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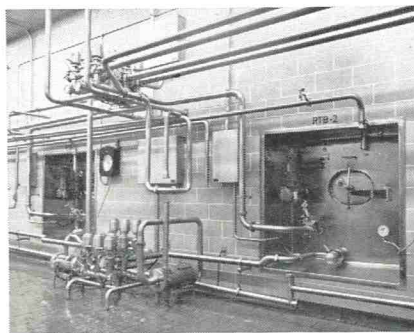
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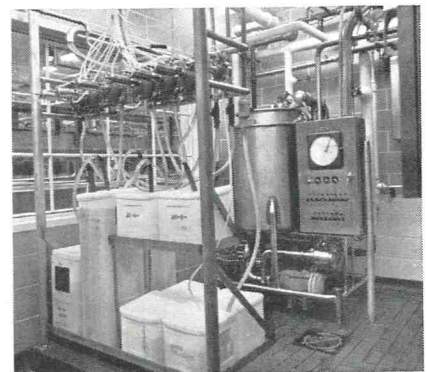
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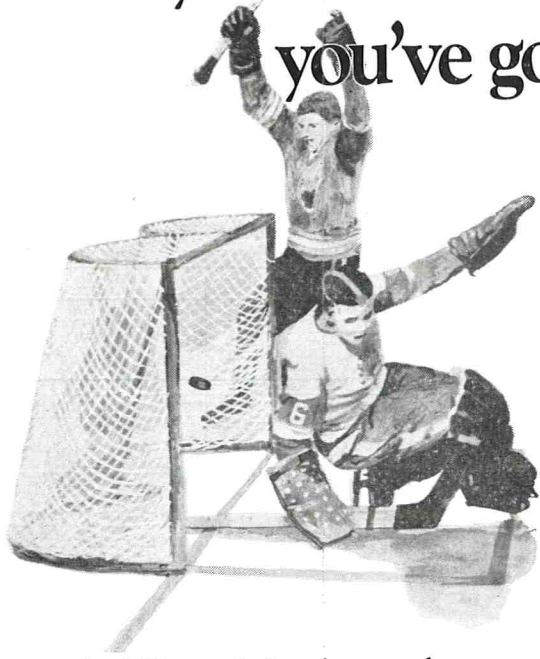Amino Acids
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METHODS FOR PRODUCTION OF HIGH QUALITY RAW MILK

(A Summary of Annual Reports Prepared From 1955 to 1970 by the IAMFES Dairy Farm Methods Commitee)

COMPILED AND EDITED BY

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THE FOOD INDUSTRY IN THE SEVENTIES[1]

WILLIAM O. BEERS[2]

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ABSTRACT

More than any other, the food industry is extensively and inextricably involved in the "growth/zero growth" or "quantity/quality" dilemma. For food, as man's own source of energy, is the determinative element in mankind's formula for survival. As the apparatus for supplying this source of energy, the food industry thus plays a crucial role in the resolution of the crisis that could eventually determine the fate of mankind.

The food industry, both in regard to the individual consumer in the store, and the world population as a whole, will have to give even greater attention to the quality of its overall contribution in terms of the ends achieved by its products. Such a change in emphasis implies a greater degree of social purpose and accomplishment, but does not mean an erosion of the profit factor. For without profits, private business—the food industry included—cannot continue to function as the most effective means of allocating resources that man has yet developed.

Trying to survey briefly the present decade of an industry that represents a segment of the U. S. economy amounting to approximately $120 billion annually or a little over 11% of the G.N.P. is a little like trying to summarize the Old and New Testaments for an article in *Readers Digest*. In addition to its staggering size and diversity, the food industry from farm field to the dinner table daily affects the well-being of each man, woman, and child in every corner of our shrinking globe. In much of the world the hand to mouth task of subsistence makes food *the* premier daily pursuit of the majority of the populations. In the more developed portions of the world where food in abundance is taken for granted, the food industry has become one of the most scrutinized, regulated and, in some quarters, maligned and misunderstood industries of our time.

The food industry, perhaps more than any other, is intimately involved in the rapidly changing attitudes and value systems that are re-shaping the fundamental underpinnings of our society. The impact of these changes—how they are perceived and managed will determine not only the shape and form of the industry by 1980 but also the rise and fall of leadership within the industry.

For hidden beneath the shock waves of rapid change and the noise and nonsense of food faddists and professional naysayers, new and very legitimate sets of demands on the part of our customers are taking shape. And they're taking shape in an environment of diminishing consumer trust and confidence about quality, safety, marketing practices, and nutritional values of our food supply. And they are emerging out of the need to close the nutrition gap —whether it is malnourishment in the developed countries or undernourishment in the underdeveloped. They are developing out of ideas expressed in the vocabulary of new concepts—"ZPG," "Ecology," "Life Support Capacity," "Energy Crisis," and "Earth as a Giant Spacecraft."

These are some of the concerns that will determine our future on this earth and will influence us as human beings involved in the essential task of providing mankind with the energy fuel to support his life. We must be careful in evaluating the changes in the priority of demands made by our customers. It's not so much that the older more traditional needs and desires have been replaced but rather that new expectations have been added. To be successful in the market place a food product still must taste good, be convenient to use, be perceived as good economic value and, in the main, make a contribution to good nutrition. There is nothing that I see on the horizon to change these basic consumer desires.

FOOD FANTASY AND NOSTALGIA

Contrary to demand for new and different performance characteristics in many products of other industries, the dream for food products is in the reverse direction—like people think it used to be in Grandma's day—down on the farm and all that. This form of food fantasy and nostalgia can be seen reflected in similar ways by many who market food products. I refer to it as a "food fantasy" not to demean the dream but to remind us that, as in many other things, the so-called good old days in food weren't so good after all.

Supposing my mother—who spent 4 to 5 hr a day preparing meals from a meager list of alternative raw materials, that were inconsistent in terms of quality, that would spoil while you looked at them, and that cost dad 43% of the family's disposable income—suppose she could visit a modern food store

and a modern home kitchen. Why, she'd think any-
one who referred to her experience as the good old
days was a practical joker, who, as she would put it,
ought to have his head examined.

J. L. Kraft, the founder of our Kraft Foods Division,
got his start by trying to solve the problem of in-
consistent quality and the poor keeping characteris-
tics of much of the cheese marketed at the turn of
the century.

But today's problems are far more complex. And
with higher levels of education, improved communi-
cations and vastly expanded knowledge, we have
mixed in with the more traditional demands relating
to food itself, those relating to other values. To
many modern consumers raised to accept the premise
of food in abundance, and who take the marvels of
modern food technology for granted—much in the
same way that we all take daylight for granted each
morning—the mere availability of food is no longer
enough.

It's unrealistic to expect today's consumer to con-
gratulate us for getting the food to market if its pro-
cessing or packaging negatively affects the environ-
ment . . . or if its performance claims exceed reality
. . . or if there's some significant question about the
long term effect or value of some ingredient used in
its preparation.

Consumer demands have become much more intri-
cate, and this is going to affect consumer purchas-
ing patterns. The proving ground for these new
concerns and concepts of value is in the market
place. The successful food marketer of the seventies
is not the one who debates with his customer about
the worth of these new expectations, but is the one
who has the ingenuity and the built-in flexibility to
move with the times. No company rigidly clinging
to the belief that last year's demands are inelastic
will survive the course.

Consumer Confidence

The second and perhaps most perplexing factor
for the food industry in the seventies is the erosion
of consumer confidence. This phenomenon of our
times seems to be present throughout our country
and is affecting our attitudes towards every institu-
tion of our society—from education, to government,
to our judicial system, to our businesses, and even
marriage and the family structure. A Harris public
opinion poll in 1966 showed that 55% of adults said
they had great confidence in the major business cor-
porations and their leaders. Following up 5 years
later, in 1971, that percentage is down to 27%. There
is some irony in all this. Since we are all a part of
one institution or another, we seem to be mistrusting
each other and ourselves. One of the overlooked as-
pects of consumer dissatisfaction is the simple fact

that with the exception of those who merely criti-
cize, we are all both consumers and producers.
Therefore, much loss of confidence can be traced to
indifferent performance when consumers are wearing
their producer hats.

Misinformation or just plain lack of information
also results in lack of consumer trust. Some of the
present criticism over food prices is a case in point.
It's no wonder when people assume profit levels
four or five times and sometimes 20 times greater
than reality, that demands for price reductions reach
ear-splitting levels. In recent months, as I've heard
false inflations of food industry profits—at farm—or
processor—or retailer level—I've thought of today's
consumer in terms of a Mark Twain comment—"It's
not what you don't know that will hurt you, but
rather what you know for sure that ain't so."

Food Prices

Very few shoppers realize that if they buy a $29
bag of groceries and tip the bag boy a quarter they
are giving him more than the store made on their
purchases. In our own company over the past dec-
ade, Kraftco profits as a food processor have amount-
ed to about 3 cents on each dollar of sales—while the
average for all industry is about 5%.

Meanwhile, the farmers' share of the food dollar
decreased 1 cent from 39 to 38 cents between 1961
and 1971 and by June 1972 had increased to only
40 cents. At the same time, the amount of dispo-
sable income spent for all food—including food eaten
away from home—has dropped from 20% in 1960 to
15.6% in 1971. Between 1960 and 1970, according
to U. S. Department of Commerce figures, increases
in the amount of personal income used for food
ranked lowest in a list of 14 categories. In the last
decade income allocated for food rose 63.7% com-
pared to 180% for education and 120% for recreation.
Personal taxes, incidentally, gobbled up an increase
of 119%.

Of course in terms of total dollars, there isn't any
question that people are spending more at the food
store these days. But increased prices are only part
of the story. For one thing, consumers have been
steadily upgrading their food purchases. The steady
increases in per capita consumption of meat is a case
in point. Furthermore, as supermarkets continue to
move further into general merchandise, many non-
food items; records, pantyhose, lawn chairs, and
shampoo, have gone into the push cart to be lumped
in the shopper's mind as part of her food bill.

Food Quality

The decline in quality of our food supply is an-
other misconception. Dr. Emily Wick, Professor of

Nutrition and Food Science at M.I.T., recently said, "We'll have to give up eating if we get much more picayune." As one writer put it—"If we are to believe all the horror stories written about food—sitting down to dinner means taking your life in your hands." The book titles alone are enough to scare you to death—*Food Pollution, The Chemical Feast, The Great American Food Hoax, Poisons in Your Food.*

Some of the accused culprits are additives used in food processing and unfortunately the extreme declarations of these nutritional soothsayers who see doom on the dining room table, get more notice than the more moderate and seasoned views expressed by doctors and scientists in the field of nutrition.

In addition, there have been accusations of nutritional deficiency, widening debate over diet-related diseases as well as those attributable to microbial contamination. Part of the problem is, of course, that there is precious little scientific data to confirm or refute once and for all the alleged hazards in our food supply. But despite legitimate questions and the search for answers, the facts tend toward a rather more optimistic and positive picture than the catastrophic scene painted by some critics.

First of all, the traditional diseases of malnutrition —pellagra and rickets—have all but disappeared in this country. Iodization of salt virtually eliminated goiter. The problem of botulism, which got widespread publicity following an unfortunate death recently, still remains primarily a problem of home canning.

E. M. Foster, Director of the Food Research Institute at my alma mater, the University of Wisconsin, in a recent article pointed out some interesting facts. "Since 1925 there have been only three outbreaks of botulism with four deaths from the consumption of commercially canned food in the U. S." . . . There was an outbreak of botulism in connection with commercial fishing operations in 1960, but Dr. Foster here is referring to commercially canned food, and I want to make that clear . . . "One in 1941, two in 1963 and one in 1971. Meanwhile in that same period—1925-1971—we Americans have consumed 775 billion containers of food canned in the U. S."

Salmonella has also come in for wide-spread attention and Dr. Foster also comments on this. "In 1970 the National Center for Disease Control listed 79 outbreaks of *Salmonella* gastroenteritis involving 3,852 people. Not a single outbreak was attributed to commercially processed food."

Dr. Foster concludes by stating, "I am convinced that the American food supply is, in fact, far safer than some would have us believe. Mishandling during preparation and serving, both in the home and in the food service establishment, is responsible for the vast majority of food poisoning in this country. Micro-organisms account for more than 90% of this."

FOOD ADDITIVES

Another area of controversy which is perhaps less clear-cut concerns additives. But what is clear is that *without* additives many foods presently being marketed simply would not exist. Dr. Emily Wick, while acknowledging that additives should be used with care and caution, said that without additives we wouldn't have good food. Much of it would spoil overnight. "Most of the big scares," Dr. Wick concluded, "have been the result of animal testing." She asks, "Is their behavior analogous to what would happen in a human being?"

Others have raised questions about dose relationship and exaggerated test conditions with animals as well as the possibility of toxicity from additives. But of course possible toxicity in foods is by no means limited to additives. Natural carcinogens have been identified in wheat flour (patulin), in cabbage and turnips (thiourea), and in spinach (nitrites). Safrole, an extract of sassafras, was found carcinogenic in 1960. Ironically sassafras tea has become a dietary favorite of some health food faddists.

Any discussion of additives must take into account that they do provide specific benefits and that the possibility of risk must be evaluated with regard to a balance between the two. Take for example, the present controversy over the use of sodium nitrite in meat packing. Nitrites in combination with certain amines may produce nitrosamine compounds which are carcinogenic. Sodium nitrite, for which there is at present no acceptable substitute, also acts to prevent development of botulism bacteria. The consumer is faced with a dilemma—the risk of cancer with the use of nitrites or the risk of botulism without it. The meat industry wonders if bacon, ham, sausage, and other cured meats will survive in the market place. The food industry in the seventies will continue to be preoccupied with the search for answers to questions just like this one.

HOW TO MEET THE NEW DEMANDS

How are we going to marshall our capabilities to do all that has to be done to meet new demand patterns, to restore consumer confidence and to close the nutrition gap? I'm going to suggest four keys to better service by the food industry.

More support for research

First, we desperately need exactly the opposite of what so many critics of our system suggest. We urgently need to funnel a great deal more of our resources into *more* scientific and technological endeavors. Yes, I said *more!* Science and technology has been the favorite whipping boy of those who

think the solution to our problems is to stop civilization in its tracks. But it just isn't so. We need more, not less—more research and development to gain a better understanding of the inter-acting function of the 50 or so known nutrients—more research to discover additional nutrients and knowledge of the role of trace minerals.

We need to improve the efficiency of photosynthesis, and we need to learn how to make better use of our water resources. Dr. Gerald Thomas, President of New Mexico State University, earlier this year noted that under current techniques it takes over a ton of water to produce a pound of bread. He also observed that if organic and "chemical free" backyard farming became the order for the world's 3-2/3 billion people that land resource requirements would triple and many would be without essential fresh water.

We need increased scientific research for better and cheaper methods to control microbial contamination in food which, it is estimated, accounts for 91% of food borne disease. In fact, Dr. Virgil Wodicka, Director of the FDA's Bureau of Foods, considers microbial contamination as the number one food safety problem. Incidentally, Dr. Wodicka places additives at the bottom of his problem priority list behind nutritional imbalances, environmental toxicology, natural toxicants, and pesticide residues.

When there is so much to do, so much to learn, so many applications to be developed, how can anyone argue for less scientific effort? In 1967, the President's Science Advisory Committee estimated the following percent increase in world needs from 1965 to 1985: (a) calories from 7.7 trillion to 11.1 trillion per day, and (b) protein need from 187,693 metric tons to 284,903 metric tons per day. We cannot give up, the increasing need for technological advancement is too great.

Regulatory procedures

The second key to better food industry service is to be found in the rationalization of regulatory procedures. The expansion and greatly increased complexity of the food industry has caused additional strain on the already inadequate resources and facilities of the Food and Drug Administration and other regulatory agencies at the state, local, and federal levels.

Despite dedication of purpose, the problems of personnel and budgetary limitations of these regulatory bodies are compounded by the need for improved organization and coordination of the activities of various agencies. Division of essentially similar functions naturally leads to duplication, waste, and inefficiency. It is apparent that overall efficiency (and ultimately the consumer) will suffer until a

greater degree of uniformity of standards and co-ordination of efforts is accomplished. This is especially true of the dairy industry, which we know something about, in that throughout the country there is a massive amount of duplication and inefficiency in the inspection of milk supplies and processing plants.

There is an important need to establish uniform regulations and reciprocity among states, counties, and municipalities not only in regard to inspection and testing standards, but also labeling of products. The number of regulatory bodies with which almost every segment of the food industry must do business not only leads to confusion and inefficiency and weakens the barriers of food safety, but it also costs the consumer, the taxpayer, and the food industry needless and substantial amounts of money every year.

If regulatory procedures are to become more efficient, we must have continued and improved co-operation between various governments and better communications between producers and regulators. A good deal of progress in this area has been made in this country more recently. One example is the work of the Grocery Manufacturers of America/Food and Drug Administrative Council. Their work last year covered a wide range of topics including: (a) Food Color Testing Program, (b) The Problem of Polychlorinated Biphenyls, (c) Food Inspection and Product Recalls, (d) A National Center for Toxicological Research, (e) Nutrition Labeling, (f) Food Plant Sanitation, and (g) Codex Alimentarius. This is not an all-inclusive list. And of course in addition, GMA, other trade associations and FDA are cooperating in an orderly review of the GRAS list—a study of the 1,810 food additives Generally Recognized As Safe. The FDA also meets regularly to discuss these problems with representatives of consumer groups.

An informed consumer

The third key to better service is a more informed consumer. We've got to take the time and money necessary to see that our consumers are well informed, not only about what we do, but how and why we do what we do.

The food industry has been too reticent to make its voice heard amid the din of its detractors. Too often food industry silence has given credence to charges of profiteering or adulterating or short-cutting safety.

We shall not regain consumer confidence until we help create a better informed consumer—a consumer who is told not only how good everything is but also, candidly, how it can be made better and how scientists and food technologists are tackling the problem. We must also assist in educating the con-

sumer about the importance of handling food to avoid contamination.

Increased productivity

The fourth and last key that I am going to suggest is increased productivity. And this goes beyond the matter of direct employee productivity. We need to take a hard look at the whole process of food growing, processing, and distributing. Relating improved productivity to inflation control, James Cook, Chairman of Penn Fruit and President of Super Market Institute, speaking before that group's recent annual meeting said, "Employee productivity is only one weapon. Improving the productivity of capital—including inventory, space and equipment—is just as important."

Not surprisingly, Mr. Cook touched on several points that are just as applicable to us as food processors as they are to his organization of food retailers. These common denominators also reach backward to the farm, which is rapidly changing from being "a-way-of-life" to a capital-intensive industry. The need for increased productivity will lead, I believe, to a closer integration of all of these food industry functions—a kind of systems approach to the whole business of converting the energy latent in the sun and the soil into food energy for man.

Indeed, I think it is essential that we begin to think of food in terms of energy conversion. Food must be included in our thinking about the total energy needs of mankind. The potential inadequacy and uneven distribution of food in global terms is part of the total energy crisis facing us on this planet.

Increasing productivity, however, does not mean that somewhere around the corner there is, waiting to be discovered, a revolutionary change that will produce dramatic results. Productivity improvement will result from an accumulation of many separate actions.

By making better use of computer technology, we should look for ways to reduce the volume of paper needed to operate and control our businesses. Similarly, if we can achieve the ideal of a checkless society, we should be able to increase the velocity of money and thereby improve the productivity of capital.

We should continue to strive for improvements in the utilization of space—not only production space but office space as well.

We've barely begun to scratch the surface in the possibilities for increasing productivity through better application of existing communications techniques . . . techniques, for instance, that can bring people together without actually having them physically transported over long distances.

People productivity can be increased by sharpening our techniques for getting square pegs in square holes—by a better method of matching skill, experience, age, and energy. For example, perhaps business operations leadership ought to come at an earlier, more energetic age while a man's imagination is most pliable and vivid, and before decades of experience have made his approach to operations more rigid. His later productive years perhaps would be more efficiently utilized in those areas where experience and knowledge and wisdom can give perspective to broad policy and longer-range functions.

IN CONCLUSION

These then are some of the tasks that I think will occupy the food industry in the seventies and beyond. The laundry list is much longer. I haven't even mentioned marketing questions like counter-advertising and the "fairness doctrine," both or either of which stand to drastically alter advertising practices.

As a closing comment and at the risk of being stoned in the marketplace by some of our social critics, I would like to reiterate what I have said about profits on several previous occasions. It is "profits" that keep our world going. No matter how strident and insistent the demands of society that we carry out the mission of providing the required quantities of good-tasting, nourishing food for as many people as possible at as low a price as possible—the food industry cannot do this unless it survives; and it cannot survive unless it makes a profit.

I'll never believe that the exercise of social responsibility and the concept of profit are incompatible. There is no better way of achieving an equitable distribution of resources, in my opinion, than the combined working of enlightened and progressive capitalism. And the fuel that keeps this wonderful machine going is profit. This is a great and legitimate form of energy in itself.

NITROSAMINES: A REVIEW[a]

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ABSTRACT

Nitrosamines have become a recent problem for the food industry since it is thought that certain nitrosamine precursors, such as sodium nitrite, which are commonly used in some foods, may lead to significant hazards in the food supply. This review draws together recent information and evaluates the problem of nitrosamines in foods. Chemistry of nitroso compounds is discussed and results of several animal studies are cited to emphasize the extreme toxic and carcinogenic potential of some of these compounds. The mechanism of action in tumor development is considered. Although not yet entirely clear, the most acceptable idea involves alkylation of cellular components such as DNA, RNA, and proteins which leads to changes in normal cell growth. Presence of nitrosamines in foods has been documented in several instances but more recent analytical techniques involving gas chromatography and mass spectrometry have indicated that levels in foods may be lower than previously believed. The potential for formation of nitrosamines from nitrite and secondary amines is discussed, in relation to food before consumption and to formation in the body, particularly the stomach, after consumption. Finally, analytical procedures are reviewed and briefly evaluated and some other possible sources of nitrosamines in the environment are considered.

Nitrosamines and their potential hazards to man have been known to the scientific community for almost 20 years, since the pioneer work of Magee and Barnes *(14, 80)*. Yet it has only been within the last few years that great concern has arisen over nitrosamines in the human food supply and their possible link to human cancer. Nitrosamines have recently been discussed in technical literature, consumer periodicals, and the popular press often with an air of unwarranted emotionalism.

There is little doubt about the danger of certain nitroso compounds, and this cannot be ignored by the food industry. The food industry, however, has an excellent history of food safety and care must be taken to avoid unwarranted action which may be based on potential rather than actual hazards.

