87 for psychrotrophic organisms (Figure 11) and 0.81 for mesophiles (Figure 15). This was probably due to some difficulty in obtaining the proper area for both chemical and microbiological determinations since it was rare to find turkey breast sections which would measure 75^2 cm. Many times an extra section was nedded to complete the 75^2 cm. A schematic for the analysis of cadaverine is shown in figure 16. Figure 17 represents a typical TLC plate after development. Spot c represents the cadaverine extracted in a sample which matches spots a and b which represent cadaverine standards.

No matter the identity of the organisms involved, the consistent increases in cadaverine concentrations above the basal 'fresh' levels are of potential value in the objective assessment of meat freshness/spoilage which presently are judged by odor, appearance, and, sometimes, on a number of bacteriological indices. Although there is no evidence that the diamines are themselves responsible for spoilage their, concentrations might serve as a warning that shelf life should not be unduly prolonged.

For people in the meat industry, the test for cadaverine presents a new and more rapid way to quantitatively measure total bacterial numbers in pork, beef and turkey. Each industry would be responsible for setting their own standards and limits for the

amounts of cadaverine present in their products. From the results obtained in this experiment, 150ug/cm^2 of cadaverine correspond to about 10^5 to 10^6 bacteria per square centimeter in pork, beef and turkey. One million bacteria (10^6) per centimeter square represents in most cases that point when spoilage characteristics start to appear (odors, flavors, color and texture changes). Therefore, 10^6 bacteria per square centimeter is usually taken as that point that represents an acceptable or a non-acceptable product. However, each user of this test is responsible for setting their own standards. From the results presented in this experiment, it would be advisable to use 150 ug/cm^2 cadaverine as that point when pork, beef or turkey would be either accepted or rejected. A schematic for the analysis of cadaverine is shown in figure 16.

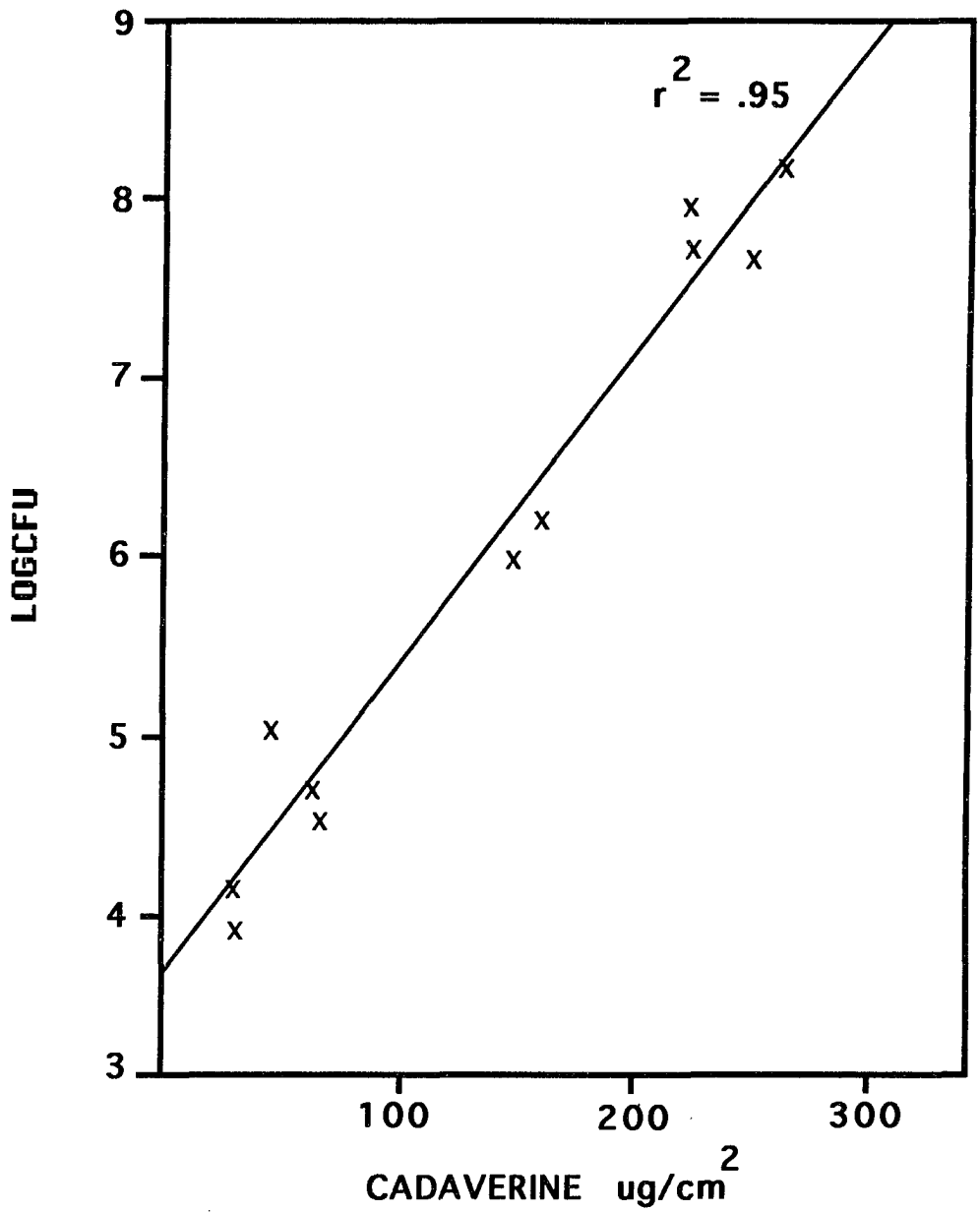

Figure 8. Correlation analysis between cadaverine and total bacterial counts in pork loins