The problem is not one of establishing the danger of nitrosamines, but rather it is one of relating nitrosamines to food hazards. The difficulties of very low concentrations; of long, laborious procedures for analysis; of a lack of a "threshold dose;" of a need to establish a benefit-risk ratio for some possible nitrosamine precursors such as nitrite; and of differences in toxicity of specific nitrosamines have all added to the complexity of the problem.

Nitrosamines, when present in foods, are found in concentrations of parts per million or even parts per billion and this necessitates very careful, well-developed methods of analysis. Although these compounds are present in very low concentrations, they are extremely potent in their attack on animal species and presumably also on man. This makes any decisions concerning threshold dosage levels, as they may be applied to man, extremely difficult. It is also very difficult to evaluate nitrosamine precursors such as nitrite, in terms of benefit-risk ratios, when so little is really known about human tolerance and exposure to nitrosamines. Finally, a complex biological system such as a food system, has the potential to form many different kinds of nitroso compounds.

Thus, the problem has far-reaching implications for the food industry. There are many questions yet to be answered and careful, diligent research designed to establish basic principles will form the basis for intelligent and logical decisions.

HISTORICAL

Nitrosamines first came to the attention of the scientific community in 1954 when Barnes and Magee *(14)* studied dimethylnitrosamine as a result of a laboratory toxicity incident. Dimethylnitrosamine was shown to be causally related to liver disease in two workers in an industrial plant where it was being used as a solvent. Shortly following, in 1956, Magee and Barnes *(80)* reported the potent hepato-carcinogenicity of dimethylnitrosamine in rats. Subsequent studies on other nitrosamines followed and Heath *(60)* and Heath and Magee *(62)* showed that dimethylnitrosamine was, overall, the most toxic and carcinogenic of this group of compounds, although over 65 different nitrosamines were shown to be carcinogenic *(82)*.

Enthusiastic work ensued in the field of cancer research as the value of nitrosamines as model compounds for induction and study of chemical carcinogenesis was recognized. General dosage levels for rats were established by Heath *(60)*, and Argus and Hoch-Ligeti *(12)*, and several ideas were advanced to explain the mode of action by which nitrosamines

induced carcinogenesis *(28, 32, 75, 76, 83, 125)*.

A sudden interest concerning nitrosamines developed after 1960 when toxic effects (hepatic lesions) were observed by Hansen *(58)* and Koppang *(71)* in mink and in ruminants, both of which consumed feeds containing fish or fish meal preserved with unusually high levels of sodium nitrite. These lesions apparently resulted from a method of preservation in which herring at peak supply periods was subjected to very high levels of nitrite and dried to preserve it until it could be processed to herring meal *(69)*.

Consequently, attention turned to determination of the amounts of nitrosamines in the human food supply. Investigations of various foods have shown several to contain some level of nitrosamines. Hedler and Marquardt *(63)* found nitrosamines in samples of wheat flour, wheat plants, milk, and cheese. Ender and Ceh *(42)* demonstrated their presence in some meat samples and mushrooms. Fazio et al. *(49)* also found positive results for various processed meats, while Sen et al. *(112)* found nitrosamines in nitrite treated fish. They have been identified in an edible South African plant by Du Plessis et al. *(39)*, and Devik *(34)* determined their formation as products of the Maillard reaction. On the other hand, Thewlis *(122)* could not find nitrosamines in wheat flour and Keybets *(69)* was unable to demonstrate their presence in spinach which contained high levels of nitrite.

The scope of investigation then expanded to include nitrosamine precursors since secondary amines and nitrite are known to be present in the food supply. Beatty *(15)*, Preusser *(97)*, and Stewart et al. *(116)* have shown that secondary amines may be present in foods and nitrite is commonly added to some foods, especially meats and fish *(5)*. It has been suggested by Ender and Ceh *(43)* and Lijinsky and Epstein *(78)* that nitrites and secondary amines can combine to form nitrosamines in foods before consumption. The possibility that such formation might occur in the stomach after ingestion has also been advanced by Sen et al. *(111)* and Sander and Burkle *(105)*.

Recent work by Hawksworth and Hill *(59)* has shown the possibility of nitrosamine formation by bacterial action in the intestine or in the bladder. Also, Tannenbaum *(119)* has noted that human saliva contains a significant amount of nitrite as well as thiocyanate, which Boyland et al. *(16)* have shown to catalyze the reaction of nitrite and secondary amines to form nitrosamines.

There has been recent interest in the geographical distribution of cancer with the intent of relating observed differences in geographical distribution of tumor incidence with environmental factors. Doll *(35)* and McGlashan *(87)* have pointed out the high incidence of liver cancer in parts of East Africa and

of cancer of the esophagus in areas of South Africa. McGlashan et al. *(88)* have implied that food or even alcoholic drinks may be a source of nitrosamines as the causative agent.

Wolff and Wasserman *(130)* have recently indicated some doubt concerning the validity of some early analytical methods since later work has shown the likelihood of artifacts and errors. This would mean that the already low levels found in many foods may in reality be even lower than previously thought.

Thus, at present, the toxic and carcinogenic properties of nitrosamines are well established. However, the gaps of knowledge concerning dose-response relationships as well as the low concentrations found in food make it difficult to evaluate the hazard to human health.

CHEMISTRY

According to Magee *(79)*, N-nitroso compounds can be divided generally into two groups. One group includes the dialkyl, alkylaryl, and diaryl nitrosamines and the other includes alkyl and aryl nitrosamides. These two groups differ chemically and in biological activities. Nitrosamines are chemically stable under physiological conditions, but will undergo photochemical decomposition when exposed to ultraviolet light. Morpholine or piperidine may contribute the amino-N to the molecule and replacing the NO group with CHO gives the corresponding formamides; Heath and Magee *(62)* have demonstrated that these compounds are not carcinogenic.

Magee *(79)* also states that the nitrosamides are of variable stability depending on pH. Alkaline conditions promote formation of diazoalkanes and this has been applied in synthetic organic chemistry. Some nitrosamides are unstable even at neutrality. N-Nitroso methyl urea, for example, has a half life of 125 hr at pH 4, 1.2 hr at pH 7, and 0.1 hr at pH 8 *(82)*.

The nature of the toxic response to the nitroso compound depends, apparently, on chemical characteristics. Magee *(79)* indicated that the dialkylnitrosamines such as dimethylnitrosamine or diethylnitrosamine, show a tendency for liver damage, generally a hemorrhagic lesion. This specificity has been explained by Magee *(79)* to be due to the requirement that these compounds be metabolized to exert their effect and that the active carcinogen is a metabolite of the original nitrosamine. Since the liver is generally highest of all organs in metabolic activity for breakdown of drugs and foreign compounds, it follows that the active carcinogen would be produced there.

Nitrosamides show local cytopathic action, in contrast to nitrosamines, presumably because of their greater chemical instability *(79)*. Some of the nitro-

samides also show a "radio-mimetic" action, causing damage to organs that have a rapid cell turnover, such as lymphoid tissues and bone marrow. This, too, is apparently related to the chemical instability of the nitrosamide, but it is not clear how these compounds cause damage to the cells (79).

A wide variety of physical properties are possible for nitroso compounds. Magee and Barnes (82) have pointed out that while dimethylamine is an oily liquid at room temperature and miscible in water, others may be liquid or solid and soluble in water or organic solvents. There are, however, some general characteristics shared by most of the nitroso compounds. They are characteristically photosensitive, and ultraviolet light will split the nitroso group to give various products. Preussmann et al. (98) showed that the nitroso group may be reduced to give hydrazine derivatives and Udenfriend et al. (123) showed that oxidation of the nitroso group gave several unidentified breakdown products. Schoental (109, 110) reported that nitroso compounds such as N-methylnitrosourethane may react with sulfhydryl groups of cysteine at neutral pH, giving complex mixtures of products but including some which may be involved in carcinogenic activity. However, dialkylnitrosamines such as dimethylnitrosamine, do not react with sulfhydryl compounds under similar conditions.

Nitrosamines are classically produced by a combination reaction of nitrite or nitrous acid with secondary amines under acid conditions (see Fig. 1).

Mirvish (90) found that this reaction depended on pH and basicity (pK) of the amine as well as relative concentrations. The chemistry of the formation of nitrosamines from nitrite and secondary amines will be discussed more thoroughly in a later section on the potential for formation in foods before ingestion.

CARCINOGENIC AND TOXIC POTENTIAL

The N-nitrosamines are considered by Magee and Barnes (82) to be among the most potent and versatile

$$\begin{matrix} R' \\ \diagdown \\ \diagup \\ R \end{matrix} NH + HNO_2 \longrightarrow \begin{matrix} R' \\ \diagdown \\ \diagup \\ R \end{matrix} N-N=O + H_2O$$
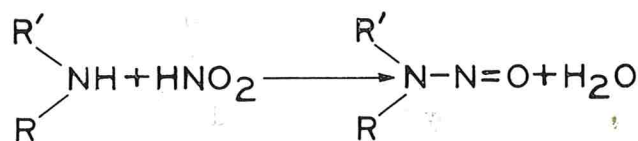
Figure 1. Reaction of nitrite with a secondary amine to form a nitrosamine.

of all chemical carcinogenic agents. They can produce tumors in many species of animals, and almost all organs are susceptible to at least one nitroso compound. Even though they are rapidly metabolized, they can even induce cancer after a single dose. Schmahl and Osswald (107) found that similar doses of diethylnitrosamine produced liver cancer in all 11 species of animals tested (see Table 1). Ashley and Halver (13) have also demonstrated similar effects on rainbow trout with dimethylnitrosamine. Thus, there seems to be little reason to believe that other species, particularly man, might be unaffected by these compounds.

The site of activity in a particular instance seems to depend on the species, diet, age, particular compound, dosage level, and route of administration, as well as dosage rate (23). Magee and Barnes (82) point out that continual feeding of dimethylnitrosamine at a level (about 50 ppm) that allows survival of rats for 30 weeks or longer results in development of only liver tumors. Terracini et al. (120) found that levels even as low as 5 ppm caused a significant (7%) incidence of tumors of the liver.

Magee (79) and Magee and Barnes (82) showed that higher levels of feeding (200 ppm) over shorter time periods also produced tumors but in the kidney rather than the liver of rats. This was also noted for single doses of a few milligrams. There appeared to be scarring of the liver in this instance, but no malignant changes. Argus and Hoch-Ligeti (12) reported, however, that when limited large doses were fed, both liver and kidney tumors could be occasionally observed. These workers also found that the incidence of only kidney tumors in this situation was

TABLE 1. THE CARCINOGENIC ACTION OF DIETHYLNITROSAMINE IN VARIOUS ANIMAL SPECIES[a, b]

Species	Mode of application	Daily dose (mg/kg)	Total dose (LD$_{50}$) mg/kg
Mouse	oral	3	871 ± 124
Rat	oral	3	700 ± 53
Hamster	oral	40 (weekly)	640
Guinea pig	oral	3	1200 ± 100
Rabbit	oral	3.4	2500
Dog	oral & S.C.[c]	3	560
Pig	oral	2-50	1400-25,700
Grass parakeet	i.m.[c]	70 (weekly)	2800 ± 400
Brachydanio rerio	oral	10-100 ppm	10-13 weeks

[a]All resulted in liver tumors of various types.
[b]From Schmahl and Osswald (107) by permission of Experientia and the authors.
[c]S.C. is subcutaneous and i.m. is intramuscular.

90% or more. Zak et al. (131) and Argus and Hoch-Ligeti (12) were able to induce lung tumors in rats by using dimethylnitrosamine.

Magee and Barnes (82) noted that there are parallels to be drawn relating structure of the nitrosamine with the target organ when the compound is administered orally. Symmetrical dialkyl nitrosamines attack the liver and asymmetrical dialkylnitrosamines select the esophagus. Cyclic nitrosamines showed a mixed response with some being non-carcinogenic. Functional group nitrosamines seemed to attack the liver or bladder.

Other organs which may develop tumors on exposure to various nitroso compounds include the nasal cavity, olfactory nerve, brain, spinal cord, and peripheral nervous system (82). Brune and Henning (21) has produced eyelid carcinomas with methylbutylnitrosamine by topical application.

That the route of administration is not the only determinant of the site of tumor development is exemplified by N-nitroso-N-butylamine. Magee and Barnes (82) point out that this compound results in bladder tumors alone when injected subcutaneously, but gives liver, esophagus, and bladder tumors with equal distribution when given orally. Also, many nitroso compounds produce esophageal cancer when fed to rats but nitrosopiperidine produces esophageal cancer upon intravenous injection. The variation of response of different organs is of interest in considering biochemical changes which may be necessary to induce carcinogenesis.

One of the most sobering facts about nitroso compounds is the capacity to induce tumors in progeny of animals treated during pregnancy. N-Nitroso-methylurea was given to rats on the 15th day of pregnancy and produced nervous system tumors in the mothers. Ivankovic and Druckrey (66) then observed that nervous system tumors were produced in the offspring. The dosages were well below that which showed a toxic effect on the mother. In another study by Druckrey et al. (36), mothers (rats) were injected (iv) with 50 mg of nitrosoethylurea/kg body weight and 162 of 179 offspring died of malignant tumors of the brain, spinal cord, trigeminal nerves, or peripheral nervous system. Nitrosamines, as opposed to the nitrosamides mentioned earlier, appear to be less able to induce transplacental carcinogenesis but have been demonstrated to do so in mice and hamsters (82). Table 2 lists some of the carcinogenic nitroso compounds along with their site of activity, species tested, and means of administration. The other major physiological effect that nitroso compounds or nitrosamines may exhibit is that of acute toxicity. As previously mentioned, Barnes and Magee (14) first studied dimethylnitrosamine as a result of poisoning in industrial laboratories. It was found in this early work on rats that single doses of 25 mg of dimethylnitrosamine/kg body weight administered orally, intravenously, intraperitoneally, or subcutaneously would manifest the toxic effect by centrilobular liver necrosis as well as hemorrhages in the liver and lungs, blood in the lumen of the gut, convulsions, and coma. Death or complete recovery usually followed within 2-4 days. Carter et al. (22) discovered that mink are extremely sensitive to the toxic activity of nitrosamines. Dietary levels of 2.5-5 ppm produced signs of toxicity in 7-11 days and death in 23-24 days. Magee (79) explained the relatively selective action of nitrosamines for the liver in the same way as the carcinogenic activity is explained. The liver is highly active in metabolizing foreign compounds and produces a nitrosamine metabolite which causes the damage and resulting toxicity symptoms. Magee (79) points out that other tissues metabolize these compounds to a varying but lesser degree, which seems to correspond to the susceptibility of the particular organ. For example, tissue slices of rat kidney have shown metabolic activity for dimethylnitrosamine second to that of the liver; lung and esophageal tissue showed some lesser activity and activity of the small intestine was low. These effects correspond to the relative susceptibility of these organs to dimethylnitrosamine.

The severity of the toxic effect depends on several factors such as species, route of administration, and type of nitroso compound considered. Magee (79) indicates that hamster tissues show the same susceptibility for dimethylnitrosamine as the rat but the diethyl compound differs. Hamster lung tissue seems to have the greatest capacity of metabolizing diethylnitrosamines while other tissues including the liver are less active.

Some of the nitroso compounds are very reactive and produce lesions at the site of entry into the body. Schoental (108) found that nitrosomethylurethane was irritating to the lungs and skin and produced severe necrotic lesions of the stomach and liver as well as congestion of the lungs when given orally to rats. An anesthetic effect after oral administration of trimethylnitrosurea for 2-3 hr was noted by Ivankovic et al. (67) but was followed by lung edema and death.

Microscopically, the necrotic effect of 20 mg of dimethylnitrosamine/kg body weight on rats is complete in 24 hr (82). In those rats which survive, the necrotic areas are receding within 72 hr and restoration of the liver is almost complete within three weeks, as noted by Magee and Barnes (80). The acute liver lesions in the rabbit, dog, and mouse follow the same pattern.

Mizrahi and Emmelot (92) discovered that the toxic effect of the alkylnitrosamines may be decreas-

TABLE 2. CARCINOGENIC ACTIVITY OF SOME NITROSO COMPOUNDS[a, b]

Compound	Species	Organ	Treatment
Dimethylnitrosamine	Rat	Liver	Feeding L.S.
	Rat	Kidney	Feeding 1-12 weeks S.C. and oral 1-10 doses
	Rat	Lung	Feeding and daily dosing P.O.
	Rat	Nasal sinus	Inhalation
	Mouse	Liver	Feeding L.S.
	Mouse	Kidney, Lung	Drinking water L.S.
	Hamster	Liver	Drinking water L.S.
	Trout	Liver	Feeding
Diethylnitrosamine	Rat	Liver	Drinking water L.S. single oral
	Rat	Kidney	Single oral or IV
	Rat	Esophagus	Drinking water L.S.
	Mouse	Liver	Drinking water L.S.
	Mouse	Stomach	Drinking water 30 weeks
	Mouse	Esophagus	Drinking water 30 weeks
	Mouse	Nose	Percut daily 6 weeks
	Guinea pig	Liver	Drinking water 30-40 wks.
	Guinea pig	Lung	Drinking water 30-40 wks.
	Rabbit	Liver	Drinking water 80 wks.
	Dog	Liver	Food and drinking water
	Monkey	Liver	Daily oral
	Fish	Liver	Water
	Hamster	Liver	Oral 2 times weekly, IP 1× wk
	Hamster	Lung	Oral 2× weekly
	Hamster	Nose	S.C., IP
Dipropylnitrosamine	Rat	Liver	Feeding L.S.
Dibutylnitrosamine	Rat	Liver	Feeding L.S.
	Rat	Bladder, esophagus	Feeding L.S.
	Rat	Bladder	S.C.
Butyl-4-hydroxy-butynitrosamine	Rat	Bladder	Drinking water L.S.
Di-n-amylnitrosamine	Rat	Liver	Drinking water L.S.
	Rat	Lung	S.C. 1× wk, 25 wk
Allylmethylnitros-amine	Rat	Nose, kidney	IV 1× wk
Butylmethyl-nitrosamine	Rat	Liver	Oral, 30 doses
Methylvinyl-nitrosamine	Rat	Nose	Single inhalation
Methylphenyl-nitrosamine	Rat	Esophagus	Feeding L.S., drinking water L.S.
Benzylmethyl-nitrosamine	Rat	Esophagus	Feeding L.S.
Ethylisopropyl-nitrosamine	Rat	Esophagus	Feeding L.S.
Butylethyl-nitrosamine	Rat	Esophagus	Feeding L.S.
Cycasin	Rat	Liver, kidney	Feeding L.S., S.C. single dose
	Rat	Colon	Feeding 13-21 days
Diazomethane	Rat	Lung	Inhalation

[a]From Magee and Barnes (82) by permission of Academic Press, Inc. and the authors.
[b]Abbreviations as follows: L.S. = life span; S.C. = subcutaneous injection; IV = intravenous injection; IP = intraperitoneal injection; percut = percutaneous and P.O. = orally.

TABLE 3. ACUTE TOXICITY OF SOME NITROSO COMPOUNDS[a, b]

Compound	Dose (mg/kg)
Dimethylnitrosamine	27-41
Diethylnitrosamine	216
Di-n-butylnitrosamine	1200
n-Butylmethylnitrosamine	130
Tert-butylmethylnitrosamine	700
Di-n-amylnitrosamine	1750
Methylphenylnitrosamine	200
Benzlmethylnitrosamine	18
Ethylisopropylnitrosamine	1100
n-Butylethylnitrosamine	380
Tert-butylethylnitrosamine	1600
Ethylvinylnitrosamine	88
Azoxyethane	530

[a] From Magee and Barnes (82) by permission of Academic Press, Inc. and the authors.
[b] All figures are for single oral LD_{50} dose for rats.

ed by some SH compounds such as cysteine. Cysteine, injected subcutaneously, apparently decreases the activity of certain liver enzymes involved with the transformation of nitrosamines.

LD_{50} data have been obtained for most nitroso compounds and are given in Table 3. Some of the compounds however have a very low acute toxicity so that accurate LD_{50} data are not available.

Interestingly enough, Magee and Barnes (82) state that there is no correlation between carcinogenic activity and acute effects of nitrosamines. Both dimethyl- and diethylnitrosamine produce lesions, predominantly in the liver, when given in toxic amounts. Diethylnitrosamine, however, has about one-eighth the acute toxicity of dimethylnitrosamine but if given continuously is probably a more active liver carcinogen.

Magee (79) believes that, with the exception of the ruminant and mink poisonings noted, environmental concentrations of nitrosamines are generally too low to constitute a toxicity hazard to human health. However, it is much more likely that carcinogenic concentrations may be present in the environment since prolonged low dosage levels to rats results in malignant liver tumors.

METABOLISM AND BIOCHEMICAL EFFECTS

Magee and Barnes (80) in their early work demonstrated that metabolism of nitrosamines in vivo takes place very rapidly and that the half life for dimethylnitrosamine in rats was about 4 hr. Even very large doses are removed completely from the body within 24 hr. This evidence suggests that there is very rapid metabolic conversion of dimethylnitrosamine. Another interesting result (82) is the lack of accumulation of dimethylnitrosamine in any one organ of the body following injection. Thus, the selectivity shown

for the liver by dimethylnitrosamine carcinogenesis seems to be caused by some metabolite of nitrosamine in the liver and not the nitrosamine itself. Work by Dutton and Heath (40) in which most of a ^{14}C label on dimethylnitrosamine was expired as CO_2 lends support to this idea. The remaining ^{14}C not expired was evenly distributed in the tissues and some (7%) was excreted in the urine, all within 6-8 hr after injection of the nitrosamine.

Heath and Dutton (61) demonstrated with ^{15}N that dimethylnitrosamine is demethylated to one carbon intermediates which are either oxidized to CO_2 or used in body metabolism. Alkylating agents such as diazomethane or a carbonium ion have been suggested as the possible active metabolic products of nitrosamines. Other suggested intermediates have been the corresponding aldehyde, nitrous acid, a hydroxylamine derivative, and a hydrazine derivative since these are all known mutagens (82). Most evidence suggests that the alkylation reaction with cellular components is most likely but Kruger et al. (72) have shown this is not true in rainbow trout, and Magee (79) and Heath (60) have claimed that there is conflicting evidence. That nucleic acids could become methylated after administration of dimethylnitrosamine was demonstrated by Craddock and Magee (28) and the methylation was found to occur primarily at the seven position of guanine. The base 7-methylguanine was then excreted in the urine and this was believed to result from excision of the base or breakdown of the nucleic acid molecule. Although other abnormal bases may be possible, Magee and Barnes (82) indicate that 7-methylguanine is overwhelmingly predominant following dimethylnitrosamine administration. Magee and Farber (83), Craddock and Magee (28), and Magee and Hultin (84) have also observed that proteins as well as RNA and DNA are methylated by dimethylnitrosamine. Brouwers and Emmelot (20) noted that dimethylnitrosamine showed an inhibitory effect on incorporation of amino acids into protein but that respiration, glycolysis, and other enzymatic functions remained normal. Protein inhibition is paralleled by breakdown of ribosomal aggregates and Villa-Trevino (125) suggested that messenger RNA is alkylated and is then destroyed or is functioning defectively.

Hypophysectomized rats are resistant to many potent liver carcinogens such as aflatoxins and azo dyes but when Lee and Goodall (77) administered dimethylnitrosamine to such rats, the usual pattern of DNA and RNA alkylation occurred along with liver tumors. Diethylnitrosamine also leads to a loss of response of the rat liver to corticosterone, according to Dalton and Snart (31). Fiume et al. (52) discovered that aminoacetonitrile prevents inhibition by dimethynitrosamine of protein synthesis and thus perhaps prevents meta-

bolism of the nitrosamine. This work supports the idea of metabolic conversion to an active carcinogenic and hepatotoxic agent.

Several other mechanisms of action have been suggested. Rondoni (102) proposed that denaturation may play a part in chemical carcinogenesis and Argus and Hoch-Ligeti (12) found that dimethylnitrosamine and some other carcinogens were able to denature proteins. However, work by Magee and Barnes (80) showed a uniform distribution of dimethylnitrosamine in the body water with tumors only in the liver, kidney, and lungs of rats after oral administration. Thus, simple protein denaturation does not appear to be the answer.

Another postulated mechanism advanced by several workers and discussed by Magee and Barnes (82) in their review, is that of active metabolites of the nitroso compound such as the corresponding aldehyde, nitrous acid, hydroxylamine derivaties, hydrazine derivaties, and alkylating intermediates, most of which have been previously mentioned.

The idea of alkylating intermediates is supported by Druckrey et al. (37) since they found, in general, that there is good correlation between the tendency for a nitrosamine to form diazoalkane and its ability to produce cancer. However, Magee and Barnes (82) indicate that this is not always true as evidenced by heterocyclic nitrosamines. N-Nitrosomorpholine, for instance, is highly active but is not believed to have the ring open to form a diazoalkane. Magee and Barnes (82) also pointed out that while several alkylating agents are carcinogenic, they vary in their potential to produce cancer. And, while there is some correlation between the organs affected and the amount of methylation of their nucleic acids, there are some exceptions. The liver undergoes a high degree of methylation upon a single dose but no tumors develop. Kidney tumors which do develop are in an area of much less methylation. Magee and Barnes (82) suggest that this may reflect the liver's greater capacity for regeneration and repair.

If one is to assume that the alkylation reaction causes carcinogenesis, then the possible cell components affected should be considered. Dalton and Snart (31) suggested that the carcinogen may cause deletion of hormone receptor proteins in the cell. If these receptor proteins are part of the protein synthesis control, then protein deletion would be of major concern. Schoental (110) emphasized the possibility of protein sulfhydryl groups becoming alkylated, particularly at points of possible links between proteins and nucleic acids. Magee and Barnes (82) also advanced the idea that while there has been little work in the area, there is little reason to believe that lipids should be totally unaffected.

Nagata and Imanura (93) calculated energy incre-

ments (ΔE) involved in each step of the metabolic conversion of alkylnitrosamines to alkylated guanine and similarly for non-carcinogenic analogs. They found that the ΔE value for the carcinogen was smaller than for the non-carcinogen only in the alkylation step occurring by bimolecular nucleophilic substitution. Thus, they concluded that this alkylation step was the essential one in the carcinogenic process (see Fig. 2).

It has long been thought that cancer may result from a change of certain genetic factors, and the idea of methylation of DNA and RNA is consistent with this. The possibility of two types of molecular change has been advanced by Brookes and Lawley (18). In the first, the amount of genetic material remains constant but the base sequence is changed. The alkylated guanine results in an increased acidity at the N-1 atom of the purine ring. Thus, at a neutral pH there is more ionized base present than before. Alkylated, ionized guanine pairs with thymine instead of the usual cytosine thus leading to a base pair change in replication of the DNA. The second type of molecular change is acutal base pair deletion since the 7-alkyl-guanine is slowly removed from the macromolecule.

Thus, the current idea with the most support is that the dialkyl nitrosamines undergo a dealkylation and an intramolecular condensation to the active diazoalkane. The diazoalkane (or a carbonium ion) then functions in alkylating nucleic acids, changing the genetic code, and interfering with protein synthesis. While there are gaps and conflicts in the evidence, this has been the most widely accepted postulation so far.
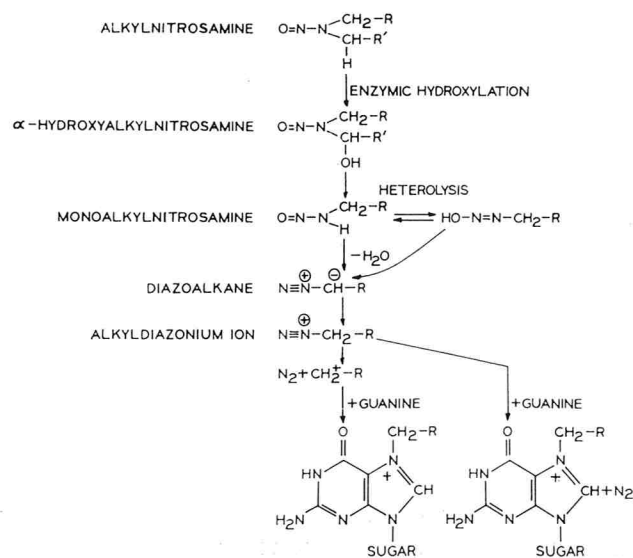

Figure 2. Scheme for alkylation of guanine. From Nagata and Imamura (93). Reprinted from Gann by permission of the copyright owner, the Japanese Cancer Association.

Occurrence of Nitrosamines in Foods

Although the initial research on nitrosamine toxicity and carcinogenicity was stimulated by human poisoning in an industrial situation (80), it was not until the 1960's that consideration was given to other possibilities for contact with nitrosamines. An outbreak of liver disease in sheep was caused by 30-100 ppm of dimethylnitrosamine present in a herring meal used in the feed (44). This raised the possibility of nitrosamines occurring in human food, particularly at concentrations low enough to produce no obvious toxic effects yet great enough to induce cancer.

There have been few instances of nitrosamines reported in raw food. Cycad nuts are toxic and Laqueur et al. (74) observed that rats fed the nuts developed liver and kidney tumors. It was suggested that the toxic principle may be very similar to alkylnitroso compounds and, in fact, structurally they are much alike (82). Cycasin, the toxic factor, is a glycoside of glucose and methylazoxymethanol. Riggs (101), Miller (89), and Matsumoto and Higa (86) showed cycasin to be a methylating agent which probably forms the same methylating intermediate as dimethylnitrosamine.

A fungus that may contain a nitroso benzaldehyde has been observed and Magee and Barnes (82) advise the examination of other microorganisms for toxic metabolic products.

Nitrosamines in the range of 0.4-30 μg/kg have been found in some samples of various mushrooms by Ender and Ceh (42) (Table 4). Fazio et al. (48) demonstrated the presence of nitrosamines in raw sable fish but only at a very low concentration of 4 ppb. Hedler and Marquardt (63) found diethylnitrosamine in samples of wheat plant, wheat grain, and wheat flour but the procedure was not quantitative nor has it been confirmed. Thewlis (122) was unable to find diethylnitrosamine in wheat flour. Du Plessis et al. (39) reported that fruit of a plant (Solanum icanum) in the Transkei area of South Africa, contained dimethylnitrosamine and its presence was correlated to a high incidence of esophageal cancer in this area.

The greatest present concern for the food industry lies with foods that are processed with nitrite which serves to inhibit Clostridium botulinum (38, 56) and which also may combine with secondary amines in a product to produce the corresponding nitrosamine (64). Consequently, investigations have centered on such products as fish, cured meats, and some kinds of cheese (when made with nitrite as in Europe and perhaps elsewhere but not in the U.S.) as well as alcoholic beverages, spinach, and milk products. Hedler and Marquardt (63) examined samples of milk and

Table 4. Nitrosamines in Some Foods[a]

Food	Nitrosamine (μg/kg)	No. of samples
Fish:		
Smoked herring	0.5-9.5	5
Kippers	0.5-2.4	4
do.	40	1
Smoked haddock (from Iceland)	15	1
Smoked mackerel	0.6	1
Meat:		
Smoked sausage	0.8-2.4	3
Bacon	0.6-6.5	3
Smoked ham (from Iceland)	5.7	1
Mushrooms:		
Polyporus ovinus	11.6	1
Boletus scaber	1.4	1
Amanita muscaris	30	1
Champignon	0.4-5	2
Hydnum imbricatum	3-15	2
Armillaria mellea	12	1
Lactarises trivialis	9.2	1
Russula emetica	10.2	1
A mixture of various edible mushrooms	14	1

[a]From Ender and Ceh (42) by permission of Pergamon Press and the authors.

Table 5. Dimethylnitrosamines (DMN) in Various Meat Products[a]

Type of product	DMN ppb
Cold cuts	0-4
Sausages	1-3
Baby foods	2-3
Canned meats	1-3
Bacon, hams and pork products	0-5
Beef products (misc.)	1-2

[a]From Fazio al. (49) by permission of J. Ass. Off. Anal. Chem. and the authors.

Tilsit cheese and found diethylnitrosamine in both. However, as with the flour samples, they were unable to quantitate the amounts present. Keybets et al. (69) examined spinach in which nitrate-reducing bacteria produced a high nitrite level but could find no nitrosamines and concluded that the pH was too high and concentration of secondary amines too low to allow significant formation of nitrosamines.

Ender and Ceh (42) found from 0.5-40 μg of nitrosamines per kilogram of smoked fish and smoked meats (Table 4). Fazio et al. (49) found evidence of nitrosamines in various meat products (Table 5) but in only one sample (a ham) was the concentration (5 ppb) high enough to confirm with mass spectrometry. All other samples contained from 0-4 ppb. Fazio et al. (48) also examined smoked marine fish and found dimethylnitrosamine in all samples of sable,

Table 6. Dimethylnitrosamine in Fish[a]

Type of process	Dimethylnitrosamine (ppb)
Sable	
Raw	4
Smoked	4-9
Smoked, nitrate treated	12-14
Smoked, nitrite treated	8-9
Smoked, nitrate and nitrite	20-26
Salmon	
Raw	0
Smoked	0-5
Smoked, nitrate treated	16-17
Smoked, nitrite treated	4-6
Shad	
Raw	0
Smoked, nitrate treated	10
Smoked, nitrite treated	12

[a]From Fazio et al. (48) by permission of J. Agr. Food Chem. and the authors.

Table 7. Values of $\overline{K}$ and K for DMN Nitrosation at Various pH Values[a]

pH	Yield of DMN (%)	$10^2 \cdot \overline{K}$	$10^{-6} \cdot K$
1.5	3.3	1.88	32.2
2.0	5.0	2.88	16.4
2.5	9.3	6.24	13.3
3.0	14.3	8.94	9.5
3.4	15.5	9.66	8.8
4.0	9.0	5.34	7.7
4.5	3.1	1.76	6.1

[a]From Mirvish (90) by permission of J. Nat. Cancer Inst. and the author.

all samples of smoked shad, and all samples of smoked salmon (Table 6). Nitrosamines have been reported in alcoholic drinks by McGlashan et al. (88), who found levels up to 3 ppm and noted a spatial correlation between positive samples from certain areas of Central Africa and incidence of esophageal cancer. Collis et al. (25) and Williams et al. (128) used more specific methods of gas chromatography and mass spectrometry but found no evidence of nitrosamines in alcoholic drinks.

Sen et al. (112) studied nitrosamines in fish with added nitrite and found that cooking led to formation of nitrosamines. The authors noted some variability and pointed out that the concentration of amines in fish depends on many factors and the amount of nitrosamines formed may depend on initial concentrations of amines. Marine fish, for example, generally have higher quantities of amines than do fresh water fish.

Fong and Walsh (54) examined Cantonese salt dried fish for nitrosamines since the Cantonese seem particularly prone to a nasopharyngeal cancer. They found both dimethyl- and diethylnitrosamine in all samples of nine species of fish purchased in the area. The concentrations found ranged from 0.6-9 ppm for dimethylnitrosamine and 1.2-21 ppm for diethylnitrosamine. The authors further investigated the presence of nitrate-reducing bacteria since the salt used contains some nitrate but no nitrite and they found that such bacteria were indeed present.

Wolff (129) summarized results of some recent studies by the USDA and FDA in which ham, frankfurters, bacon, and other cured meats were examined for nitrosamines. Only a very small number of samples were positive with concentrations ranging from 5-106 ppb. It appeared that nitrosamines were erratically produced in meat products and the conclusion was that they may indicate localized areas in a product where conditions (pH, temperature, amine, and NO_2 concentrations) are favorable to nitrosamine formation. Fiddler et al. (51) reported that nitrite could result in nitrosamine formation during frankfurter processing but only at concentrations higher than normally used.

Interestingly enough, even though nitrosamines have been demonstrated in several food products, Wolff and Wasserman (130) note that the validity of some earlier reports is questionable since improved analytical procedures have brought recognition of artifacts in many earlier procedures.

Nitrosamine Precursors and Potential for Formation in Foods Before Consumption

There is little doubt that presence of any significant amount of the alkylnitrosamines in human food constitutes a hazard to human health. Thus, it becomes of critical importance to examine the likelihood that nitrosamine precursors occur in our food and the conditions which may favor nitrosamine formation. As mentioned previously, nitrosamines can be formed by reaction of secondary amines with nitrous acid or nitrite under acidic conditions (Fig. 1). Nitrite is used for curing meat and fish, to preserve color, enhance flavor, and protect against the danger of botulism (5, 38, 56, 126). In addition, Magee and Barnes (82) and Wolff (129) point out that nitrates are widespread in our environment in vegetables such as spinach, beets, celery, and lettuce as well as in some well-water supplies. Thus, it is possible to obtain significant nitrite concentrations in these sources from microbial reduction of nitrate (96, 130).

Amines or amine precursors such as proteins, amino acids, phospholipids, or other compounds are present in most foods and may be available for reaction with nitrite. In fact, Phillips (96), Wick et al. (127), Preusser (97), and Stewart et al. (116) have reported high levels of secondary amines in foods such as fish, vegetables, and fruit juices and, in certain instances,

a single meal may have as much as 100 mg of secondary amines. It has also been noted by Malins et al. (85) that trimethylamine concentrations in chub can reach 25 ppm, trimethylamine oxide can reach 107 ppm, and dimethylamine can reach 2 ppm, all of which the authors believed to be too low for significant formation of nitrosamines. They were unable to demonstrate formation of nitrosamines under conditions similar to but more severe than those found in normal processing. As noted before, however, quantities of secondary and tertiary amines can vary greatly in fish (112). Fiddler et al. (50) found that quaternary ammonium compounds such as neurine, choline, acetylcholine, carnitine, and betaine all formed trace amounts of nitrosamines. Also, some tertiary amines were found to form nitrosamines under similar conditions. Thus, there may be potential nitrosamine precursors in different types of basic substances present in foods.

Various studies have been made on systems which contain nitrite and secondary amines to evaluate the potential for nitrosamine formation. Keybets et al. (69) examined spinach which developed significant concentrations of nitrite after storage but found no evidence of nitrosamines under normal conditions. However, upon adding high levels of diethylamine and lowering the pH to about 3, trace amounts of diethylnitrosamine appeared. Further studies by these authors indicated that nitrosation generally occurs below pH 4.5. If the nitrite concentration is very high and a long reaction time allowed, traces of nitrosamines may be found in even weakly alkaline conditions (pH 7.6). Malins et al. (85) studied the possible nitrosation of amines in model systems and used reaction conditions more severe than those used for commercial processing of nitrite-treated chub. They concluded that commercial processing would not result in more than 10 ppb of dimethylnitrosamine in smoked chub.

Archer et al. (10) studied the reaction of nitrite with creatine and creatinine (present in meats, milk, and some vegetable matter) and found that nitrate and creatine can form the carcinogenic nitrososarcosine under acid conditions while creatinine formed creatinine-5-oxime and 1-methylhydantoin-5-oxime of which the toxicity is undetermined.

Nitrosation of dimethylamine has been studied by Mirvish (90) who used tritium labeled amine in buffered aqueous solutions. He found that formation of dimethylnitrosamine was directly proportional to the dimethylamine concentration and to the square of the nitrite concentration. He points out that the chief nitrosating agent at pH 1 is nitrous anhydride (N_2O_3) which forms reversibly from two HNO_2 molecules. The rate of reaction is proportional to the N_2O_3 concentration and thus to the square of the

HNO_2 concentration (Fig. 3). Mirvish also noted that this applied to the nonionized species.

However, as Mirvish indicates, it is more convenient to use total concentrations of amine and nitrite rather than those of nonionized species. The proportions of total nitrite and total amine as HNO_2 and Me_2NH are constant at a constant pH, and therefore equation 2 may be used.

The only difference between equations 1 and 2 is that $\overline{K}$ (equation 2) is pH dependent while in equation 1, K is independent of pH (Table 7). It was also found that maximum yield of the nitrosamines occurred at pH 3.4.

Mirvish explains the pH curve (Fig. 4) as follows: the concentration of nonionized Me_2NH decreases about 10-fold for each unit drop in pH between 9 to 5, and the nonionized HNO_2 increases by a like factor. Since the reaction rate is proportional to the square of HNO_2 concentration, the rate increases about 10-fold for each drop of one unit in pH. However, the pK of HNO_2 is 3.36 so that below this pH, the nitrite becomes almost completely nonionized and the principle effect of dropping the pH is a decrease in Me_2NH concentration which slows the reaction.

Mirvish concluded from these studies that, with certain assumptions, rough estimates could be made as to how much dimethylnitrosamine formation may occur in a food of known dimethylamine and nitrite content. Hypothetical calculations based on a situation similar to that in a food product before or after ingestion, suggest that the concentrations of dimethylnitrosamine would be too low to be carcinogenic. The concentration of nitrites and especially secondary amines in foods is not, however, clearly determined. It was pointed out that nitrosation of amines other than dimethylamine could be a more serious problem in foods since the rate of nitrosation also depends on the basicity of the amine, increasing 1,000-fold as basicity decreases from dimethylamine to the aromatic amines.

Ender and Ceh (43) studied the nitrosation of amines and found similar results in which the amount of nitrosamine formed increased with the temperature and time of reaction. They found that significant nitrosation could occur even at −18 C.

From a very recent paper by Mirvish et al. (91), the effect of ascorbate could be used as an effective blocking agent to prevent nitrosation of amines by nitrite. Action of ascorbate in preventing formation of nitrosamines was found to result from its direct reaction with nitrite. This may well be a method to reduce the risk of nitrosamine formation in certain drugs and foods to which ascorbate is not already added.

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$Me_2NH + N_2O_3 \longrightarrow Me_2NNO + HNO_2$$

$$RATE = K\left[Me_2NH\right]\left[HNO_2\right]^2 \quad (1)$$

OR FOR TOTAL CONCENTRATIONS

OF AMINE AND NITRITE,

$$RATE = \bar{K}\left[TOTAL\ DIMETHYLAMINE\right]\left[TOTAL\ NITRITE\right]^2 \quad (2)$$
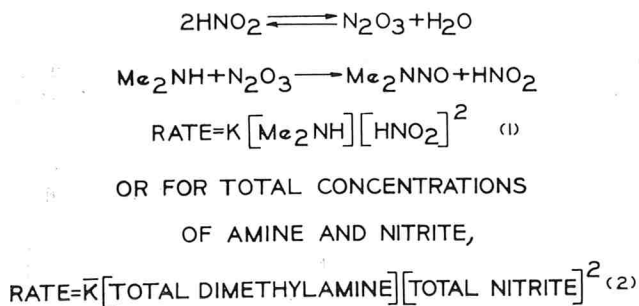
Figure 3. Kinetics of nitrosamine formation. From Mirvish (90). Reproduced by permission of J. Nat. Cancer Inst. and the author.
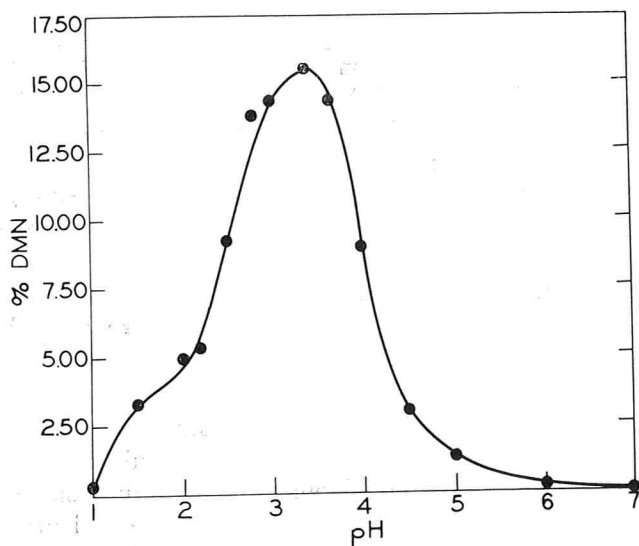
Figure 4. Dependence of DMN formation on pH. From Mirvish (90). Reproduced by permission of J. Nat. Cancer Inst. and by the author.
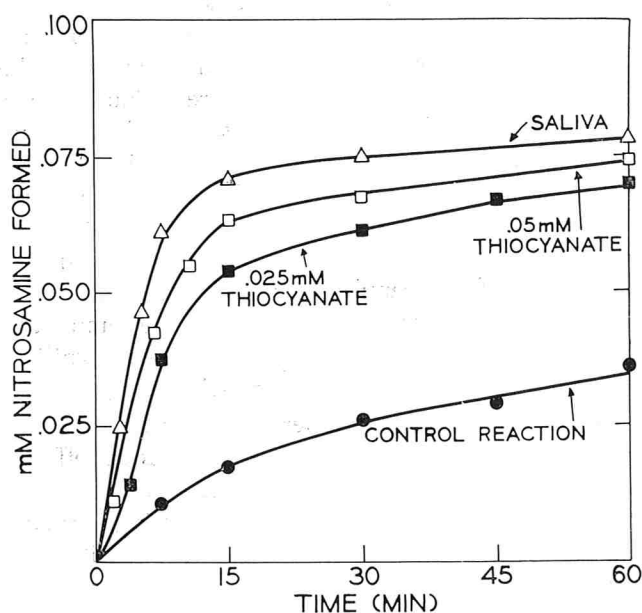
Figure 5. Catalysis of nitrosation by thiocyanate. From Boyland et al. (16) by permission of Food Cosmet. Toxicol. and the authors.