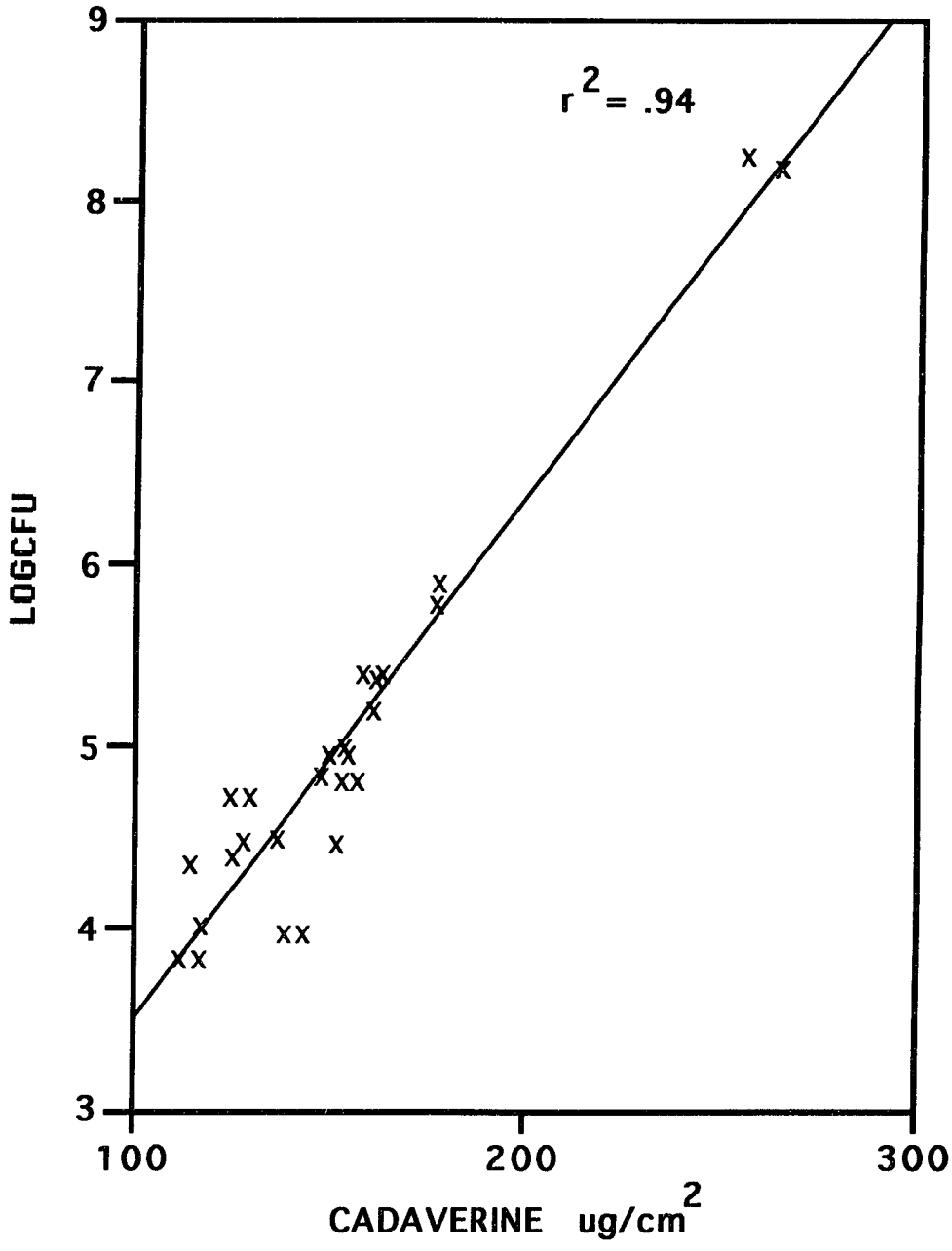

Figure 9. Correlation analysis between cadaverine and total bacterial counts in beef loins and rounds

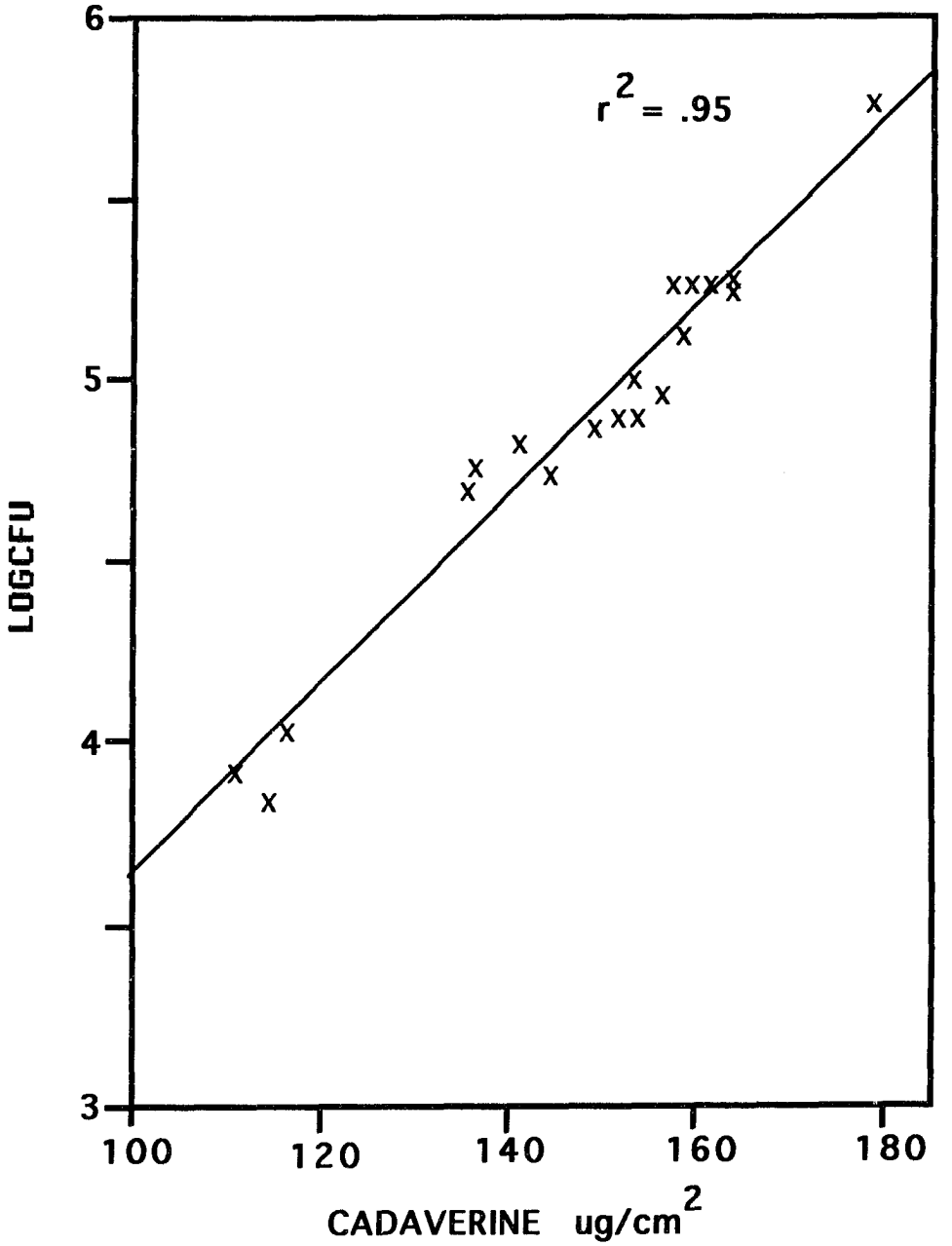

Figure 10. Correlation analysis between cadaverine and total bacterial counts in beef loins