POTENTIAL FOR IN VIVO FORMATION OF NITROSAMINES

The implications of nitrosamines in human cancer was broadened by the suggestion of Druckrey et al. (37) that nitrites and secondary amines might react under the acidic conditions of the stomach to form nitrosamines. Sander et al. (106) incubated nitrite and various secondary amines with human gastric juice at pH 1.5 and demonstrated nitrosamine formation. Sen et al. (111) attempted a more quantitative study and found that incubating diethylamine and sodium nitrite in vitro with gastric juice from rats, rabbits, cats, dogs, and man resulted in formation of diethylnitrosamine. Human and rabbit gastric juice produced more diethylnitrosamine than that of rats because of the lower pH (pH 1 to 2 for rabbits and man while rat gastric juice is pH 4 to 5). These authors suggested that for test purposes, the rabbit, cat, or ferret would be a more appropriate test species than the rat because of the differences in gastric juice pH. It was concluded that formation of trace amounts of nitrosamines in the human stomach over a long time period could be hazardous. Sander et al. (106) concluded that the ease of formation depends on the basicity of the amines. Formation could be demonstrated for weakly basic amines but not for the strongly basic amines like diethylamine. Greenblatt et al. (57) reported that when secondary amines and nitrite were administered to mice, a highly significant increase in lung cancer was noted. This was concluded to result from nitrosation of amines in vivo, presumably in the stomach. Dimethylamine was not effective in this instance and this was assumed to be caused by the strong basicity of this amine.

It has also been noted that nitrosamines could be formed in vivo by bacterial action. Hawksworth and Hill (59) confirmed earlier work of Sander (104) showing that strains of Escherichia coli from the human intestinal tract could enzymatically produce nitrosamines from nitrate and secondary amines at neutral pH values. They also demonstrated that some non-nitrate reducing strains or species of lactobaccili, group D streptococci, clostridia, bacteroides, and bifidobacteria can nitrosate secondary amines with nitrite at neutral pH values. These authors suggested that bacterial nitrosation may occur in the intestine from ingestion of nitrate and amines or in the urine of people with urinary tract infections (which often are caused by a nitrosating organism, E. coli). They feel, however, that nitrosation in urine is likely to occur only in areas where drinking water is high in nitrate since the nitrate content of normal urine seems too low to constitute a hazard.

Attention has been recently brought to the fact that human saliva contains a significant amount of nitrite (1-10 ppm). Tannenbaum (119) has shown

that, with the large volumes of saliva produced, this could amount to 6-12 mg of nitrite daily. This nitrite is presumed to be formed by bacteria.

Boyland et al. (16) studied the effect of thiocyanate, which is a normal constituent of human saliva, and found that this compound is an effective catalyst for nitrosation of secondary amines by nitrite to form nitrosamines (Fig. 5). They concluded that after ingestion of secondary amines and nitrite, presence of saliva in the stomach may result in greater potential for formation of nitrosamines than previously suspected.

ANALYTICAL PROCEDURES AND PROBLEMS

Many analytical procedures to detect nitrosamines have been devised. Malins et al. (85) and others have used polarography, but Wolff (129) points out that pyrazines, formed from thermal degradation of amino acids and sugars heated above 100 C in food, often interfere with this method as well as with some gas chromatographic procedures. Thin layer chromatography with color forming spray reagents has been used by Preussman et al. (98). In this method nitrite is released from the nitrosamine by ultraviolet light after the nitrosamines are separated and the color developed results from nitrite interacting with the reagent. However, Wolff (129) again points out that some of the color forming reagents used can also react with fatty acids, tocopherols, pigments, and other compounds in foods which may have the same Rf as the nitrosamines. Eisenbrand et al. (41) described a method in which nitrosamines are oxidized to nitramines and identified with an electron capture detector but this technique has not been widely used. Ender et al. (45) used various spectrophotometric procedures, and Du Plessis et al. (39) used nuclear magnetic resonance and infrared spectrophotometry for dimethylnitrosamine determinations. These authors indicate, however, that exact levels cannot be determined and that there is a possibility of artifacts and contamination.

Howard et al. (65) reported a gas chromatographic method sensitive to 10 ppb of dimethylnitrosamine when used to test smoked chub. They also indicated that the identity of the nitrosamine could be confirmed by mass spectrometry at levels as low as 10 ppb.

It appears that gas chromatography followed by mass spectrometry is the most acceptable procedure; this technique has been used by Howard et al. (65) and Fazio et al. (48) for fish, by Fazio et al. (49) for meat products and by Collis et al (25) for alcoholic drinks. Wolff and Wasserman (130) have, in fact, noted that this improved procedure has cast a shadow of doubt on the earlier findings of nitrosamines in foods since many of the other methods can easily introduce errors through contamination and artifacts.

The total analytical procedure usually involves an extraction step, followed by distillation, partitioning with solvents, a clean-up step with columns or thin layer chromatography, and finally separation, detection, and confirmation.

Initial extracts from the food are usually made with water, aqueous or methanolic alkali, or sometimes organic solvents (43, 70, 111, 112, 129). This is followed in most procedures by a distillation step, usually with a basic solution since Wolff (129) indicates that an acid pH tends to degrade nitrosamines present or can lead to formation of nitrosamines from nitrite and amines present. Methylene chloride is used as a solvent to extract nitrosamines in partition with water (48, 111, 112, 129). A clean-up procedure using ion exchange resins, or celite or alumina columns is usually employed to remove interfering substances (48, 129). Detection and confirmation are most often done with gas chromatography and mass spectrometry.

Since nitrosamines are usually present in very small amounts, procedures concerned with their analysis must be very carefully done and it is hardly surprising that a long tedious series of steps is necessary. Wolff (129) indicated that this general procedure requires just over 2 days per sample and requires the labor of 1 man-day per sample, including a spiked control sample which is usually tested side by side with the experimental sample.

Most nitrosamine concentrations in foods such as meats have been in the area of 5 ppb (49). To obtain reliable, accurate values at this concentration, scrupulous attention and meticulous care are necessary at all times and at all stages of the analysis.

SOME OTHER SOURCES OF NITROSAMINES

There are two other possible sources of nitrosamines for humans, one of which has been quite extensively examined while the other has been largely ignored.

The fact that nitrosamines could be present in tobacco and could be the carcinogenic factor of tobacco smoke has received much attention. Tobacco has several secondary amines and up to 1,000 ppm of oxides of nitrogen, as noted by Serfontein and Hurter (113). Whenever these two occur together, there is a possibility of nitrosamine formation. Serfontein and Hurter (113) have indicated that at least three nitrosamines are probably present in tobacco smoke. Serfontein and Smit (114) also presented evidence that nitrosamines were present in unburned tobacco. Johnson et al. (68) concluded that the evidence for nitrosamines in tobacco smoke is not definite in light of the possibilities of artifacts being produced in the collection of smoke for analysis. However, Rhoades and Johnson (100) recently identified

dimethylnitrosamine in tobacco smoke condensates and found that growing conditions greatly affect the amount found in tobacco. Thus, while nitrosamines in tobacco smoke seem to be a very distinct possibility, it is not clear what role they may play in human lung cancer caused by smoking.

The other possible source of nitrosamines for human exposure comes from nitrogen compounds of polluted air. Although largely ignored, it is conceivable that volatile carcinogenic nitroso compounds could be present or nitrogen oxides may be available for various reactions in the body or when in contact with foods to produce carcinogenic compounds such as the nitrosamines.

SUMMARY AND CONCLUSIONS

There is little doubt that nitrosamines, particularly dimethyl- and diethylnitrosamine, are extremely dangerous compounds and if they are present in significant amounts in the human food supply, they do constitute a definite hazard to human health. Although carcinogenesis has not been directly demonstrated in man, in view of the large number of animal species affected, there is no reason to believe that man should somehow be immune to the attack of nitrosamines.

The problem, then, does not lie in establishing the hazard of nitrosamines but rather, it lies in establishing the hazard in the environment and, more specifically, in the food supply. The possibility of a hazard exists, of course, but whether it ever has, or will become, a reality is more the question. Evidence for the presence of nitrosamines in our food supply is somewhat limited particularly since some of the earlier reports may have been based on misleading analytical procedures.

The extremely low levels of nitrosamines in foods, when found, present a problem. Not only are the analytical methods made more tedious with greater possibilities of artifacts and error when working with parts-per-billion concentrations, but the hazard to man is extremely difficult to evaluate since dose response relationships and pharmacological data at these low concentrations have not been documented.

Risk-benefit ratios must also be considered for such possible nitrosamine precursors as nitrite which is used for color, flavor, and protection against clostridia and food poisoning in cured meats and other products. In light of limited scientific evidence available, decisions should not be too hastily made to eliminate nitrite since a possible hazard may be substituted with a more serious one. It is interesting that ascorbate, as mentioned earlier in this review, may prevent nitrosation of amines by nitrite. Ascorbate is often used in meat products.

The only logical and realistic way to resolve the question of nitrosamines and their possible precursors in foods is by definite research, carefully done over an extended period of time. This is necessary to clearly establish (a) whether there are significant amounts of nitrosamines in our food supply (either natural or formed before consumption), (b) whether their precursors are present in significant amounts to form nitrosamines in our foods, (c) if nitrosamines can be formed *in vivo* under practical, realistic conditions and, (d) if so, whether the ones formed are of quantitative and toxicological significance. Research in many of these areas has been stimulated by the recent concern and answers may soon be available.

ADDENDUM

The following references are not cited in the text but are listed for the benefit of readers who may wish additional information: *1, 2, 3, 4, 6, 7, 8, 9, 11, 17, 19, 24, 26, 27, 29, 30, 33, 46, 47, 53, 55, 73, 81, 94, 95, 99, 103, 115, 117, 118, 121, and 124.*

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DAIRY PROCESSING AND QUALITY CONTROL CONFERENCE

A conference for dairy processing, quality control and sales personnel has been scheduled for April 9-12, 1973 at The Pennsylvania State University. It is conducted by the staff of the Food Science Department with the assistance of the Pennsylvania Department of Agriculture.

Primary emphasis is on shelf life, flavor and nutritional value of dairy products. Processing procedures, cleaning and sanitizing, refrigeration, frozen dessert mixes, waste disposal and sanitary regulations will also be covered.

For information contact: Agricultural Conference Coordinator, Room 410, J. O. Keller Building, The Pennsylvania State University, University Park, Pennsylvania 16802.

CORRELATION STUDIES OF MILKFAT CONTENT AS MEASURED BY THE MILKO-TESTER, BABCOCK, AND MOJONNIER METHODS[1,2]

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Abstract

To evaluate some of the methods used to estimate milk-fat content in raw milk, producer's milk samples were tested by the Milko-Tester, Babcock, and Mojonnier methods. One hundred eighty-eight individual producer's raw milk samples were analyzed in duplicate by the Milko-Tester and Babcock methods. In addition, 34 of these samples were randomly selected and analyzed in duplicate by the Mojonnier method. The correlation coefficient for the 188 samples tested by the Babcock and Milko-Tester methods was 0.95. A prediction equation for calculating Babcock results based on Milko-Tester readings was developed. Statistical analysis of the results of the three methods showed a highly significant ($P < .01$) correlation coefficient between each pair of the methods. An analysis of variance showed no significant differences ($P > .05$) among the three methods. Average variation between duplicate samples showed results from the Milko-Tester to have the least variation, while those from the Babcock procedure varied the most between duplicate determinations.

Of the instrumental methods used to determine milkfat, the Milko-Tester has received the most attention and has been subjected to the most comparisons with older accepted methods. According to Foss America Inc. (3), the Mark II instrument measures the light transmitted through a measured, heated, and homogenized sample of milk and diluent. The light passing through the sample is measured by a photocell and results are read from a galvanometer scale graduated in percent fat. Murphy and McGann (6) have shown that the Milko-Tester is satisfactory for measuring the milkfat content of herd milks. An analysis of 1,457 fresh raw milk samples, by Ginn and Packard (4) showed an average reading for the Milko-Tester of 3.788% and 3.809% for the Babcock, thus indicating a close relationship between the two methods. Appleman and Laben (2) found that similar results were obtained with Babcock, Mojonnier, and Milko-Tester methods when raw milk refrigerated for 24 hr at 5C was tested, however, they observed differences when certain preservatives were used. Khalil and Layton (5)

analyzed 400 samples of milk from incoming tank trucks by the Milko-Tester and Babcock methods. The Milko-Tester averaged 3.88% fat and the Babcock, 3.87% with a correlation coefficient of 0.8951. To use the Milko-Tester it is necessary that it be properly calibrated and checked periodically against a reference. Shipe (7) has recommended that the differences between averages for the Milko-Tester and the reference should not exceed 0.04% on 20 samples. He suggested specific steps for calibrating and verifying the accuracy of the Milko-Tester.

The Dairy Science Department at Mississippi State University obtained a Mark II Milko-Tester in 1968. This instrument has proven to yield highly reproducible results, and requires less than 30 sec to make a fat determination on a sample of milk. It has been field tested in Mississippi, Tennessee, and Kentucky and used for analytical work on dairy production research. The question of standardization of the machine has been posed as a limitation to the utility of the method. The Mojonnier method, when used by an experienced analyst, is very accurate and could well serve as a basis for standardization of this machine. Wildasin (9) reported that the Milk Industry Foundation and the International Association of Ice Cream Manufacturers urge that "all testing procedures requiring calibration be standardized against the ether extract method." The present research developed a procedure for standardization and checking the accuracy of the Milko-Tester, including a prediction equation for converting the values from one method to another.

Materials and Methods

A standard curve was prepared using skimmilk and cream to give milk with fat levels of about 2, 3, 4, 5 and 6%. These samples were analyzed in duplicate by the Mojonnier procedure (1) and the results used to calibrate the Mark II Foss Electric Milko-Tester. Routinely every 25th determination by the Milko-Tester was a control standard of homogenized milk.

Individual producer's milk samples were collected at the rate of about 48 samples per week in two lots of 24 each for a period of four weeks. These were collected in two whirl-pak bags by the milk tank truck driver and then the samples were packed in ice until received in the laboratory. Upon receipt, samples were stored at 40 F and analyzed within

TABLE 1. MEANS AND STANDARD ERROR FOR THREE METHODS OF ANALYSIS OF MILKFAT CONTENT ON 34 SAMPLES AND THE AVERAGE VARIATION IN PERCENT BETWEEN DUPLICATE DETERMINATIONS

Method	Mean (%)	S.E. of mean (%)	Avg. variation between duplicate determinations (%)
Milko-Tester	3.71	.050	0.31
Babcock	3.67	.053	1.22
Mojonnier	3.69	.055	0.55

TABLE 2. CORRELATION COEFFICIENT FOR THREE METHODS OF MILKFAT ANALYSIS

Method	Correlation coefficient
Milko-Tester × Babcock	0.91**
Milko-Tester × Mojonnier	0.87**
Babcock × Mojonnier	0.95**

**Highly significant (P <.01)

TABLE 3. MEANS AND CORRELATION COEFFICIENT OF THE MILKO-TESTER AND THE BABCOCK METHOD ON 188 SAMPLES

Method	$\overline{X}$ (Mean) (%)	Correlation coefficient
Babcock	3.695	
Milko-Tester	3.755	Babcock × Milko-Tester = 0.95**

**Highly significant (P <.01)

24 hr. Before analysis, milk in the two whirl-pak bags was heated in the bags to 100 F, commingled and mixed well before aliquots were removed for duplicate Babcock analysis (1). The remaining portion was analyzed in duplicate by the Milko-Tester. In addition, 20% of the samples selected at random were tested in duplicate by the Mojonnier method. Each of the three tests was done by different laboratory personnel independent of each other. The data were transferred to computer punch cards and the means, percentage of variation, correlation coefficients, analysis of variance, and a regression equation were computed using a Univac Model 1106.

RESULTS AND DISCUSSION

A comparison of the means and percentage of variation for the three methods for analysis of milk fat content is shown in Table 1 for 34 samples. The average for these samples was 3.71% milkfat for the Milko-Tester, 3.67% for the Babcock method, and 3.69% for the Mojonnier. The standard errors for these three methods were 0.050, 0.053, and 0.055%, respectively. Shipe (8) has recently reported results, which show smaller standard deviations than these, but nonetheless follow the same pattern reported here. The Babcock method showed the greatest variation between duplicate determinations, while the Milko-Tester had the least variation. In Table 2, the correlation coefficients revealed a highly significant correlation between all possible pairs of the three methods with a correlation coefficient between the Milko-Tester and Babcock of 0.91. An analysis of variance (F-test) showed no significant difference among the three methods, when tested at the 5% level of probability. When a total of 188

samples were analyzed, Table 3, it was found that the mean milkfat content by the Babcock method was 3.70% compared to 3.76% for the Milko-Tester. A highly significant correlation coefficient of 0.95 was shown for the two methods. A regression equation for calculating Babcock results based on the MSU Mark II Milko-Tester readings was developed using 188 samples. The following equation resulted: Babcock = −0.00266 + 0.985 (Milko-Tester reading).

These results are in agreement with those cited earlier, and confirm the excellent agreement between the Milko-Tester and the Babcock methods. By using the results obtained by the Mojonnier method to calibrate the Milko-Tester, this apparently contributed to the slightly higher readings by the Milko-Tester, when compared to the Babcock. As shown in Table 1, the mean value for percentage of milkfat by the Mojonnier falls half way between the values obtained by the other two methods. The Milko-Tester can, of course, be standardized downward to correct for the higher readings and could result in an even higher correlation coefficient than we have reported here. Based on this work and research reported by others the Milko-Tester has been officially adopted as an alternate method for analysis of milkfat content in raw milk in the state of Mississippi.

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COMPARISON OF FOUR METHODS FOR ENUMERATING SOMATIC CELLS IN MILK WITH AN ELECTRONIC COUNTER

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ABSTRACT

Four chemical fat dispersion methods for conducting the Electronic Somatic Cell Count of milk were studied. The four methods were Rapid/Fixation/Dispersion, Somafix/Somaton, and two methods reported by the International Dairy Federation. Results were critically affected by variations in several procedural factors: type of test tube used for cell fixation and fat dispersion; ratio of milk to diluent; duration of cellular fixation; temperature and duration of fat dispersion; aging of milk samples before analysis; aging of fixed and dispersed samples; and method to agitate samples after fixation and dispersion.

After standardization of procedures, the four methods were used, under regulatory testing conditions, to determine the cellular content of three groups of 50 samples of bulk herd milk. A Wisconsin Mastitis Test was done on each sample and a Direct Microscopic Somatic Cell Count was done on the latter two groups of samples. Attempts to fix cells and disperse fat simultaneously with a Rapid/Fixation/Dispersion method were unsatisfactory. The somatic cell estimates obtained with the other three methods were in good agreement with the direct microscopic results; correlation coefficients ranged from r = 0.854 to 0.977. Merits of each of the four methods are discussed.

Milk somatic cells are comprised of leucocytes from the blood and body cells from the mammary epithelium. An increased concentration of somatic cells in milk is accepted as evidence that the milk is abnormal. Cells may be present as a result of injury to the udder, inclusion of colostrum or stripper milk, presence of a disease condition having systemic involvements, or, most commonly, from a bacterial infection of the udder resulting in a subclinical mastitis. Substantial progress has been made toward abating subclinical mastitis in many herds, but a significant number of herds continues to experience a high incidence of the disease.

The United States Public Health Service has recognized the need to prevent abnormal milk from being processed for human consumption (18). Additionally, the National Conference on Interstate Milk Shipments has promulgated a control program requiring that all raw milk to be pasteurized by interstate milk shippers be screened routinely for somatic cell content. The increased attention given to monitoring of somatic cells in milk has created the need for more rapid and precise methods to enumerate cells.

Electronic counters have been used successfully by several workers to estimate milk somatic cells. Several variations have been used with the basic difference being the means by which milk fat was removed. Removal of milk fat globules before counting is necessary because the size range of the fat globules overlaps that of somatic cells.

Cullen (1, 2) used several combinations of physical and chemical methods to remove milk fat and found centrifugation of a milk/saline mixture in a special centrifuge tube to be the most desirable procedure. Phipps and Newbould (11) concluded that chemical methods to separate somatic cells from milk fat held little promise and devised a centrifugal procedure yielding a linear relationship between electronic and direct microscopic results. Mitchell et al. (6), using the procedure of Phipps and Newbould, obtained a correlation coefficient of 0.978 between electronic and microscopic methods on samples from bulk milk tanks. They further observed the slope of the regression line to be quite dissimilar from that reported by Phipps and Newbould for fresh quarter or cow composite samples. A centrifugal procedure was also developed by Read et al. (14) which yielded a correlation coefficient of 0.997 between electronic and microscopic methods. The application of this procedure has been investigated extensively (12, 13, 15).

A suitable procedure for leaving the milk somatic cells intact while chemically dispersing fat globules was first accomplished by Tolle et al. (17). Cells were stabilized by treating the milk sample with formalin (1:500) for 24 hr at room temperature. The fixed milk was subsequently diluted with a saline/ethanol/formalin/Triton-X 100 mixture and incubated for 10 min at 80 C in a water bath to disperse fat globules. A correlation coefficient of 0.97 was obtained between the electronic and microscopic methods. These workers reported that two or three technicians could process approximately 1,000 samples per day. The chemical procedure of Tolle et al. was evaluated by other workers (3, 9, 10) and the basic methodology was largely confirmed. Pearson et al. (9) reported that the chemical method yielded a higher correlation coefficient with direct microscopic results than did the centrifugal method, i.e., 0.966 vs. 0.930. The correlation between the two electronic methods was 0.988.

The method of Tolle et al. was adopted nationally

in the United Kingdom and was selected by the International Dairy Federation (5) as the most valuable procedure presently available for the routine testing of large numbers of milk samples for somatic cells.

The study reported here was conducted in two phases. The purpose of the first was to determine the effects on the ESCC of altering procedural factors common to four methods of enumerating somatic cells in milk with an electronic counter. The second phase was conducted to determine the relative accuracy and merits of each of the four methods when used under regulatory testing conditions to determine the somatic cell content of bulk milk samples.