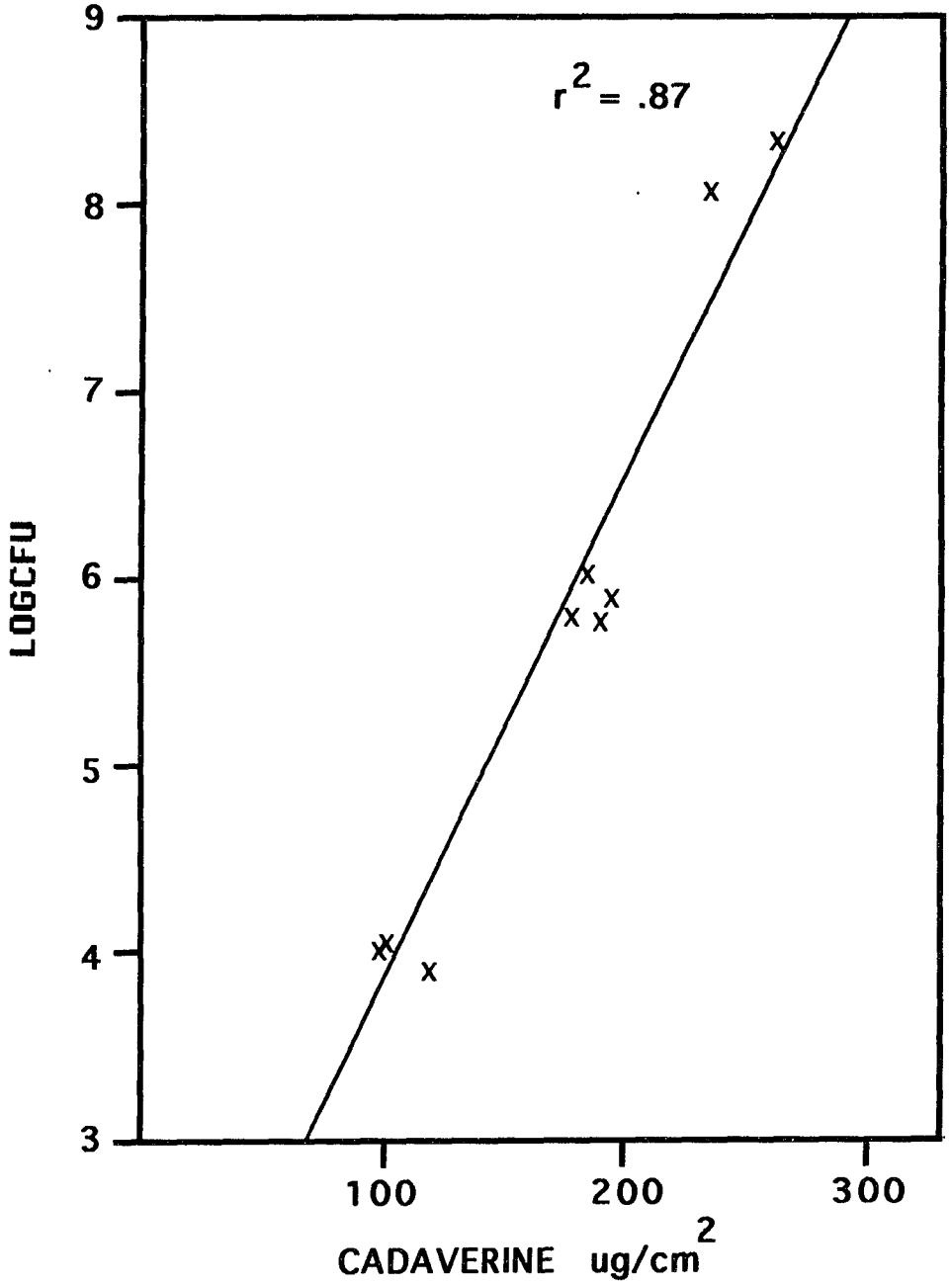

Figure 11. Correlation analysis between cadaverine and total bacterial counts in turkey

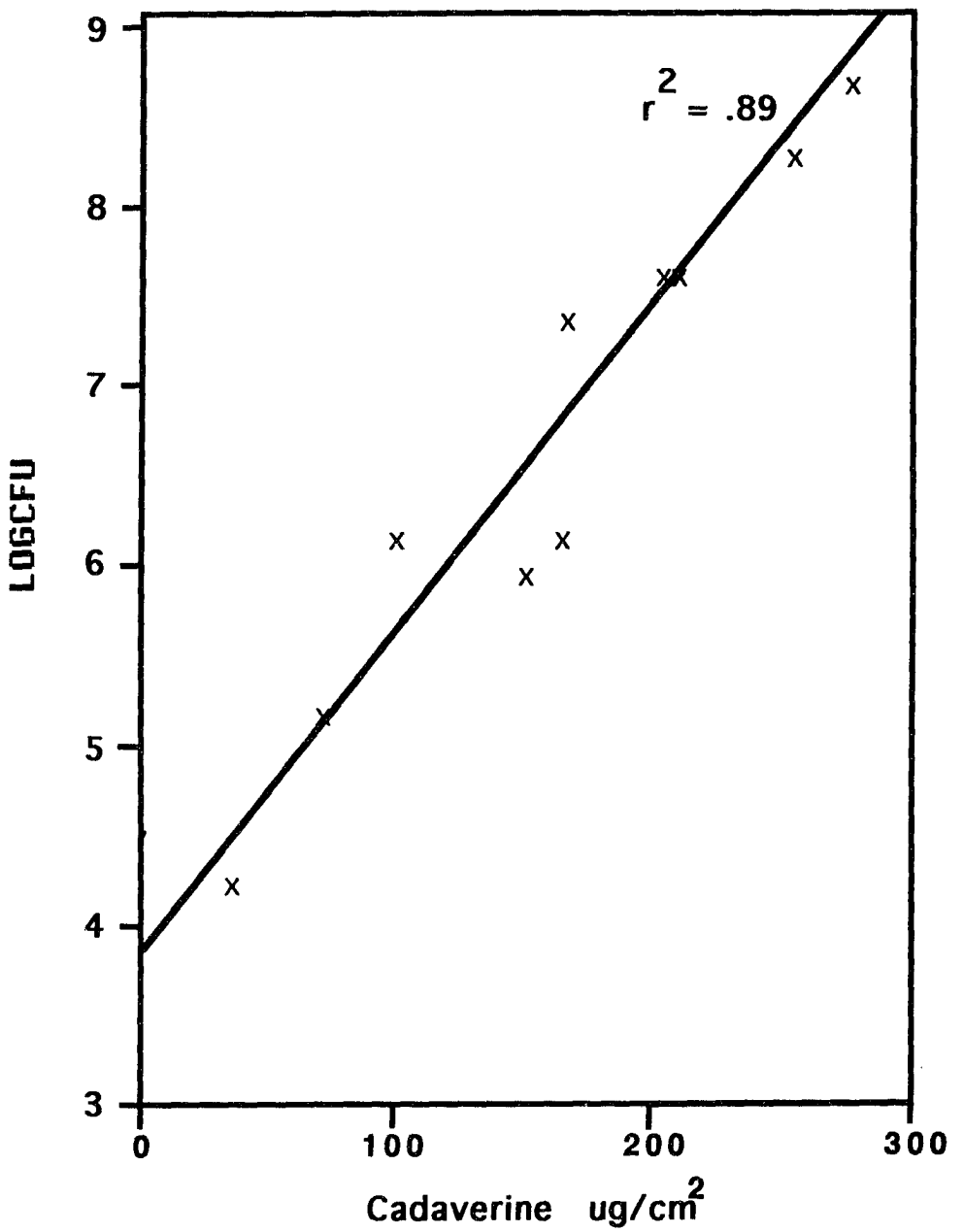

Figure 12. Correlation analysis between cadaverine and LogCFU of mesophilic counts in pork loins

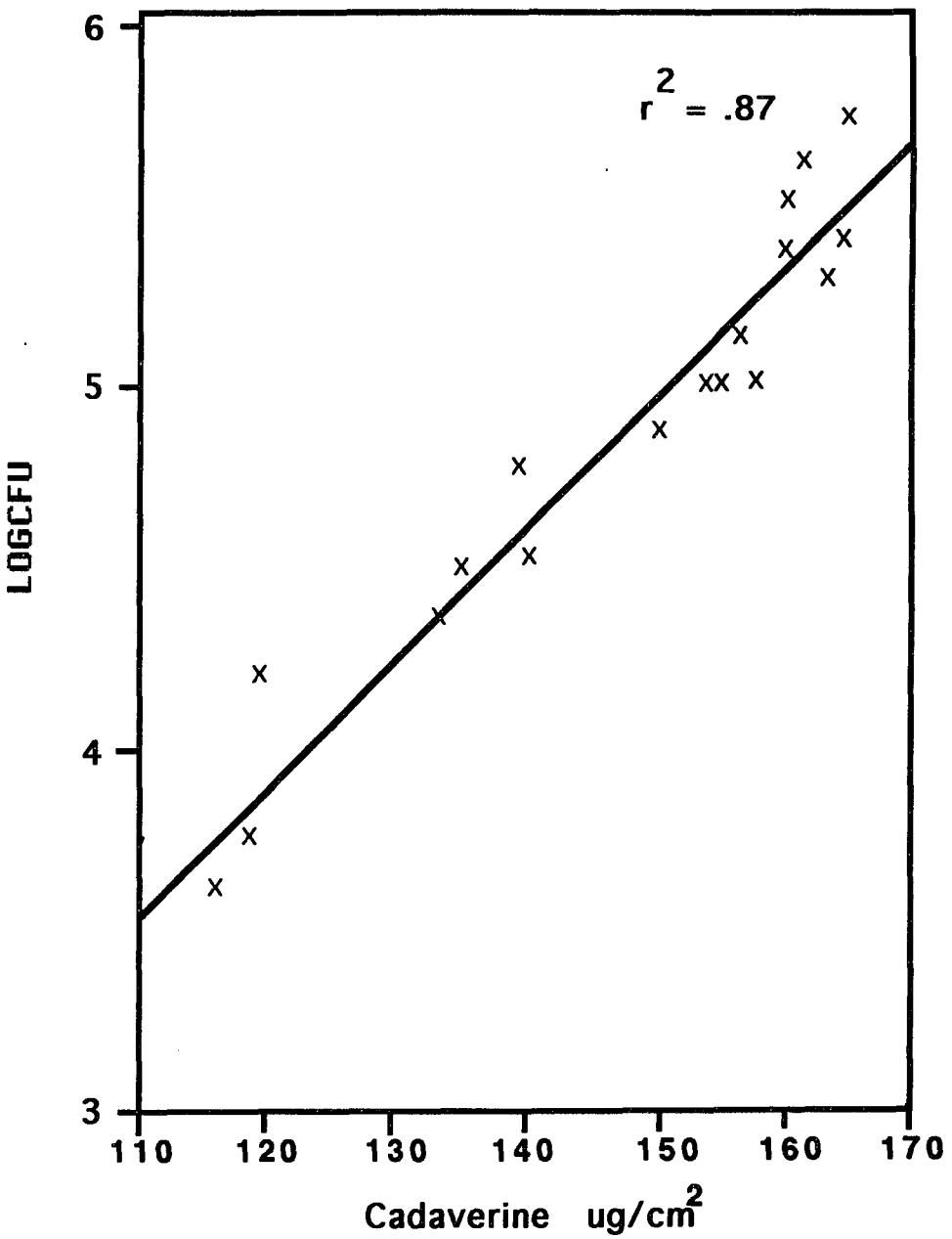

Figure 13. Correlation analysis between cadaverine and LogCFU of mesophilic counts in beef loins