Materials and Methods

Phase I

This phase of the work was conducted in our laboratory over a period of approximately 1 year. Milk samples were collected from our farm bulk milk tank and sample preparation was begun immediately. Tests were done with Coulter's Model Z_BI and Model B electronic counters. Each was fitted with a 100-μ diameter aperture tube calibrated periodically with latex particles having an average spherical diameter of 3.49 μ. Aperture and amplification settings were, respectively, ½ and ¼ for the Model Z_BI and ¼ and ¼ for the Model B. At these settings one dial division of the lower threshold (T_L) control corresponded to 1.05 μ^3 for the Model Z_BI and 1.55 μ^3 for the Model B.

The four methods evaluated in this study were the Rapid/Fixation/Dispersion (RFD) method described by Orr (8) and modified by Hein (4). Somafix/Somaton[1] (SS) method, International Dairy Federation-30 min (IDF30M) method (5), and International Dairy Federation-21 hr (IDF21H) method (5).

The procedural variables studied were a comparison of glass vs. plastic test tubes for fixation and dispersion, ratio of milk to diluent, duration of cellular fixation, temperature and duration of fat dispersion, storage of diluent at different temperatures, temperature of diluent at time of use, method of collecting and storing milk samples before analysis, aging of samples following fixation, aging of dispersed samples, and method and duration of agitating samples following fixation and dispersion.

Phase II

This phase was a collaborative study conducted at the Louisiana State Board of Health Regional Laboratory, Shreveport. Three trials were executed during three successive months. The milk samples were collected routinely from farm bulk milk tanks by drivers of milk transport trucks, refrigerated, and delivered to the designated milk processing plant where they were assembled by the local sanitarian, within 8 hr of collection, and submitted to the regulatory laboratory for testing within 18 hr of receipt. Emphasis was placed on maintaining the samples at temperatures below 4 C from collection to testing. Personnel of the Health Department Laboratory agitated each sample before doing routine analyses. Immediately thereafter, specific samples were selected for our study giving a balanced range of Wisconsin Mastitis Test (WMT) scores. Fifty samples were selected during each trial and the somatic cell content of each sample

was estimated by each of the four electronic methods. The Model Z_BI electronic counter was used in Trials 1 and 2 and the Model B was used in Trial 3.

Certain procedural factors were common to each of the four methods evaluated. The procedures outlined below yielded the most consistent and precise results during Phase I of this work.

Disposable glass test tubes (16 × 125 mm) capped with parafilm were used for fixation and fat dispersion. Following fixation, the samples were cooled to 23 C, mixed on the Vortex shaker for 3 sec, diluted (1:100) in duplicate with a dual diluter, and placed in the dispersion bath, the level of the milk sample in the tube being ¾ inch below the water level in the bath. After dispersion, samples were placed in ice water for 2 to 3 min and allowed to stand at room temperature for a maximum of 45 min before counting.

Samples were finally prepared for cellular enumeration by mixing on the Vortex shaker for 3 sec, pouring the contents of the duplicate tubes into a disposable plastic vial, placing the vial under the aperture tube of the electronic counter (allowing approximately 10 sec for air bubbles to disperse and the flow of diluent through the orifice to become stabilized), and counting the cells in a 0.5-ml aliquot of the sample. Duplicate counts were averaged. A third count was made on the infrequent occasions when the initial readings varied by more than 3%. A corrected ESCC/ml of milk was obtained by taking the reading from the electronic counter, subtracting the background reading for a sample of heated diluent that was included with each batch of samples, and multiplying by the working factor of 200. Corrections for coincidence losses were not applied. Caution was exercised during dilution to prevent any milk droplets from adhering to the wall of the test tube at a point above the level of the milk/diluent mixture because undispersed fat globules contained therein would result in counting errors.

ESCC methods

RFD method. Cells were fixed and fat dispersed simultaneously with this method. The diluent was prepared by adding to 500 ml Isoton[1]: 1.5 g dibasic sodium phosphate, 5.5 g monobasic potassium phosphate, 10 ml formalin, 200 ml methanol, 7 ml 10% sodium hydroxide, and 50 ml Tergitol 15-S-9; diluting to 1 liter with Isoton and mixing well; adjusting the pH to 7.2 with 10% sodium hydroxide; and filtering through 0.45-μ filter paper to reduce the particle count to <50/ml at a lower threshold setting equivalent to 20 μ^3. Milk samples were prepared for counting by diluting with the above fixative/diluent and heating in a water bath at 60 C for 10 min.

SS method. The fixative and diluent used in this procedure were obtained commercially[1]. Cells were fixed by adding 3 drops of Somafix to 10 ml of milk and heating in a water bath at 60 C for 5 min. Milk fat was dispersed by diluting with Somaton and heating the samples in a water bath at 80 C for 10 min.

IDF30M method. The fixative and diluent were prepared according to the method published by the International Dairy Federation (5). Cells were fixed by adding 3 drops of fixative to 10 ml of milk and heating in a water bath at 60 C for 30 min. Fat dispersion was accomplished by diluting the samples and placing them in a water bath at 80 C for 10 min.

IDF21H method. This method was identical to the IDF 30M method with the exception that cells were fixed at 23 C for 21 hr.

Wisconsin Mastitis Test. The WMT was conducted according to the method outlined by Thompson (16).

Direct Microscopic Somatic Cell Count. Duplicate smears

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[1]Trade name. Coulter Electronics, Inc. 590 W. 20th St., Hialeah, Florida 33010.

TABLE 1. MEANS OF THE WISCONSIN MASTITIS TEST SCORES, DIRECT MICROSCOPIC SOMATIC CELL COUNTS, AND THE ELECTRONIC SOMATIC CELL COUNTS BY THE RFD, SS, IDF30M, AND IDF21H METHODS

Trial no.	WMT score	DMSCC	ESCC method							
			(RFD)		(SS)		(IDF30M)		(IDF21H)	
	(mm)		(OCC)							
1	15.8	--	--	464(20.0)ᵃ	--	540(54.4)	--	618(54.4)	--	731(54.4)
2	16.5	779	1010(15.6)	718(18.8)	717(44.6)	577(54.4)	788(44.6)	720(54.4)	--	541(54.4)
3	16.3	759	700(17.2)	476(20.6)	753(41.6)	642(54.4)	700(41.6)	613(54.4)	918(41.6)	763(54.4)

ᵃFigures in parenthesis denote lower threshold setting at which ESCC determinations were made.

were prepared on the original samples (Trials 2 and 3) immediately before subsampling for ESCC determinations. The strip-reticle method outlined by the National Mastitis Council Subcommittee on Screening Tests was followed (7).

RESULTS

Phase I

Data collected during this phase were assembled in 21 tables and, for reasons of brevity, are not included in this article. Copies are available from this laboratory upon request.

Glass vs. plastic test tubes. Attempts to use snap cap disposable plastic tubes for fixation and dispersion were unsuccessful because the inner lining of the tubes frequently separated during heat treatment and the resulting debris caused excessive blockage of the orifice.

Ratio of milk to diluent. Initially, all samples were diluted 1:50. It was observed, however, that the milk fat was often incompletely dispersed by the SS method on bulk milk samples from our Jersey dairy herd. This problem was resolved by diluting the milk 1:100. Subsequently, all milk samples tested by each of the four different methods were diluted 1:100.

Duration of cellular fixation. With the RFD method the ESCC results were elevated slightly at 6 min, comparable at 8 to 14 min, and significantly higher at 16 min. The SS ESCC increased with fixation times from 5 to 30 min and decreased slightly from 30 to 60 min. The IDF30M ESCC increased steadily from 5 to 60 min, though the increase from 30 to 60 min was slight. Results were inconsistent from 18 to 40 hr for the IDF21H method.

Temperature and duration of fat dispersion. The RFD ESCC decreased steadily as the temperature was increased from 50 to 68 C. There was a steady decrease in the ESCC obtained with the SS and IDF30M methods as the temperature was increased from 76 to 84 C. The cell estimates obtained by the SS and IDF methods were not affected when the dispersion time was varied from 8 to 12 min.

Storage of diluents. The ESCC data were erratic when RFD and IDF diluents were stored at 5 C vs. 23 C for 1 to 7 days. As well, when the temperature of diluents stored at 5 C was 10 C at dilution the

RFD ESCC was decreased and the results from both IDF methods were increased compared to results obtained using split samples of the same diluents maintained at a constant temperature of 23 C.

Method of collecting and storing milk samples before analysis. A comparison was made of the ESCC of split milk samples collected in glass bottles vs. plastic Whirl-Pak bags following overnight storage at 5 C and in ice water. When samples were stored at 5 C, those in glass bottles consistently yielded higher cellular estimates by each of the four electronic methods than the samples collected in plastic bags. When stored in ice water, those in glass yielded higher estimates by the SS and IDF methods but those stored in plastic bags gave higher scores by the RFD method.

Aging of fixed samples. Following fixation/dispersion by the RFD method, samples were aged for 15 to 120 min in ice water, at 5 C, and at room temperature. The ESCC of samples aged in ice water decreased between 15 and 45 min, but showed a significant increase at 120 min. When stored at 5 C, the results increased steadily from 15 to 120 min. Storage at room temperature resulted in a significant decrease from 15 to 45 min, followed by a slight increase from 45 to 120 min.

Samples fixed by the SS and IDF methods were aged at 5 C and at 23 C for 0 to 7 days. The SS ESCC results were erratic when the samples were aged at 5 C, but when aging occurred at 23 C there was a significant increase from 0 to 1 day and a slight, but consistent, increase from 1 to 7 days. The IDF30M ESCC increased steadily following aging

TABLE 2. CORRELATION COEFFICIENTS BETWEEN DIFFERENT ESCC METHODS

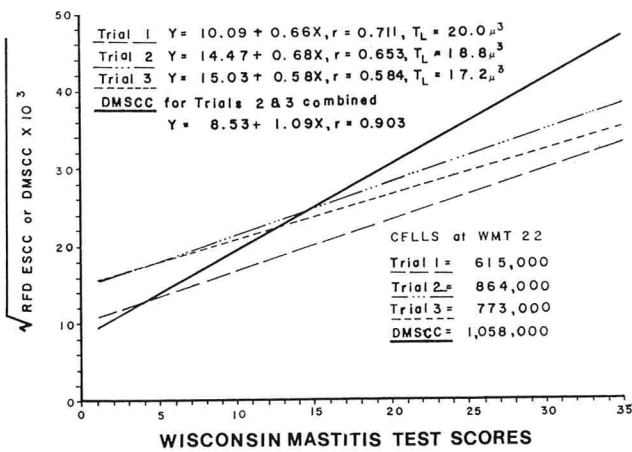| Methods	Trial		
	1	2	3
	(r)		
RFD vs. SS	0.843	0.849	0.538
RFD vs. IDF30M	0.842	0.851	0.587
RFD vs. IDF21H	0.758	0.782	0.589
SS vs. IDF30M	0.976	0.998	0.892
SS vs. IDF21H	0.934	0.887	0.920
IDF30M vs. IDF21H	0.938	0.882	0.950

Figure 1. Regression of the square root of the RFD ESCC for Trials 1, 2, and 3 and of the DMSCC for Trials 2 and 3 combined on the WMT scores.
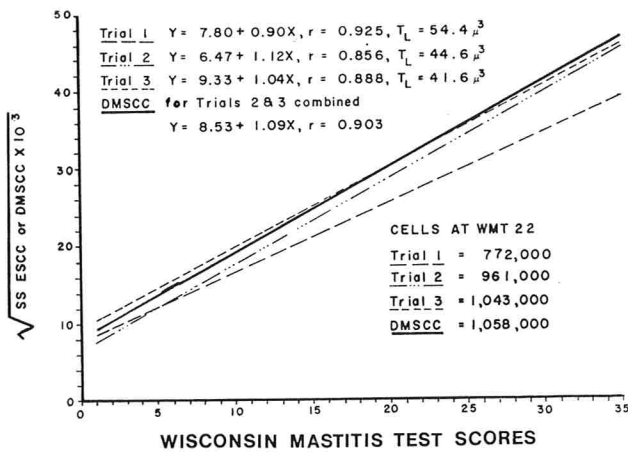

Figure 2. Regression of the square root of the SS ESCC for Trials 1, 2, and 3 and of the DMSCC for Trials 2 and 3 combined on the WMT scores.
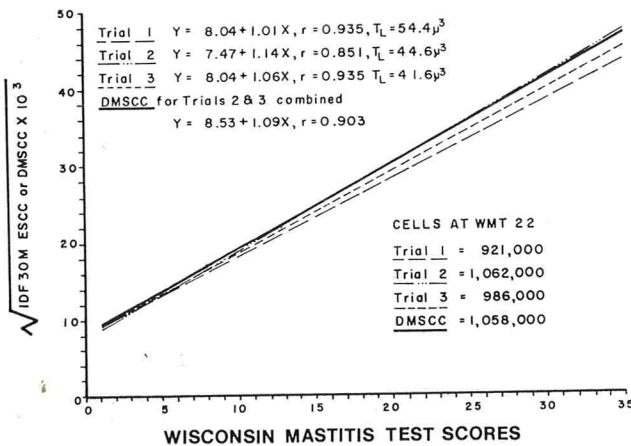

Figure 3. Regression of the square root of the IDF30M ESCC for Trials 1, 2, and 3 and of the DMSCC for Trials 2 and 3 combined on the WMT scores.

at 5 C for 1 to 7 days. Results were erratic from samples fixed by the IDF21H method and stored at 5 C. The ESCC increased for both IDF methods when samples were stored at 23 C for 0 to 7 days.

Aging of dispersed samples. The SS ESCC increased when dispersed samples were aged for 10 to 120 min at 5 C. An increase was also noted when aging occurred in ice water for 3 min plus 7 to 117 min at 23 C. Results were erratic from 10 to 120 min when the SS samples were aged continuously in ice water or at 23 C. Estimates from the IDF-30M method decreased from 10 to 120 min when dispersed samples were aged at 5 C or in ice water. Results from the same method were erratic following aging at 23 C. Samples fixed by the IDF21H method yielded a steadily decreasing ESCC when stored at 23 C for 10 to 120 min.

Method and duration of agitation following fixation and dispersion. The ESCC estimates were slightly higher with each of the electronic methods when samples were agitated for 3 sec on a Vortex mixer as opposed to hand mixing for 5 sec. The ESCC also increased for each procedure when the Vortex mixing time was increased from 3 to 10 sec.

Phase II

RFD method. Data obtained using this procedure were highly inconsistent both within and between trials (Table 1 and Fig 1). The linear correlation coefficients between the RFD ESCC and the WMT were 0.711, 0.653, and 0.584, respectively, for Trials 1, 2, and 3 at lower threshold settings of 20.0, 18.8, and 17.2 μ^3. At WMT 22 mm the equivalent ESCC $\times$ 10^3 was 615, 864, and 773, respectively, for the three trials as compared to an average DMSCC of 1,058 $\times$ 10^3 at the same WMT level. The RFD method consistently yielded a higher ESCC estimate than the corresponding DMSCC in samples with a low cell content, while the reverse situation prevailed with samples having a high concentration of cells.

SS method. Estimates were made in all three trials of the cellular content of each milk sample at a lower threshold setting equivalent to 54.4 μ^3 (4.7 μ diameter). The means of the ESCC values obtained at this threshold were consistently lower than the mean of the corresponding DMSCC (Table 1). The ESCC was also determined in Trials 2 and 3 at lower threshold settings of 44.6 and 41.6 μ^3, respectively. These settings were equivalent to spherical particle diameters of 4.4 and 4.3 μ. Regression of the square roots of the SS ESCC values on the WMT scores revealed that a threshold of 41.6 μ^3 yielded cell estimates essentially comparable to those obtained when the square root of the DMSCC was regressed on the WMT score (Fig. 2). However, the regression of the DMSCC on the SS ESCC values revealed 44.6 μ^3 to be the preferred lower threshold setting

for this procedure (Fig. 5). Correlation coefficients between the square roots of the SS ESCC and the WMT scores for Trials 1, 2, and 3 were 0.925, 0.856, and 0.880, respectively. Correlations between the DMSCC and the ESCC were 0.977 for Trial 2 and 0.854 for Trial 3.

IDF30M method. The ESCC estimates obtained with this procedure at a setting of 54.4 μ^3 were consistently lower than those of the corresponding DMSCC (Table 1). Regression of the square roots of the ESCC values obtained at threshold settings of 44.6 and 41.6 μ^3 on the WMT scores indicated cell equivalents closely approximating the DMSCC (Fig. 3). Subsequent regression of the DMSCC on the ESCC revealed, however, that the ESCC estimates were approximately 5 to 10% lower than the DMSCC. It seemed likely that a setting of 38.8 μ^3 (4.2-μ diameter) would yield values more closely approximating the DMSCC. This thesis was subsequently confirmed. Correlations between the square root of the IDF30M ESCC and the WMT were 0.935, 0.851, and 0.935, respectively, for the three trials. Correlations between the DMSCC and the IDF30M ESCC were 0.977 for Trial 2 and 0.934 for Trial 3.

IDF21H method. Regression of the square root of the ESCC at 54.4 μ^3 on the WMT yielded cell equivalents comparing favorably to the DMSCC values obtained in Trials 1 and 3 (Table 1 and Fig. 4 and 6). For reasons that were not apparent, the ESCC results obtained during Trial 2 were unusually low (Fig. 5). Respective correlations with the WMT for the three trials were 0.890, 0.837, and 0.953. Regression of the DMSCC on the ESCC for Trial 3 yielded a regression slope of 1.0, indicating that the two procedures were comparable. Correlation was 0.949.

Correlations between different methods. Regression of the square roots of the DMSCC on the WMT was conducted on the combined data of Trials 2 and 3 (Fig. 7). A unit slope of 1.09 was obtained with a correlation of 0.903. The cell equivalent at WMT 22 mm was 1,058 × 10³. Correlations for the various combinations of procedures are presented in Table 2. The correlation coefficients involving the RFD ESCC ranged from 0.538 to 0.851 while those between the SS, IDF30M, and IDF21H ESCC methods ranged from 0.882 to 0.998.

DISCUSSION

Results obtained with each ESCC method were critically affected by several procedural variables. If accurate results are to be obtained with these methods it is imperative that extreme caution be exercised in following standardized methodology within each laboratory. In fact, it seems likely that each laboratory will need to establish parameters for
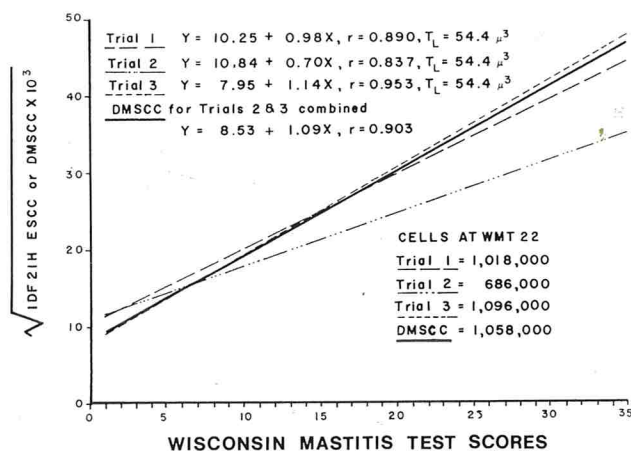

Figure 4. Regression of the square root of the IDF21H ESCC for Trials 1, 2, and 3 and of the DMSCC for Trials 2 and 3 combined on the WMT scores.
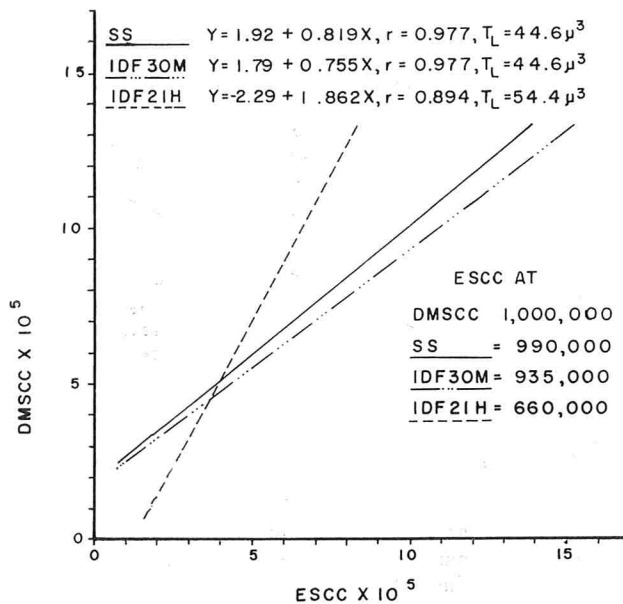

Figure 5. Regression of the DMSCC on the SS, IDF30M, and IDF21H ESCC - Trial 2.

a given method before it is adopted in that specific laboratory as a confirmatory test for milk somatic cells.

The optimal lower threshold setting suggested by Orr *(8)* for the RFD method was 22.0 μ^3. Our work with this method, though failing to define the preferred setting, suggests that the optimal level is more likely to be in the range of 17.0 to 19.0 μ^3. More work will be required with this procedure before it can be adopted as a confirmatory method for somatic cells in milk. The difficulty encountered in obtaining reproducible results with this method probably resulted because cells were being fixed and fat was dispersed simultaneously.

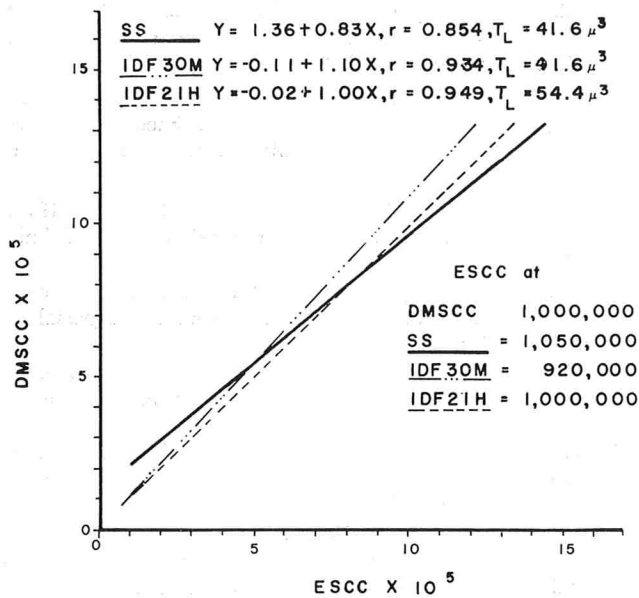The threshold setting recommended for use with the SS method was 54.4 μ^3. As previously noted, the

$$SS \quad Y = 1.36 + 0.83X, r = 0.854, T_L = 41.6 \mu^3$$
$$IDF\,30M \quad Y = -0.11 + 1.10X, r = 0.934, T_L = 41.6 \mu^3$$
$$IDF\,21H \quad Y = -0.02 + 1.00X, r = 0.949, T_L = 54.4 \mu^3$$

ESCC at

DMSCC	1,000,000
SS	= 1,050,000
IDF 30M	= 920,000
IDF 21H	= 1,000,000

Figure 6. Regression of the DMSCC on the SS, IDF30M, and IDF21H ESCC - Trial 3.

$Y = 8.53 + 1.09X, r = 0.903$

CELLS AT WMT 22
DMSCC = 1,058,000
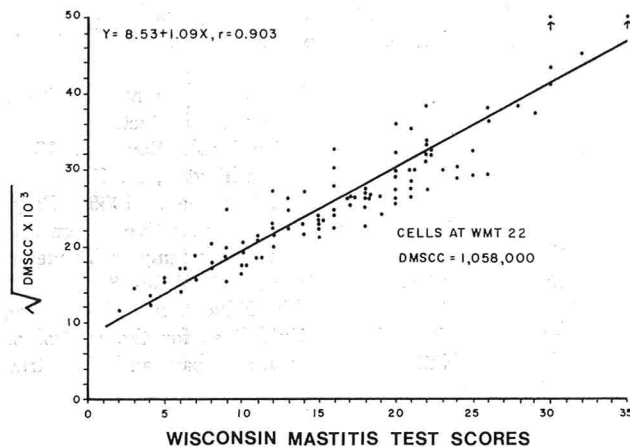
Figure 7. Regression of the square root of the DMSCC on the WMT scores - Trials 2 and 3 combined.

optimal setting determined in this study was 44.6 μ^3. Tolle et al. *(17)* did not recommend a precise setting for the procedures designated here as IDF30-M and IDF21H, but Phipps *(10)* suggested that the setting likely was 47.7 to 65.5 μ^3 (4.5 to 5.0 μ diameter). Dijkman et al. *(3)* reported good graphical agreement with the direct microscopic method when a setting of 65.5 μ^3 was used. Pearson et al. *(9)* and the International Dairy Federation *(5)* have recommended a setting of 54.4 μ^3 for use with the two IDF methods. Our findings seem to confirm this setting for use for the IDF21H procedure, though some variation was observed, particularly in Trial 2.

Tolle et al. *(17)* reported that if testing were urgent and samples fresh, fixation of the cells could be accomplished by treating the formalized milk sample for 30 min in a water bath at 60 C. Dijkman et al

(3) reported similar findings. The term "fresh sample" was not clearly defined by Tolle et al. but since the milk used in our work was refrigerated from the time of milking to the time of testing it was felt that the IDF30M procedure would be applicable. The data confirmed that this was indeed the case, though the optimal lower threshold setting more closely approximated 38.8 μ^3 than 54.4 μ^3 as previously reported *(5, 17)*. Our work confirms the observation of Tolle et al. *(17)* that "duration and temperature are decisive for the degree of fixation" and that "modifications in these factors results in a drifting of the counting plateau."

Blockage of the orifice occurred with approximately one in every eight samples. No difference was observed among the four methods. When blockage occurred, debris was removed with a small brush and air bubbles were allowed to disperse before counting was recommenced.

The problem encountered with the SS method's not dispersing all of the fat globules at a dilution ratio of 1:50 was also encountered by Tolle et al. *(17)* and Dijkman et al. *(4)* while using the IDF methods. Besides diluting to 1:100, this problem could perhaps be overcome by adding additional fat dispersant to the diluent. This would permit technicians to dilute samples 1:50 and read the ESCC in thousands directly from the electronic counter.

The International Dairy Federation *(5)* reported that it was advisable, following a test on a suspension of high cell concentration, to rinse the aperture tube with diluent to prevent transference of cells to the succeeding sample. Though we systemically rinsed the aperture tube following the testing of each sample having a high concentration of cells, our own evaluations on this variable indicated that it was not of practical consequence. For example, when a sample containing 2.4×10^5 cells/ml was reexamined (without rinsing of the aperture tube) following the testing of a sample containing 2.4×10^6 cells, the ESCC was increased to only 2.5×10^5. Similar results were obtained in other comparisons.

The necessity of preparing diluents for use with the RFD and IDF methods constitutes a decided disadvantage in that an opportunity is provided to introduce error. Each step in the ESCC methodology should be standardized as carefully as possible, and use of commercially-prepared reagents offers one means of minimizing errors within a laboratory as well as differences between laboratories. Based upon the data collected and the availability of commercially standardized reagents, the SS method is considered the method of choice.

Our work with the SS and IDF30M procedures revealed that a trained technician could process approximately 200 samples per day when specific modi-

fications in methodology were accomplished, e.g., covering a rack of 40 tubes with a single sheet of parafilm measuring 150×250 mm rather than covering each tube separately. When a tube was removed from the rack a corner of the sheet of parafilm was torn off by grasping it between the thumb and the top of the tube, providing a cover for the sample tube during subsequent agitation on the Vortex mixer. The 200 samples per day obtainable with the SS and IDF30M methods represented approximately a three-fold increase over the number of samples processed by the DMSCC method in a comparable period of time.

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1973 DAIRY AND FOOD ENGINEERING CONFERENCE

Through a cooperative arrangement between faculty at Purdue, Ohio State and Michigan State Universities this conference will be held at Purdue University in 1973 at Ohio State University in 1974 and at Michigan State University in 1975. The 1973 conference will be held on April 3 and 4 at the Stewart Center at Purdue University. Inquiries on additional information related to the conference should be directed to Dr. V. E. Sweat, Assistant Professor, Agricultural Engineering Department, Purdue University, Lafayette, Indiana.

Program themes for six one-half day sessions have been established. The half day sessions will deal with the following topics:

a) Use of Computers and Systems in Plant Management
b) Recent Developments in Dairy and Egg Processing
c) Recent Developments in Fruit and Vegetable Processing
d) Recent Developments in Meat and Poultry Processing
e) Waste Management in Food Plants
f) New Concepts in Food Packaging

Sessions (b), (c), and (d) will be concurrent on the afternoon of April 3.

A Research Note

A STUDY OF METHODS TO ENUMERATE THE MICROBIAL FLORA OF THE AVIAN EGG SHELL[1]

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Abstract

Experiments were done to compare results from three different methods used to enumerate the microbial flora of avian egg shells. The methods compared were: (a) swabbing the shell surface, (b) rinsing the shell surface, and (c) blending the entire shell and membranes after removal of the contents. Sanitized eggs held at 25 C were inoculated with a 24-hr old culture of *Pseudomonas fluorescens* by immersion for 5 min at 25 C. In the swab method, the entire surface of the inoculated egg was swabbed by rolling the swab in either direction twice as each area was swabbed. For the rinse method, the inoculated egg was held using a metal holder and rinsed with 100 ml of 0.1% peptone solution. With the blender method, the entire emptied inoculated egg shell and shell membranes were blended using a Sorvall Omni-Mixer to which a presterilized Mason jar was attached. As expected, the blender method gave significantly higher counts ($P<0.05$) than either the swab or rinse methods. There was no significant difference ($P<0.05$) between counts obtained by the swab and rinse methods.

This study was undertaken to compare a newly devised rinse method with that of the swabbing method used by Rizk et al. (4) and a modification of the blending method used by Board et al. (2) to enumerate the microbial flora of the avian egg shell.

Materials and Methods

The experiment consisted of four replicates conducted during four different weeks. For each replicate eggs were gathered from the Washington State University White Leghorn flock and those weighing 61 g were stored at 1.1 C for 24 hr before using for inoculation. The surface area of a 61-g egg was calculated using the formula of Besch et al. (1). Eggs were sanitized by immersing in 70% ethyl alcohol for 5 min. They were then aseptically transferred onto a sterile board which had pointed wire nail ends to support the eggs, and were allowed to air dry at room temperature (25 C). The eggs were then flamed to remove any excess alcohol and allowed to cool at 25 C for ca. 2 hr. The eggs were then inoculated with a 24-hr old culture of *Pseudomonas fluorescens* by immersion for 5 min at 25 C. These eggs were then transferred to the sterile board as described earlier and left to air dry at 25 C.

The inoculum was prepared in 100 ml of nutrient broth (Difco) and incubated at 25 C for 24 hr. It was then diluted with sterile distilled water until a transmittancy reading of 20% was observed. The average number of organisms at this transmittancy was 4.4×10^7 organisms/ml.

Swab method

The inoculated egg was aseptically held in position between the left thumb and index finger using sterile surgical gloves. A swab made as described by Mallman et al. (3) and moistened in 0.1% sterile peptone solution was used to swab the entire surface of the egg by rolling the swab in either direction twice for each area swabbed. The swab was then broken into 99 ml of 0.1% peptone solution. Serial dilutions were then made and plated using Standard Methods Agar (BBL). Plates were incubated at 25 C for 72 hr.

Rinse method

One inoculated egg was aseptically held small end downwards using a sterile metal holder. The holder was made from stainless steel wire 1.5 mm in diameter bent into a triangular shaped base with a handle extending upward from one corner of this triangle. For rinsing, the holder and egg were held over a sterile funnel leading into a sterile bottle. The egg was rinsed with 100 ml of sterile 0.1% peptone solution which was dispensed using a Brewer automatic pipetter, the whole delivery system of which had been previously sterilized. Serial dilutions were made and plated using Standard Methods Agar (BBL) and then incubated at 25 C for 72 hr.

Blending method

One hundred milliliter dilution blanks of 0.1% peptone solution were made in six regular one-pint Mason jars. A 0.1 N solution of sodium thiosulfate was prepared and 240 ml of this solution were placed in each of six one-pint Mason jars. Also, 240 ml of distilled water were placed in each of six one-pint Mason jars. All the above 18 Mason jars were autoclaved at 15 psi for 15 min. At the same time 240 ml solution containing 200 ppm available chlorine were transferred to each of six previously sterilized one-pint Mason jars which were free of any organic material.

A Sorvall Omni-Mixer[2] with Mason jar adaptor was used for blending in this method. The rotor knife blade was sanitized before use and between counts in the following manner. Using sterile disposable tissue wipers any dust particles and any organic material on the knife blade assembly were physically removed. The Mason jar containing 200 ppm available chlorine was attached to the adaptor ring. The mixer was operated for 1 min at maximum speed. Next, the jar containing 0.1N sodium thiosulfate solution was attached to the mixer and the mixer operated for 1 min at maximum speed. This was repeated after attaching the Mason jar containing sterile distilled water.

Each egg was aseptically broken and the contents were removed. The shell and shell membranes were then placed in a one-pint Mason jar containing the sterilized 0.1% peptone solution. The jar was attached to the blender and the blender operated for 45 sec at maximum speed. The Mason jar was removed, its mouth flamed, and its lid replaced. Using sterile disposable tissue wipers all adhering shell membranes and

TABLE 1. AVERAGE[a] NUMBER OF *Pseudomonas Fluorescens* ORGANISMS PER SQUARE CENTIMETER OF AVIAN EGG SHELL PER REPLICATE

Replicate number	Method		
	Swab	Rinse	Blender
	———————— ($\times 10^2$) ————————		
1	5.9	8.2	89
2	7.7	6.8	99
3	31.0	23.0	190
4	10.0	11.0	170

[a]Average of five eggs.

shell were removed from the rotor knife blade which was then sanitized in the manner described above. A sample from the final rinse of distilled water was plated using Standard Methods Agar (BBL) to test for complete sanitization. The blended sample was serially diluted, and plates were poured using Standard Methods Agar (BBL). Plates were incubated at 25 C for 72 hr.

RESULTS AND DISCUSSION

Results obtained in this study (Table 1) show that the blender method gave higher counts than the rinse or swab methods. The results were statistically analysed using an analysis of variance. When Duncan's new multiple range test was applied, results showed a significant difference between the blender method and the other two methods at the 5% level, but there was no significant difference between the swab method and rinse method at the 5% level.

The blender method is useful to enumerate the microbial flora of the entire shell and membranes, while the swab and rinse methods are useful for making counts of the shell exterior only. Therefore, the marked difference between the results of the blender method and the other two methods can be attributed largely to the fact that the entire shell and shell membranes were macerated when using the blender method, thus giving a higher recovery of microorganisms. This seems reasonable, because by swabbing and rinsing, all organisms may not be removed from the surface of the egg shell. When swabbing, there is a possibility of error from rolling the swab in the same area or from not swabbing a particular area. In the rinse method microorganisms which may tend to adhere to the shell surface may not be removed. Also, the removal of organisms may depend on the force with which the egg is rinsed.

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AMENDMENT TO THE 3-A SANITARY STANDARDS FOR INSTRUMENT FITTINGS AND CONNECTIONS USED ON MILK AND MILK PRODUCTS EQUIPMENT

Serial #0906

Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Public Health Service
The Dairy Industry Committee

The "3-A Sanitary Standards for Instrument Fittings Used on Milk and Milk Products Equipment, Serial #0900" as amended are hereby further amended as indicated below.

The following is substituted for the paragraph on page 1 preceding the list of drawings:

The following is a list of drawings included in this standard. Dimensions and the contour of fittings and connections shown on the drawings are for reference only and may be changed if they do not affect cleanability. Instrument fittings and connections not illustrated in the drawings shall be considered as being included in this standard, provided they conform to the provisions of this standard with respect to material, finish, construction and use of gaskets and have no special requirements for construction or installation.

This amendment is effective April 22, 1973.

SHELF LIFE OF FRESH SEPARATOR CHEESE[1]

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ABSTRACT

Samples of non-fat and 9%-fat fresh cheese obtained from two commercial dairies were stored at 3, 10, 20, and 37 C; they decreased in numbers of coliform bacteria and increased in acidity. These changes occured rapidly at higher storage temperatures. At 10 C, spoilage resulted from mold growth; at higher storage temperatures it was caused by acid development. Keeping quality did not correlate with initial coliform bacterial counts, kinetics of the coliform population, or acidity.

Behavior of coliform bacteria in *Streptococcus lactis* based fresh cheeses seems to be somewhat variable. Skelton and Harmon (7) reported considerable increases in coliform counts in cottage cheese stored at 13 C, while Goel et al. (4) noted decreases in most,

but not in all samples of cottage cheese stored at 7 C. Kempinski (5) reported that the main contaminants of quarg were coliform bacteria, yeasts, and mold; these stemming mainly from the cream. Frank (3) found little significant change in counts of coliform bacteria, molds, or yeasts during storage of quarg at 4 C but rapid increases in yeast and mold counts at higher storage temperatures. On inoculation of *E. coli* into whole or skim milk at various stages of quarg manufacture, proliferation was reported until cutting of the coagulum at pH 5.0 - 5.2 (8).

The present study reports on the development of acidity and the changes in coliform bacteria in relation to the shelf life of non-fat and 9%-fat fresh separator cheese.
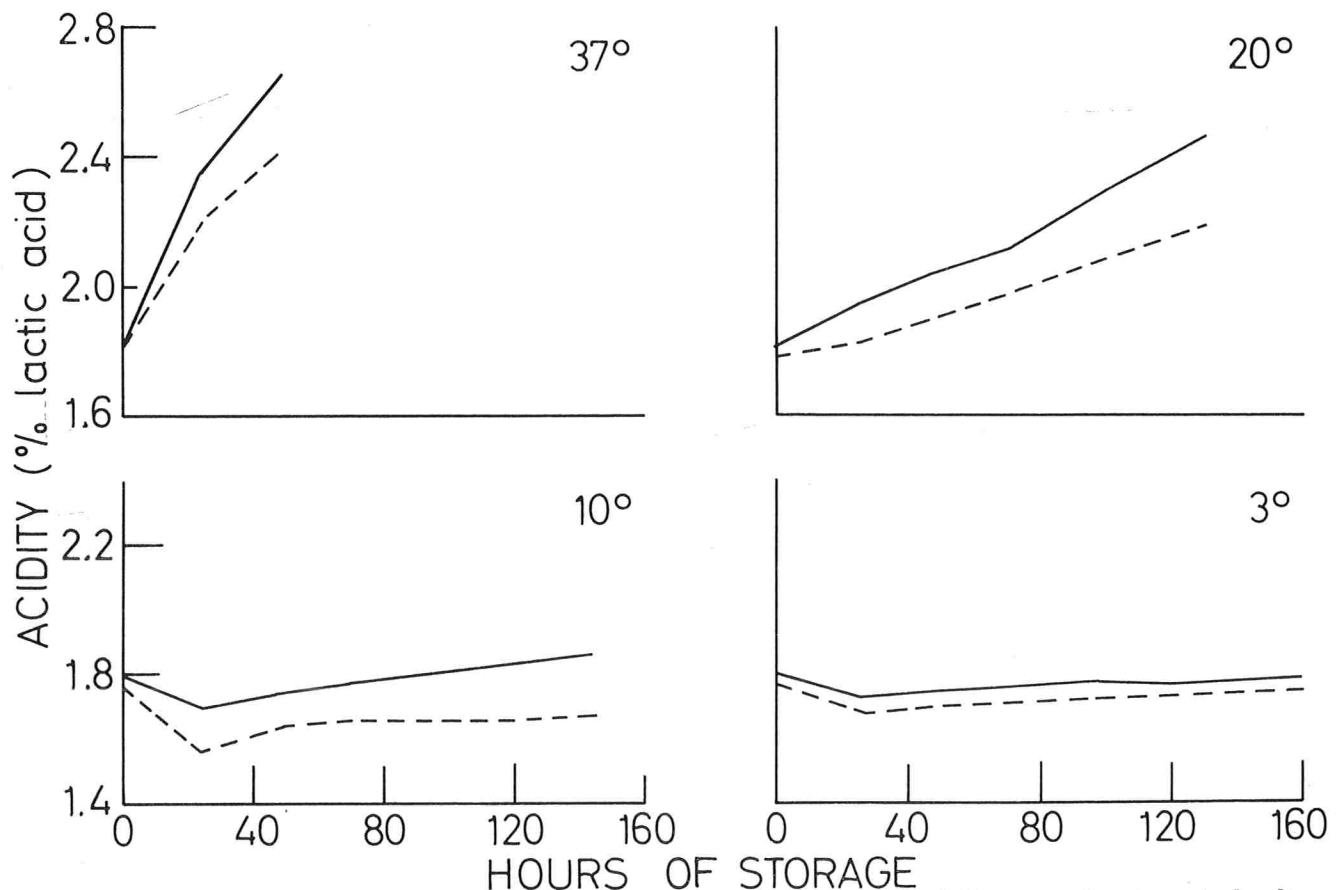
Figure 1. Mean changes in acidity on storage of cheese at several temperatures. Solid lines: non-fat cheese; broken lines: 9%-fat cheese.

METHODS

Samples of fresh cheese, from two commercial dairies, were prepared as follows: skim milk was pasteurized at 75 C for 16 sec and ripened with *Streptococcus lactis* and aroma bacteria at 22-29 C for 10 - 15 hr to pH 4.6. The curd was
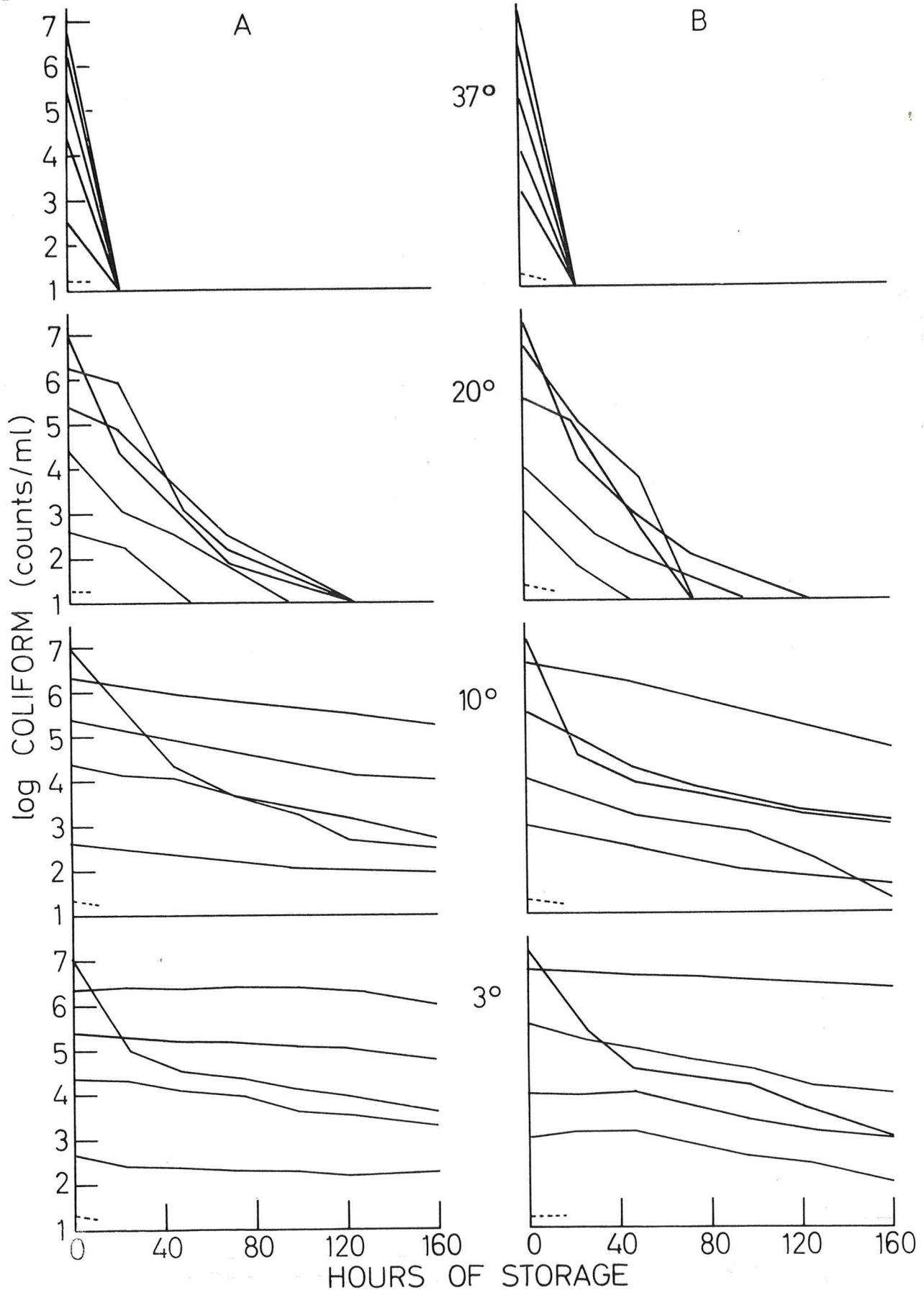
POTASHNIK ET. AL.