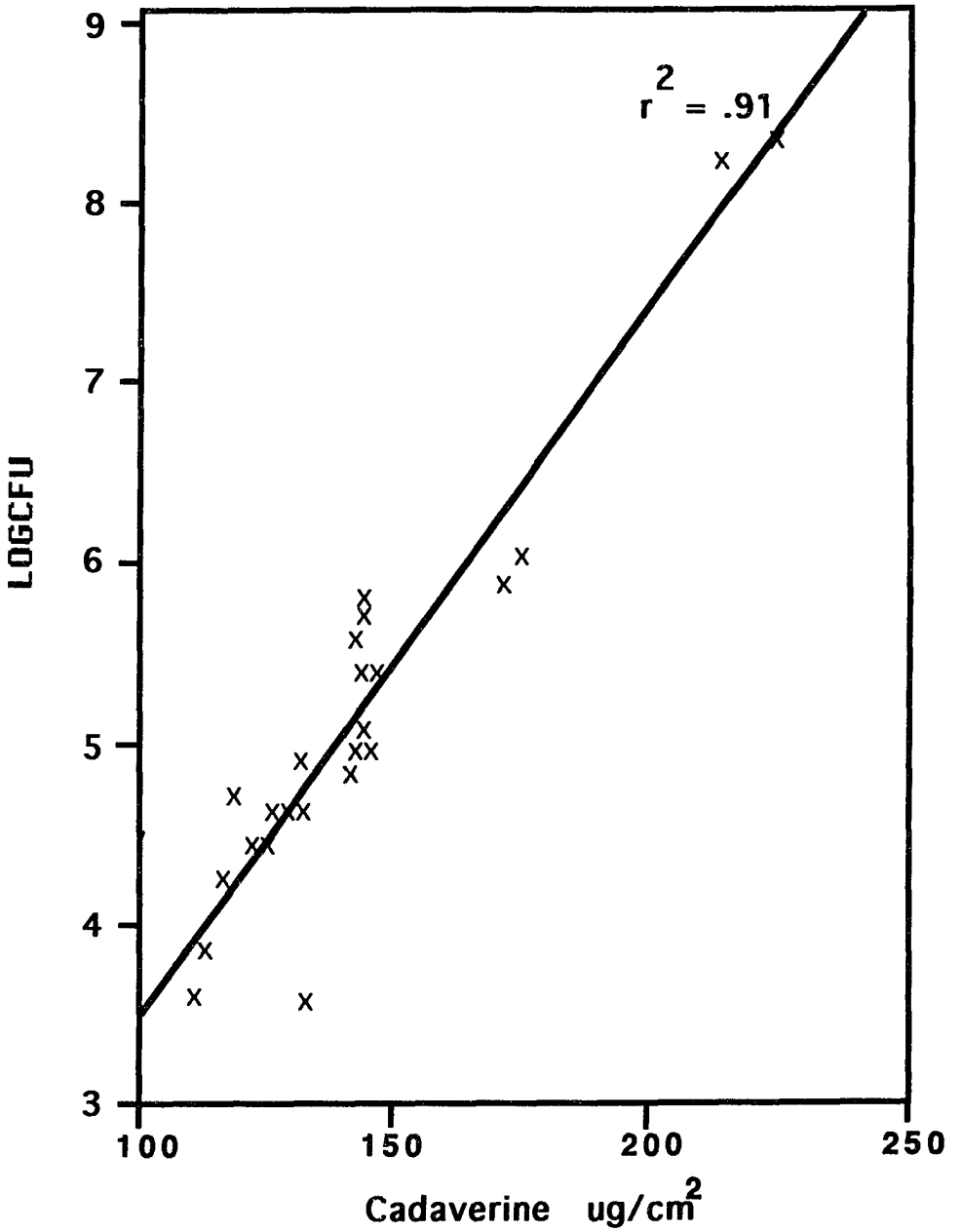

Figure 14. Correlation analysis between cadaverine and LogCFU of mesophilic counts in pork loins and rounds