Figure 2. Changes in coliform bacterial counts on storage of cheese at several temperatures. Each line represents the mean of duplicate determinations on two samples having a similar number of coliform bacteria. The broken lines indicate samples containing 10 coliforms per gram. Data labeled "A" were obtained with 9%-fat cheese and those labeled "B" are results from non-fat cheese.

TABLE 1. SHELF-LIFE AND CAUSE OF REJECTION OF SAMPLES STORED AT SEVERAL TEMPERATURES

Temperature of storage (C)	Sample	Shelf life	Cause of rejection
3	Fat	> 8 days	—
10	Non-fat	> 8	—
10	Fat	5.7[a]	mold (70)[b]
	Non-fat	4.3	mold (80)
20	Fat	1.7[a]	acid (100)
	Non-fat	1.1	acid (90)
37	Fat	1	acid (100)
	Non-fat	1	acid (100)

[a]Significantly different (P < 0.05) from other value at this storage temperature.

[b]Figures in parentheses indicate the percentage of samples rejected for the cause stated.

then separated in a Westphalia centrifuge to 16-18% total solids and the non-fat product packed at this point. The 9%-fat cheese was manufactured by metering salted, cultured, pasteurized cream (prepared using the same culture) into the curd and packaging.

Samples were transferred to the laboratory and stored at 3, 10, 20, and 37 C. Acidity (1), pH, and coliform bacteria (2) were determined and organoleptic acceptability evaluated (6). Each trial lasted 8 days and involved daily duplicate examination of two samples at each temperature, and six coliform levels were examined.

RESULTS AND DISCUSSION

The mean changes in acidity and changes in coliform counts at the temperatures of storage are shown in Fig. 1 and 2. Table 1 summarizes shelf life and the reasons for rejection of product.

Samples stored at 3 C remained edible after 8 days of storage. The predominant reason for spoilage at 10 C was mold growth, which caused rejection of the non-fat cheeses after 4.3 days and the 9%-fat cheeses after 5.7 days (significant difference, P < 0.05). At higher storage temperatures spoilage resulted from excess acid development.

Coliform bacterial counts decreased at all storage temperatures, the decrease was slight at 3 C and very rapid at 37 C. This confirms previous indications concerning the behavior of coliform bacteria in quarg (8) and other reports on S. lactis based cultured milk products (4, 6).

The rate of acid development $(\frac{dA}{dT})$ at 10 C was significantly greater (P < 0.05) by some 10-20% in the non-fat cheese than the 9%-fat cheese, and the rate of decline of the coliform bacteria counts $(\frac{dC}{dT})$ was also significantly faster (P < 0.1) by some 10% in the non-fat product. At 20 C, $\frac{dA}{dT}$ was about 15-20% greater (P < 0.05) and $\frac{dC}{dT}$ some 10-20% greater (P < 0.05) and at 37 C, $\frac{dA}{dT}$ was also significantly greater (P < 0.05) in the non-fat cheese. Factors serving to protect the coliforms and reduce the rate of acid development in the 9%-fat cheese and which probably cause the longer shelf life are the subject of further investigation.

No correlation was found between any derived function of the coliform bacteria and acid development, or between either of these two parameters and shelf life. Some factor other than the increase in titratable acidity seems to be responsible for the decline in coliform bacteria. Use of a fixed number of coliform bacteria as an indicator of hygienic parameters in this product is only justified when the time of manufacturing and testing are strictly defined.

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USE OF CULPAK-KITS TO TRANSPORT MILK SAMPLES FOR CULTURE

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Abstract

Culpak-kits were examined for their usefulness in mailing milk samples to diagnostic laboratories for recovery of mastitis pathogens. Certain mastitis pathogens survived on Culpak-kits stored at room temperature for 7 days. Culpak-kits proved to be efficient for transporting aseptically collected quarter milk samples, but inefficient for use with milk samples which contained contaminants, such as found in bulk milk samples.

Unpreserved milk samples mailed to diagnostic laboratories for bacteriologic culture frequently are delivered in useless condition. A practical and convenient container is needed to mail milk samples for bacteriologic culture. Such a container would be useful for practicing veterinarians, dairymen, and others concerned with mastitis diagnosis, treatment, and control.

This is a report of a study of the usefulness of Culpak-kits[2] for transporting milk samples for culture. A Culpak-kit contains a sterile filter paper strip (54×22 mm), in a sterile carrier paper sealed in a paper envelope. It was developed for delayed recovery of streptococci from human throat swabs (2, 4, 5).

McFarland et al. (5) reported that the filter paper strip technique provided a safe method to mail specimens for culture, and this method was identified with a higher recovery of group A streptococci than was obtained by several other commonly used methods. Group A streptococci survived on a dried filter paper strip for several days (2, 5). In our laboratory, Field (1) reported on the use of Culpak-kits to transport milk samples by mail from regional laboratories to the central laboratory for duplicate examination. He reported results which compared favorably with cultures of vial milk samples.

[2]Culpak-kit is a trade mark of Culpak Products Inc. Culpak-kits are distributed by Falcon Plastic Division of B-D Laboratories, Inc., 5500 West 83rd Street, Los Angeles, California 90045.

Materials and Methods

Collection and transport of milk samples

To compare the efficiency of recovery of organisms from vial milk samples and Culpak-kit milk samples, multiple milk samples were collected on several farms during routine surveys in the New York State Mastitis Control Program. Milk samples were collected mid-to-late morning, and following a strip plate examination. Techniques for collection of uncontaminated samples were used. Collecting milk directly from a cow onto a Culpak-kit offers special opportunities for contamination, so extra precaution was required to collect useful samples. Mild restraint of some cows helped to prevent tail switching and stepping. Approximately 5 drops were milked directly onto the filter strips. If excess milk was collected it was permitted to drain off before the kit was closed.

Quarter milk. Triplicate quarter milk samples were taken; one vial and two Culpak-kits. Vial samples were collected during routine surveys and were transported in an insulated ice chest. One of the Culpak-kits was transported in the ice chest with the vial samples. The second Culpak-kit was transported by first class mail, posted at the farm. This sample was not refrigerated. Delivery time from posting to laboratory ranged from 1 to 5 days.

Bulk milk. Bulk milk supplies were mixed for 5 min and samples were collected from farm bulk tanks by single-use pipettes. Triplicate bulk milk samples were taken and transported as for quarter milk samples.

Bacteriology

Milk samples in vials. Milk samples were cultured on aesculin blood agar incubated at 37 C and examined at 24 and 48 hr. Quarter samples were inoculated on quadrants of plates and bulk milk samples were inoculated on entire plates.

Culpak-kit samples. The carrier paper was removed from the Culpak envelope using alcohol-flamed forceps. Forceps were again alcohol-flamed before removing the filter paper strip which contained the milk sample. The filter paper strip was placed face down and pressed firmly against the medium near the outer edge of the culture plate. The strip was then returned to the carrier paper. The inoculum was spread to the remainder of the plate with a bacteriology loop. Milk samples from Culpak-kits that had been transported and stored under refrigeration were inoculated onto half plates. Milk samples from Culpak-kits which were mailed were spread onto entire plates.

Identification. Organisms were identified by characteristics of colonies and hemolysis on aesculin blood agar. Any isolates which remained unidentified at 48 hr were subjected to biochemical tests, serological tests, cultured on selective media, and examined microscopically.

Survival time of pathogens

To determine survival times of organisms in vial milk samples and in samples on Culpak-kits, three vial milk samples were collected from each of several quarters on farms. In the laboratory one of each set of vial milk samples

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TABLE 1. NUMBER OF QUARTER MILK SAMPLES WHICH YIELDED CERTAIN PATHOGENS FOLLOWING TRANSPORT BY THREE METHODS. N = 807.

Organism[1]	Vial	Culpak-kit carried	Culpak-kit mailed
S. agalactiae	219	205	174
Other streptococci	72	178	155
Hemolytic staphylococci	75	94	94
Misc.	15	2	4
Negative[2]	449	372	414

[1]Streptococcus agalactiae, streptococci that are not agalactiae, hemolytic staphylococci (presumably Staphylococcus aureus) and miscellaneous (Coliform and other gram-negative rods, yeast, etc.). Both single and multiple isolations were recorded.
[2]No mastitis pathogens recovered.

TABLE 2. NUMBER OF BULK MILK SAMPLES WHICH YIELDED CERTAIN PATHOGENS FOLLOWING TRANSPORT BY THREE METHODS. N = 64.

Organism[1]	Vial	Culpak-kit carried	Culpak-kit mailed
S. agalactiae	43	34	21
Other streptococci	63	58	51
Hemolytic staphylococci	52	45	32
Misc.	1	1	3

[1]Streptococcus agalactiae, streptococci that are not agalactiae, hemolytic staphylococci (presumably Staphylococcus aureus) and miscellaneous (Coliform and other gram negative rods, yeast, etc.). Both single and multiple isolations were recorded.

was stored 7 days at 4 C. The second of each set of vial milk samples was stored 7 days at room temperature. Milk from the third vial of each set was used as inoculum for 14 Culpak-kits, seven stored at 4 C and seven stored at room temperature for 7 days. All sub-samples were cultured daily for 7 days. Numbers of pathogens and leukocytes in these milk samples were not determined.

Recovery of Streptococcus agalactiae from bulk milk from a herd with a low prevalence of infection

In a 25-cow experimental herd that was free of *Streptococcus agalactiae* infection, one quarter of one cow was infected with a field strain of S. *agalactiae*. After the infection was firmly established, daily bulk tank milk samples from this herd were examined for 54 days. Approximately 150 ml of bulk milk were collected daily following the evening milking. Approximately 0.05 ml of each sample was inoculated onto aesculin blood agar. Gravity cream permitted to collect

overnight on these bulk milk samples was transferred by a swab to Culpak-kits. In addition, bulk milk was transferred directly from the bulk tank to a Culpak-kit using a sterile cotton swab. A second quarter of the same cow was then infected with the same strain so that two quarters in a herd of 25 cows were infected with S. *agalactiae*. The sampling regime continued as above for 34 days. In this exercise milk and gravity cream from all Culpak-kits were cultured on each of three selective media: TKT/FC medium (10), Thallium Crystal Violet medium[3] (TCV) and TCV medium modified by the addition of crude beta hemolysin (3, 9).

Suspected S. *agalactiae* isolates recovered from the selective media were presumptively confirmed on the CAMP test (6). In this study, staphylococci exhibiting alpha or beta hemolysis were identified presumptively as *Staphylococcus aureus*.

RESULTS

Comparison of efficiency of isolation from vial milk samples and Culpak-kit samples

Quarter milk samples. The results of culture of 807 quarter milk smples from 57 herds are presented in Table 1. These samples were transported by three methods. There were a few more isolations of S. *agalactiae* from vial milk samples than from Culpak-kits. Streptococci other than S. *agalactiae* and S. *aureus* were isolated more often from both Culpak-kits than from vial samples. Relatively few gram-negative rods (i.e. coliforms, etc.) were isolated from Culpak-kits.

Bulk milk sample. Sixty-four bulk milk samples in triplicate were cultured on aesculin blood agar and results are in Table 2. Streptococci and staphylococci were isolated more frequently from vial samples than from either Culpak-kits. Because of growth of contaminants, it was rather difficult to identify pathogens from bulk milk which had been transported on Culpak-kits by mail.

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[3]Thallium Crystal Violet (TCV) Medium

Extract agar	35.0 g
Thallium Sulfate	0.3 g
Aesculin	1.0 g
Crystal Violet	0.0013 g
Ferric Citrate	0.01 g
Dist. water	1,000 ml

Add bovine blood to 5% for pouring plates.

TABLE 3. ISOLATION RATE (NO. OF SAMPLES WHICH PERMITTED RECOVERY/NO. OF TRIALS) OF *Streptococcus agalactiae* FROM BULK MILK FROM EXPERIMENTAL HERD WITH APPROXIMATELY 1% OF QUARTERS INFECTED (1 QUARTER IN 25 COWS).

	Bulk tank				Quarter
Medium[1]	Gravity cream from sample	Gravity cream on Culpak-kit 24 hr[2]	Milk from sample	Milk on Culpak-kit[3]	#1
Aesculin blood agar	25/54	3/54	12/54	18/59	54/54
T.K.T.	21/27	14/28	Not cultured	13/26	Not cultured
TCV	27/54	23/54	Not cultured	18/54	Not cultured

[1]Media described in text footnote.
[2]Gravity cream from sample was transferred to Culpak-kit and stored at room temperature for 24 hr before culturing.
[3]Milk was transferred directly from bulk tank to Culpak-kit by sterile swab.

TABLE 4. ISOLATION RATE (NO. OF SAMPLES WHICH PERMITTED RECOVERY/NO. OF TRIALS) OF *Streptococcus agalactiae* FROM BULK MILK FROM HERD WITH APPROXIMATELY TWO PERCENT OF QUARTER INFECTED (2 QUARTERS IN 25 COWS).

Medium[1]	Bulk tank				Quarters	
	Gravity cream from sample	Gravity cream on Culpak-kit 24 hr[2]	Milk from sample	Milk on Culpak-kit[3]	#1	#2
Aesculin					34/34	34/34
blood agar	15/33	0/33	16/33	12/33		
T.K.T.	26/33	12/33	Not cultured	14/33	Not cultured	
TCV	7/11	3/11	Not cultured	1/12	Not cultured	
TCV modified	16/22	3/22	Not cultured	12/22	Not cultured	

[1]Media described in text.
[2]Gravity cream from sample was transferred to Culpak-kit and stored at room temperature for 24 hr before culturing.
[3]Milk was transferred directly from bulk tank to Culpak-kit by sterile swab.

Survival time of organisms. Fourteen milk samples which contained known mastitis pathogens were subdivided and stored under four different conditions for 7 days: Vial samples at 4 C and at room temperature, and on Culpak-kits at 4 C and at room temperature. Of the 14 samples, six contained S. *aureus*, five contained S. *agalactiae*, two contained other streptococci, and one sample contained a mixture of S. *agalactiae* and other streptococci. All subsamples were cultured daily for 7 days. Thus, there were 392 attempts to recover pathogens from stored milk samples.

Streptococcus agalactiae was isolated daily from all 5 samples stored under each of four conditions. Similarly, other streptococci were recovered from each subsample of the 2 samples containing these organisms. All subsamples of the 6 samples which contained S. *aureus* yielded these organisms daily through day four. All subsamples which were expected to contain both S. *agalactiae* and other streptococci yielded these organisms through day three. A few samples failed to yield S. *aureus* or streptococci starting with day four. A high percent (96%) of all culturing attempts yielded some pathogens through seven test days.

Recovery of S. agalactiae from bulk milk from a herd with a low prevalence of infection:

Results from the culture of bulk tank samples for isolation of S. *agalactiae* from a herd of 25 cows are in Tables 3 and 4. With approximately 1% of the quarters infected with S. *agalactiae*, this organism was recovered from nearly half of the gravity cream samples using a non-selective medium. The percent recovery was improved by use of selective media. Very little improvement in the rate of isolation was found when two quarters (2% of the quarters) of the same cow were infected (Table 4). Gravity cream appeared to concentrate all organisms present in the bulk tank, so it was difficult to identify S. *agalactiae* from gravity cream cultured on a non-selective medium. When either milk or gravity cream was transferred to a Culpak and then cultured, the efficiency of recovery of S. *agalactiae* was seriously reduced.

CONCLUSION

It was demonstrated that the primary etiologic agents of bovine mastitis could survive in and be recovered from a filter paper strip during the period required for delivery of most first class mail, even if mail were delayed because of a weekend or a holiday. It was also shown that Culpak-kits can be used efficiently to transport quarter milk samples for identification of common mastitis pathogens. Thus it would appear that the Culpak-kit provides a useful tool to transport carefully collected quarter milk samples for culture purposes.

Samples that contain contaminating organisms, and which are not refrigerated, rapidly lose their usefulness for diagnostic purposes. Thus it was found that Culpak-kits were inefficient when used for the recovery of pathogens from either bulk milk samples or quarter milk samples which became contaminated during collection.

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EVALUATION OF A SURVEY OFFICER

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ABSTRACT

To acknowledge and justify the worth of any health program, the justification and reliability of the report has to come from the Survey Officer. Thus, it is through the efforts of the dedicated Survey Officer that the true story of local programs is brought to the attention of local officials, the enforcement officer, and the consumer. It is with this in mind, that we sanitarians should look to the "Survey" as a means of increasing the efficiency and resourcefulness of our efforts on the local level.

The purpose of this evaluation is to provide an unbiased opinion of the qualifications the Survey Officer must possess to render a thorough and accurate report on all items projected by the Ordinance and Code on any given survey that comes under his jurisdiction.

Furthermore, the evaluation of any given survey should not only be accurate and thorough, it should become a tool in the hand of the Local Enforcement Officer. If used wisely and intelligently, it can be of value to such a person in communicating with his constituents. He need not, in every instance, require pressure tactics to accomplish what has been called to his attention by the Survey Officer. Many Local Enforcement Officials have been guilty of oversight, not by the sin of commission, but by the sin of remission. Therefore, what has been called to his attention now can be corrected because he realizes the violation does exist. Perhaps, too, enforcement people have put the wrong interpretation on the violation involved. This can be corrected through knowledge of the Code and Ordinance by an intelligent and practical Survey Officer.

Thus the remarks in this paper are not to justify the acts of a Survey Officer, but rather to clarify the purpose and intent of any survey and what it should portray to the consumer and local, state, and Federal officials when completed. Such information should be made readily available through local news media to the public at large.

CHARACTERISTICS OF THE IDEAL SURVEY OFFICER

1. He must be intelligent. His knowledge of the Ordinances and Codes is revealed through his vocabulary. His practical interpretation of the Ordinances and Codes and his reasoning for justification for any violation must be evident.

2. His reference to Code information is quick and exact when needed for proof.

3. His temperament is such that he isn't easily disturbed. He is able to maintain an unruffled composure, even under the most rigid and adverse circumstances.

4. He must not be biased pro or con. He must indicate violations by logic and proper interpretation.

5. He must prove himself adequate in the presence of the people with whom he works and associates.

6. He must have the ability to admit and concede when proven in error.

7. His job is a challenge to him, and as a result, *his learning process never ceases!*

8. His integrity is above reproach. Everything that he does relevant to his job is done without fanfare and without expectation of reward, such as honor, glory, or remuneration.

9. He must be a good conversationalist. He must have the ability to expound and explain his ideas without searching for words of explanation.

10. His stature, as a Survey Officer, must be recognized by Federal, state, and local people.

11. He must recognize the *purpose* for which the survey of any given program is intended.

VIBRIO PARAHAEMOLYTICUS:
AN INTRODUCTORY STATEMENT[1]

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Last Fall (1971) investigations by the State of Maryland and the Food and Drug Administration established for the first time on record that *Vibrio parahaemolyticus* was responsible for outbreaks of foodborne illness in the United States. These findings supported and gave immediate relevancy to the concern of public health officials over the past several years that *V. parahaemolyticus* posed a potential threat to the public health in this country. A summary of the epidemiological studies made in connection with the Maryland outbreaks will be presented in the paper by Dr. T. A. Dadisman and his associates.

The first outbreak of disease for which *V. parahaemolyticus* is known to be responsible occurred in Osaka, Japan on October 21-22, 1950. Thus, this organism is a relative "newcomer" to the list of foodborne disease organisms. Its importance in Japan is evident by the fact that over the years it has caused 40-70% of all reported illnesses in that country from bacterial causes. Foods involved in outbreaks have been almost exclusively raw seafoods or seafood products. Many investigators have contributed to our present knowledge of the distribution of *V. parahaemolyticus* in the natural environment. Dr. Liston and Dr. Baross at the University of Washington were the first to report its presence in U.S. coastal waters. Their paper will discuss its distribution in environments throughout the world.

Fortunately, we were able to look to the Japanese for methods of isolation and identification of *V. parahaemolyticus*. Methods development is a never end-ing task. Workers at the University of Washington and at the Food and Drug Administration were among the first in the United States to apply the Japanese experience and expand methods development. Dr. Fishbein in his paper will discuss methods of isolation from seafoods.

Of great current interest is the question: "Is parahaemolyticus food poisoning an infectious disease or is it due to ingestion of a preformed toxin?" The question is far from resolution. Dr. Twedt will address himself to this question.

The first indication that parahaemolyticus food poisoning might be classed among the zoonoses was given by Dr. Colwell and her associates as a result of finding *V. parahaemolyticus* associated with dead and moribund crabs in Chesapeake Bay. More recently, it has been shown that *V. parahaemolyticus* may be a significant threat to successful production of pond raised shrimp. In their paper Dr. Vanderzant and Dr. Nickelson will discuss problems in mariculture that may relate specifically to *V. parahaemolyticus*.

The taxonomic position of *V. parahaemolyticus* is not yet entirely clear. Currently Dr. Colwell and Dr. Baumann are deeply involved in such studies. Their views while somewhat divergent are contributing significantly to our knowledge of the taxonomy of the marine vibrios including *V. parahaemolyticus*. Their papers will conclude the series.

It is hoped that the seminar and publication of the papers will result in greater awareness and knowledge of this foodborne disease. Hopefully this in turn will result in including methods for recovery of *V. parahaemolyticus* among the battery of analytical procedures applied to foods incriminated in gastrointestinal illness. The greater the effort in this regard the sooner we will know the relative importance of this organism among those responsible for foodborne disease in the United States.

VIBRIO PARAHAEMOLYTICUS GASTROENTERITIS IN MARYLAND: CLINICAL AND EPIDEMIOLOGIC ASPECTS[1]

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ABSTRACT

During August, 1971, three separate outbreaks of foodborne disease occurred in Maryland and involved about 425 (57%) of an estimated 745 at risk. Steamed crabs or crabmeat was the suspected vehicle of disease transmission in each outbreak. *Vibrio parahaemolyticus* was isolated from the crab and from stools of some ill persons in each outbreak.

During August, 1971, three separate foodborne disease outbreaks occurred in Maryland, involving about 425 (57%) persons of an estimated 745 at risk. In each outbreak, steamed crabs or crabmeat was the suspected vehicle of disease transmission. From the crab and from the stools of some ill persons in each outbreak, *Vibrio parahaemolyticus* was isolated.