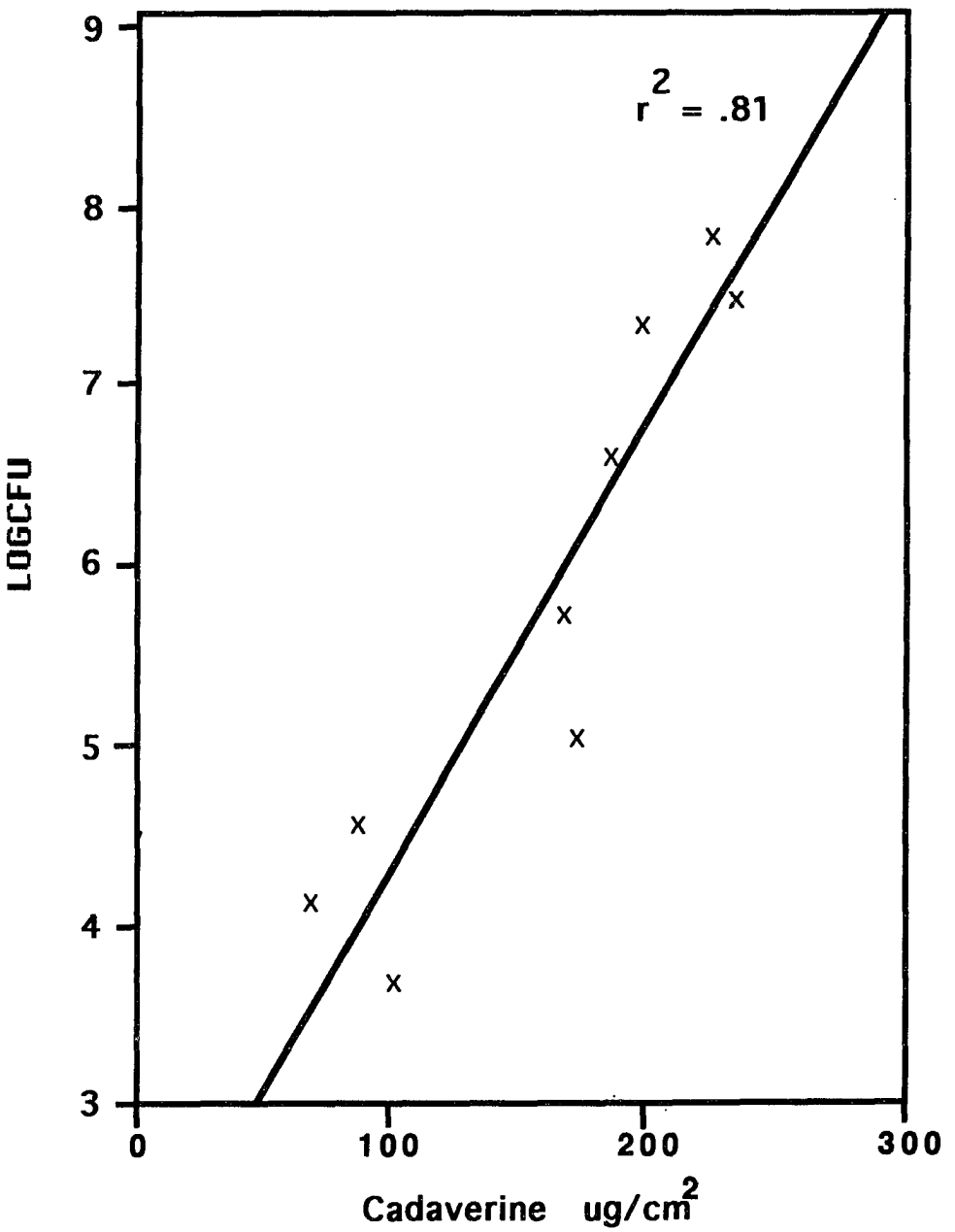

Figure 15. Correlation analysis between cadaverine and LogCFU of mesophilic counts in turkey

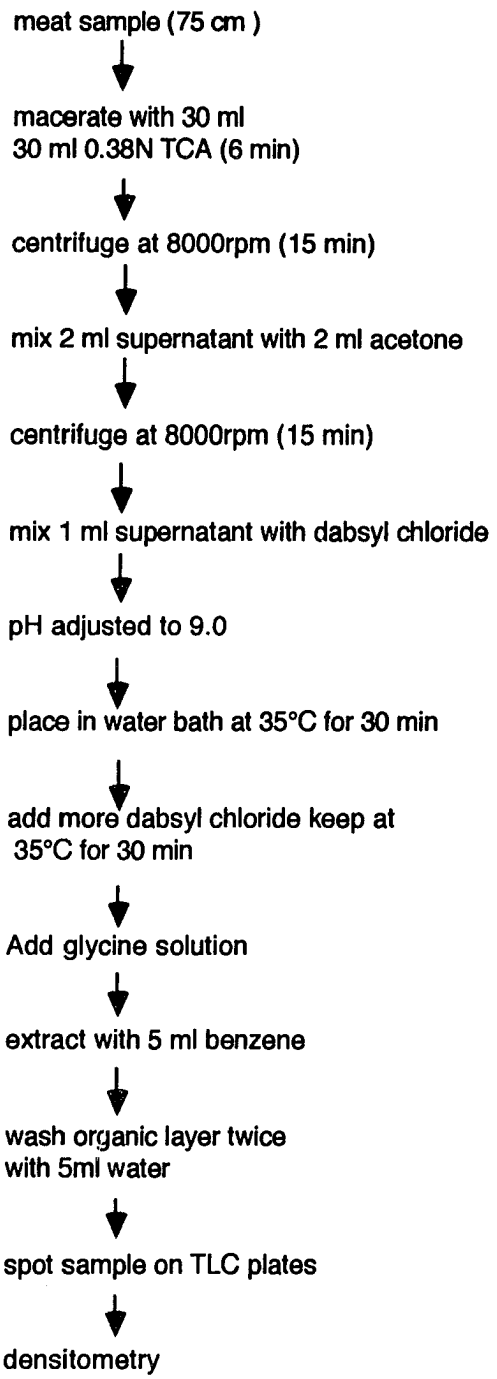

Figure 16. Schematic for analysis of cadaverine

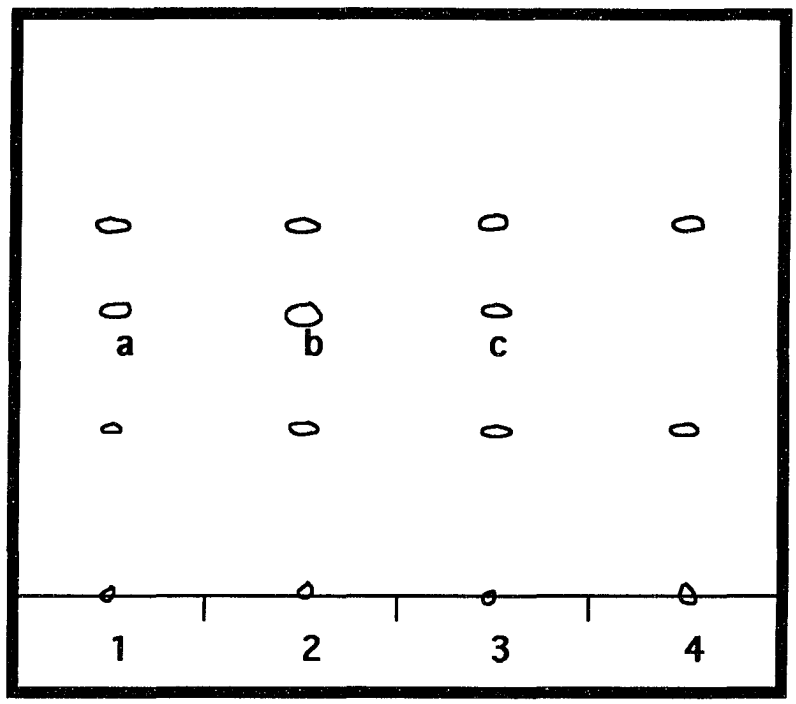

Figure 17. TLC plate drawing
1 and 2 = cadaverine
3 = sample
4 = dabsyl chloride

GENERAL SUMMARY

The first part of this study was undertaken to determine the possibility of developing a rapid method for the enumeration of bacteria based on the analysis of muramic acid in pork and beef under both aerobic and anaerobic storage conditions. There is a need for a rapid method for bacterial enumeration in meats because classical plate counting methods understate the number of microorganisms and is a very slow procedure. Plate counting results take days to obtain because of the incubation time. The method used for the analysis of muramic acid is based on the release of lactate from the muramic acid molecule followed by the actual analysis of lactate. A correlation value of 0.98 was found between muramic acid and total psychrotrophic bacterial loads in pork stored under aerobic conditions and a correlation value of 0.84 for pork stored under vacuum-packaging conditions. Our correlation between total bacterial counts and muramic acid in both beef treatments was low. From the analysis of all data, it can be concluded that there is substantial error (variation) in the muramic acid measurement. The average of multiple measurements predicts total bacterial counts but a single value is not overly reliable. No difference was found when the measurement of muramic acid was performed on the same sample three times in both pork and beef. Also, when performing

the measurement using a piece of sample instead of using swabs little difference was found on the amounts of muramic acid in both pork and beef.

In the second part of this study, meat freshness was compared to the content of the amine cadaverine and total bacterial numbers throughout aerobic chilled storage of commercial fresh pork, beef, and turkey. The range for bacterial counts went from 10^3 to 10^9 bacteria /cm^2 for psychrotrophic bacteria. In pork loins, a correlation of 0.95 was found for cadaverine and total bacterial counts. The level of cadaverine ranges from about 100 ug/cm^2 when counts were at about log5 and went as high as about 280 ug/cm^2 for log8. In the samples obtained from local supermarkets a correlation between cadaverine and total counts for both beef rounds and beef loins was 0.94. Bacterial loads in beef ranged from 10^3 to 10^8 bacteria/cm^2 and cadaverine levels ranged from about 110 ug/cm^2 to about 200 ug/cm^2. These results clearly indicate that cadaverine can be used as a quality indicator not only in pork but also in beef. The lowest correlation (0.87) was found on turkey meat.

Future work and recommendations

Cadaverine is not the only biogenic amine which is formed in meat by bacteria. Other amines such as spermine, spermidine

and putrescine are also formed. It would be interesting to study these other amines and see if there is also a correlation between them and a combination of them and total bacterial numbers or any other specific bacteria in meats. Another interesting study would be to investigate if these amines could be used to assess quality of processed meats, such as hams, cold cuts or any other meat product which have gone through any processing. Many of these amines should be present in the final product specially on dry and semi-dry sausages and on country-cured hams since amine build-up generally occurs in products undergoing lactic fermentation or long term aging (Rice et al., 1975).

In addition to what was previously mentioned, there might be other routes to make an even faster method to detect amines in fresh meats. One of such routes is by the use of the compound fluorescamine which we tried in our laboratory for a short period of time. We were able to conjugate cadaverine to fluorescamine and see the fluorescent spots on reverse-phase TLC plates; however, we were not able to separate any spots on meat samples. Maybe instead of analyzing only one amine one could analyze total amine production and use fluorescamine as our fluorescent marker. This could perhaps be done in test tubes, followed by a fluorescence reading on a fluorometer. Another route may be by the use of dansyl chloride or with 1,2-benzene dicarbonal as the detection agents.

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Dennis Olson and Dr. Henry Stahr for their guidance, patience and encouragement during the course of my doctorate program. Their concern for me as an individual and their interest for my future have given me the courage during many discouraging hours, specially during the past year.

Special acknowledgement is due to Dr. Homer Walker, Dr. Joe Sebranek, Dr. Susan Lamont and Dr. Patricia Murphy for their willingness to serve on my graduate committee. I am also grateful to Steve Niebuhr for his laboratory assistance.

Special mention also goes to my friends and graduate students, Sharon Kotinek, Zoraida DeFreitas, Rodrigo Tarte and Wireko Manu for their help and humor given during my graduate studies.

I would also like to express my appreciation to my wife, Lourdes, for her patience, understanding, encouragement and help throughout this past years.

Finally, my heartfelt thanks go across the Atlantic Ocean, to Puerto Rico, and to my mother and father, brother and sisters, all of whom sacrificied the joys of family closeness and supported me with words of love and encouragement.