OUTBREAKS 1, 1A, AND 1B

The first outbreak, outbreak 1, occurred on August 14, 1971, following a picnic attended by about 550 people. After a median incubation period of 15 hr, about 320 (58%) of these persons developed acute gastroenteritis. One hundred and twenty-one persons responded to our questionnaire. Most noted symptoms of nausea, vomiting, abdominal cramps, and diarrhea, and about 25% of the ill persons suffered fever and chills. The median duration of illness in these people was between 3 and 4 days; over one-half of them sought medical attention. Food histories from both ill and well picnic participants showed a significant difference in attack rates of illness between those who did and did not eat steamed crabs. In addition, of the ill persons interviewed, 27 had eaten only crab at the picnic.

Furthermore, several picnickers took crabs home for friends and relatives—we know of at least 43 of these

people who became ill (outbreak 1a). The incubation period, duration of illness, and symptoms of people in this outbreak matched those in outbreak 1.

Another outbreak (outbreak 1b) occurred the same day, involving 15 (71%) of the 21 persons at another picnic, who ate 2 bushels of crabs from the original lot of 85 bushels reserved for the outbreak 1 picnic. Only 80 of these bushels were consumed in outbreaks 1 and 1a; 2 went to outbreak 1b, and 3 bushels were sold over the counter. The characteristics of this outbreak were similar to those of outbreak 1 and 1a.

Routine bacterial cultures of stool specimens from ill persons and of crab in outbreak 1 were negative for enteric pathogens. At this point, *Vibrio parahaemolyticus* was considered as a possible pathogen in this outbreak, and further laboratory investigation was conducted.

Stool specimens were obtained from two ill persons in outbreak 1 and two ill persons in outbreak 1b; some crabs were on hand in the laboratory from outbreak 1b. From all these specimens, *Vibrio parahaemolyticus* was isolated—serotype 04:K11 from each of the stools; and 03:K30, and an untypable strain from the crabs.

The crabs were steamed at a plant near their point of harvest in the lower Chesapeake Bay. Contamination occurred during the transport and storage of these crabs with baskets of live crabs, under conditions of inadequate or absent refrigeration.

OUTBREAKS 2 AND 2B

On August 28, 1971, two more outbreaks occurred (2, 2a), involving 39 of 60 people in outbreak 2, and 8 of 15 in 2a. Steamed crabs were again the vehicle, although too few people were interviewed to allow for statistical analysis of food histories. As in the first group of outbreaks, crabs were taken home from one picnic to another, and accounted for the "satellite" outbreak, 2a. The character of the illness in outbreaks 2 and 2a closely resembled that in the first series of outbreaks. From outbreak 2a, *V. parahaemolyticus*, 04:K11 was recovered from one stool sample and 02:K28 was recovered from a sample of

the crab.

The crabs served at these picnics came from several areas of Maryland and Virginia waters of Chesapeake Bay. Contamination of the crabs seems to have occurred after they were steamed, when they were placed back into the baskets in which they had been before cooking, and when they were stored with baskets of live crabs.

OUTBREAK 3

On August 31, 1971, 24 of 100 employees eating the noon meal at a state hospital developed acute gastroenteritis (outbreak 3). The meal included crab salad, prepared from canned crabmeat. The illness resembled those previously described; median incubation period was 18 hr, and the median duration of illness was about 3 days.

Although the same meal had been eaten by the 251 patients in residence at the time, there had been no increased incidence of diarrhea among these people. For several weeks before this outbreak, 4%-6% of patients had had diarrhea at one time or another. However, at the time of investigation 11 patients (4.4%) had had the onset of diarrhea after the suspect meal. Culture survey of these patients showed 3 with V. parahaemolyticus in their stools—2 type 04: K11; 1 type 03:K30. In addition, cultures of ill employees yielded 3 persons with V. parahaemolyticus in their stools—1 type 03:K33; 1 type 04:K11; and 1 untypable. The remaining crab salad yielded an untypable strain of V. parahaemolyticus.

The crabmeat had come from a nearby packing house, where, after steaming, the crabs were picked into cans, which were then sealed and marketed. Methods of food handling of the state hospital were satisfactory, and it is presumed that contamination occurred during the picking process, when the workers have the opportunity to come in contact with both live and cooked crabs.

Following the outbreaks, samples of live crabs and water from Chesapeake Bay were cultured. The crab yielded V. parahaemolyticus type 03:K31; 04: K45; and an untypable strain. From the water, type 07:K19 was recovered.

All isolates of V. parahaemolyticus from crab and Bay water were Kanagawa-negative; all isolates from people, save one, were Kanagawa-positive, findings reflected in the extensive Japanese experience with this organism.

These foodborne disease outbreaks definitely seem to have been caused by V. parahaemolyticus. The clinical illness closely resembled descriptions in the literature; other recognized bacterial enteric pathogens were sought and not found in either stool or food samples; in each instance, a seafood, crab or crabmeat, was implicated as the food vehicle of disease transmission; from stools of ill persons and from the crab in each outbreak various serotypes of V. parahaemolyticus were isolated; and from live crabs and water of Chesapeake Bay, the organism was recovered.

V. parahaemolyticus has been known to be present in most, if not all, United States coastal waters and shellfish, and now is known capable of producing gastroenteric illness in this country. *Accordingly, it should be considered a potential pathogen in foodborne disease outbreaks. Specimens of stool and food should be cultured on thiosulfate citrate bile salts (TCBS) agar to recover this organism, as routine enteric media do not possess the requisite salt concentration, and will not support the growth of V. parahaemolyticus.*

DISTRIBUTION OF *VIBRIO PARAHAEMOLYTICUS* IN THE NATURAL ENVIRONMENT[1]

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Abstract

Vibrio parahaemolyticus has been isolated widely from marine environments but appears to be most abundant in inshore and estuarine areas where ambient temperatures rise seasonally to levels permitting growth of the organism. Japanese and U. S. studies of coastal areas have shown a direct relationship between temperature and abundance of *V. parahaemolyticus* and this correlates with the seasonal incidence of *V. parahaemolyticus* food poisoning in Japan. The organism has been isolated from water, sediment, plankton, fish, and shellfish. In North America it seems to be most abundant in molluscan shellfish and in waters of high organic content. Counts of 10-200/ml of water, 1-7/g of sediment and up to 10^5/g of oyster tissue have been reported for North American inshore areas. Limited information on market seafood samples indicates very low incidence of *V. parahaemolyticus* on fin fish in Europe and North America and high incidence in Japan during summer months. Limited data on market samples of frozen and fresh shellfish in U.S.A. suggest sporadically high incidence on shrimp, crabmeat, oysters, and clams.

There can be little doubt that the principal source of *Vibrio parahaemolyticus* is the sea. The organism is mildly halophilic and in its role as a human pathogen is mostly isolated from patients who have become sick after eating uncooked or lightly processed fish and shellfish. Moreover, strains of the same species have been identified as the cause of disease in crabs, shrimp, and possibly even oysters and fish. Unfortunately there is very little published information on the incidence of *V. parahaemolyticus* in truly terrigenous environments.

Seasonal Variations

It is not surprising, in view of the importance of *V. parahaemolyticus* food poisoning in Japan, that the bulk of information on the organism has been published by Japanese scientists. The early identification of halophilic bacteria by Fujino in 1951 *(16)* and later and independently by Takikawa in 1955 *(29)* as the agents of gastroenteritis in people who had eaten raw or semi-raw fish, rapidly led Japanese

workers to the conclusion that the bacteria were of marine origin. It is true that on occasion foods of terrestrial origin such as brined cucumbers, meats, eggs, etc. have been implicated in food poisoning outbreaks due to *V. parahaemolyticus (1, 23)*, but these are presumed to have been ultimately contaminated from a marine source. In 1965 Terayama *(30)* reported that 34% of the marine products sampled in Tokyo shops were contaminated with *V. parahaemolyticus* during the summer, and Zenyoji et al. *(35)* reported a general Japanese average of 13%, also in summer. These figures correlate well, of course, with the seasonal incidence of *V. parahaemolyticus* gastroenteritis in Japan, which is high in summer and very low or completely absent in winter. This in turn accords well with the unusual (for a "marine" bacterium) sensitivity of *V. parahaemolyticus* to cold *(5)* and its very high growth rate at higher temperatures *(22)*. While *V. parahaemolyticus* seems to be quite generally present in the coastal waters, sediments, and plankton *(4, 19)*, the abundance of these organisms is strictly seasonal. This is clear from the work of Miyamoto et al. *(25)* who showed low incidence when water temperatures were cold and highest incidence when they were near 30 C (see Table 1). A separate study of incidence in a river near Kobe, with concomitant recording of the incidence of *V. parahaemolyticus* in feces of healthy people in the same city, results of which are summarized in Table 2, are even more revealing. Results of studies of incidence of *V. parahaemolyticus* in oysters and other marine environments of Puget Sound also indicate a seasonal temperature-related variation in abundance *(7, 8)*, and so do those of Bartley and Slanetz *(10)* in New Hampshire. Inshore waters are shallow and do show marked temperature

TABLE 1. Seasonal occurrence of *Vibrio parahaemolyticus* in Tokyo Bay water

Date	Temperature (C)	Positive samples	Mean MPN/100 ml
December, 1959	18-20	7	2
March, 1960	10-16	4	4
June, 1960	18-21	16	78
September, 1960	26-30	14	93

All numbers rounded to nearest whole number.

fluctuations with season, reaching high levels in most temperate regions during July, August, and September. As can be seen from data in Table 3, V. parahaemolyticus, as defined biochemically, has been isolated from many coastal and estuarine marine environments around the world.

OCCURRENCE IN PELAGIC AREAS

The relatively few studies of the occurrence of V. parahaemolyticus in pelagic (high sea) areas generally indicate a lower frequency of occurrence. Thus Hechelmann et al. (18) could find no V. parahaemolyticus in pelagic samples from the Baltic, and Horie et al. (20) found no true V. parahaemolyticus (biotype 1) in high sea samples taken off Japan. On the other hand, Aoki et al. (4) obtained V. parahaemolyticus from 8 of 100 water samples, 20 of 88 plankton samples, and 7 of 21 fish samples taken from pelagic areas in the Indian Ocean, the China Sea, and the Pacific Ocean. It was pointed out by Baross and Liston (8) that the incidence of V. parahaemolyticus seemed to diminish as deeper water samples were tested, and in their limited offshore studies they found low incidence of V. parahaemolyticus in deeper sediments, as compared to shallow inshore regions. It seems probable that the relatively low occurrence of V. parahaemolyticus in high seas samples results from the consistently low temperatures in many of these areas. However, nutrient supply may also be a factor, since there is some evidence (5, 8) that the organism is found in greatest numbers in waters of high organic content, typical of inshore, estuarine conditions (Table 4).

SUBENVIRONMENTS FOR THE ORGANISM

Accepting for the moment that the sea, and particularly the coastal sea and estuaries, is the common habitat of V. parahaemolyticus, this still leaves in question which, if any, of the several subenvironments, including water, sediment, fish, shellfish, and plankton, the organism favors. The evidence on this point is by no means clear-cut. Unfortunately most studies have been qualitative in nature, or at best incidence surveys which are hard to interpret quantitatively. Japanese workers report incidence of V. parahaemolyticus on a wide variety of market seafoods. Only mackerel among the free-swimming fishes, has been reported to carry large numbers of V. parahaemolyticus. Baross and Liston (8) reported low numbers of V. parahaemolyticus on 15 different species of fish and modest numbers in waters (mostly ca. 10/ml) and sediment (1-7/g), but relatively large numbers in clams and oysters. The same authors reported counts in the range 10^2 to 10^5 for shellfish purchased from retail stores (Table 5). Other North

TABLE 2. COUNTS OF Vibrio parahaemolyticus IN WATER NEAR KOBE AND INCIDENCE OF THE ORGANISM IN FECES FROM HEALTHY INDIVIDUALS

Month	Counts in water[1]			Incidence in feces[2]
	a	b	c	
Jan.-May	—	+	—	0.18
June	—	+	—	0.16
July	+	+	+	0.58
August	115	137	112	0.35
September	156	59	85	0.23
October	23	49	46	
Nov.-Dec.	—	—	—	

[1]Counts as (mean) MPN/100 ml at three stations on a small river.
[2]Based on approximately 1,000 samples each month.

TABLE 3. GEOGRAPHICAL DISTRIBUTION OF Vibrio parahaemolyticus.

Isolations from marine sources and fish		Isolations from humans	
	Reference		Reference
Japan	1, 4, 5, 19, 20, 25, 28, 35	Japan	1, 4, 15, 23, 28, 29, 35
Korea	4	Korea	4, 13
Taiwan	4	Philippines	4, 28
Singapore	4	India	12, 28
Hong Kong	4	U.S.A.	3, 6
Ceylon	4	Australia (?)	11
Hawaii	4	Mainland China	4
Indian Ocean	4		
Pacific Ocean	4		
N. America: Pac. Coast	7, 8		
N. America Atlantic Coast	4, 10, 15, 17, 24, 31, 34		
N. America: Gulf of Mexico	33, 34		
Germany	18		
Denmark	18		
Holland	21		
Britain	9		
Baltic Sea	18		
Adriatic Sea	18		
France	14		
Spain	14		

TABLE 4. INCIDENCE OF Vibrio parahaemolyticus IN SHELLFISH AND WATER SAMPLES OBTAINED FROM A NONPOLLUTED AND A FECAL-POLLUTED SHELLFISH-GROWING AREA IN PUGET SOUND

	Sampling Area			
	Nonpolluted (Purdy, Wash.)		Polluted (Bellingham Bay, Wash.)	
Season	Water[1]	Shellfish[1]	Water	Shellfish
Summer	10	500	200	70,000
Fall	10	50	15	10,000

[1]Counts expressed per milliliter of water and per gram of shellfish meat; counts represent the mean count from several replicate samples.

TABLE 5. COUNTS OF *Vibrio parahaemolyticus* IN MARKET SAMPLES OF SEAFOODS[1]

Product	Samples tested	Mean count	Low	High
Shucked oysters	9	5×10^4	100	10^5
Packaged crab meat	5	1×10^3	<10	4.5×10^3
King crab meat	1	<10	—	—
Frozen breaded shrimp	2	—	1.5×10^3	$>10^5$
Frozen breaded oyster	1	25	—	—
Fresh clams	2	1.5×10^3	6.0×10^2	2.5×10^3

[1] All counts based on biochemical identification of *Vibrio parahaemolyticus*.

American authors have indicated that shellfish may carry significant numbers of V. *parahaemolyticus* (10, 15, 31) and so, too, have Barrow and Miller (9) in Britain. These data would suggest that perhaps shellfish are at least a major habitat of V. *parahaemolyticus* in coastal waters. It is interesting that bacteriophage lytic towards V. *parahaemolyticus* can be isolated readily from oysters, particularly if an enrichment technique is used (8). Bacteriophage have not been isolated with the same frequency from other marine environments (Baross, *unpublished*). Incidence of V. *parahaemolyticus* on market fish in Europe has been reported to be quite low (21). However, it is not clear whether this results from a low incidence on the living fish or to treatment (particularly chilling) of the fish after capture. It may be relevant that Hechelmann et al. (18) reported no isolations from 356 Baltic Sea and 200 North Sea fish. On the other hand, experiments in our laboratories with fish fillets show that V. *parahaemolyticus* virtually dies out in just over 10 days at +1 C. Much more quantitative information is needed on the actual incidence, on a seasonal basis, of V. *parahaemolyticus* on live and fresh landed fish and shellfish. This is most important in assessing the hazard to the public of V. *parahaemolyticus* poisoning. This is well seen in the cases which occurred in Washington State in 1969 (6), where foreknowledge of a high count situation in clams, the incriminated food, could have led to regulatory closure of the bed, at least temporarily. Moreover, since processing techniques change rapidly nowadays, it is essential to know whether the apparent low incidence of V. *parahaemolyticus* food poisoning in the U.S.A. is caused by low initial levels of contamination of our seafoods or by effective processing procedures.

V. *parahaemolyticus* has been identified in other pathogenic roles than as a food poisoning agent. In man these include co-occurrence with cholera vibrios in patients suffering from cholera (12, 28). The organism has also been encountered quite frequently alone during cholera outbreaks in India. However, there is no evidence of the origin of such organisms. Again, V. *parahaemolyticus* has been isolated from wound infections (32). There is some evidence that these infections were secondary infections of wounds,

picked up by bathing in the sea. This is similar to the serious leg infection acquired by a clam digger in New England, described by Roland (27).

It has also been reported that V. *parahaemolyticus* causes disease in shrimp (33), blue crab (24), and possibly also in oysters (Baross, *unpublished*). This pathogenic role seems most likely to be a consequence of an opportunistic invasion of an animal which has been injured or is physiologically debilitated because of adverse environmental conditions. Alternatively, there may be particularly virulent or invasive strains of V. *parahaemolyticus* which occur from time to time.

To the marine microbiologist V. *parahaemolyticus* is one of a group of phenotypically very similar mildly halophilic, mesophilic vibrios which occur frequently in warm weather in inshore and estuarine waters, and particularly in areas of high organic content or in shellfish beds. The group is difficult to separate into clearly distinguishable species, and many types mimic V. *parahaemolyticus* closely. Even organisms which seem to meet the biochemical criteria for V. *parahaemolyticus* have sometimes proved on genetic analysis not to be this organism (2). Consequently nearly all the published accounts of incidence and distribution of V. *parahaemolyticus* should be read with caution. Most Japanese authors have stated that the organisms isolated from marine samples differ from the strains isolated from human feces in being unable to hemolyze human blood in the so-called Kanagawa phenomenon (26). Feeding experiments with human volunteers have established that Kanagawa-negative V. *parahaemolyticus* do not cause the typical sickness. However, there is not complete unanimity on this point, since Kanagawa-positive strains have apparently been isolated from marine samples, and Kanagawa-negative authentic food poisoning V. *parahaemolyticus* are known to exist. The ultimate proof of identity is probably genetic, but procedures involved are too complex for many laboratories. It would seem best to follow Sakazaki (28) and accept as V. *parahaemolyticus* organisms which grow on TCBS, grow at 43 C, fail to grow in 10% NaCl broth, and do not ferment sucrose. Regarding the last point, however, it should be noted that Baross and Liston (8) reported 7 out of 40 Jap-

anese *V. parahaemolyticus* strains isolated from outbreaks did, in fact, ferment sucrose.

However, even allowing for possible errors in identification of the organism by different authors, the evidence available from published reports suggests that *V. parahaemolyticus* is widely distributed in marine environments throughout the world. In coastal and estuarine waters the organism is apparently more abundant during warm periods of the year and present in very small numbers, or even completely absent, when water temperatures are low. In North America, *V. parahaemolyticus* is apparently most frequently recovered from shellfish which must thus be considered as the prime suspect food in cases of *V. parahaemolyticus* food poisoning.

ACKNOWLEDGMENT

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E. H. MARTH
Editor
Journal of Milk and Food Technology

VIBRIO PARAHAEMOLYTICUS METHODOLOGY FOR ISOLATION FROM SEAFOODS AND EPIDEMIC SPECIMENS[1]

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ABSTRACT

The newly revised procedure in the Bacteriological Analytical Manual of the Food and Drug Administration for the analysis of *Vibrio parahaemolyticus* in seafood samples and epidemic specimens is reviewed. Details of sample preparation of seafoods and epidemic specimens, media and test reagent formulations, enrichment and isolation procedures, and the biochemical and serological diagnostic criteria of *V. parahaemolyticus* are presented. The biochemical schema for the separation of other interfering marine vibrios is included.

The identity of *Vibrio parahaemolyticus* was established by Sakazaki and coworkers in 1963 *(6)*. It is an enteropathogenic, halophilic, gram-negative facultative anaerobe occurring in most of the marine waters of the world. In Japan it is the major bacterial cause of epidemic gastroenteritis. The organism occurs naturally in many species of marine fauna; in the U.S., *V. parahaemolyticus* has been recovered from a great variety of seafoods such as fish, shellfish, and crustaceans harvested along the Atlantic, Pacific, and Gulf Coasts. In the summer of 1971, a succession of four foodborne outbreaks of *V. parahaemolyticus* occurred in Maryland following the serving of steamed crab and crab salad. These episodes were the first examples of parahaemolyticus food poisoning in the U.S. in which the causative bacterium was recovered from both patients and contaminated foods.

The Division of Microbiology, Food and Drug Administration, collaborated with the Maryland State Board of Health in the isolation and identification of these organisms. The procedure routinely employed in our laboratories to recover *V. parahaemolyticus* from seafoods and from epidemic specimens is described below.

Figure 1 depicts the general procedure of the FDA Bacteriological Analytical Manual *(2)* for *V. parahaemolyticus*. Both seafood samples and epidemic specimens are processed in essentially the same fashion except that the epidemic specimens may lack an enrichment phase depending upon circumstances. A 50-g sample is weighed into a blender jar together

with 450 ml of a 3% sodium chloride diluent and blended for 1 min. From this suspension all dilutions are prepared and inoculated in triplicate into glucose salt Teepol broth (GSTP) on the first day. By employing the multiple tube technic, *V. parahaemolyticus* can be enumerated by means of the Most Probable Number tables. The enrichment broth tubes are incubated overnight at 35 C. On the second day, the three highest dilutions showing growth are streaked onto thiosulfate-citrate-bile salts-sucrose agar (TCBS). The TCBS plates are incubated overnight at 35 C.

Figure 2 portrays the typical appearance of *V. parahaemolyticus* on these plates (left) as round colonies 3-5 mm in diameter, with green or blue centers. On the right is a mixture of *V. parahaemolyticus* and *V. alginolyticus* colonies. The latter are easily differentiated by the formation of yellow colonies and halos. The *V. parahaemolyticus* colonies are fished and inoculated into the following screening media: triple sugar iron agar (TSI), motility agar, trypticase soy agar and trypticase soy broth. After suitable incubation and examination of the results, an isolate culture which proves to be a gram-negative, polarly flagellated, motile rod producing an alkaline slant and acid butt on TSI and which is negative for hydrogen sulfide and gas production, is examined further biochemically as illustrated in Table 1.

In Table 1 is a list of the criteria after Sakazaki *(6)* which establish the identity of *V. parahaemolyticus*. By this means a suspicious isolate is effectively separated from closely related marine vibrios with which it may easily be confused. In addition to the above screening tests, the criteria include: Hugh-Liefson glucose O/F medium-fermentation (+), gas production (−); cytochrome oxidase test (+); Taylor modified decarboxylation media, arginine dihydrolase (−) and lysine decarboxylase (+); nutrient gelatin (+); salt trypticase soy broth containing 6% and 8% sodium chloride (+) but containing 0% and 10% sodium chloride (−); growth in trypticase soy broth at 42 C (+); Voges-Proskauer test (−) and indole (+); in carbohydrate media, cellobiose (−) and sucrose (−) but maltose (+), mannitol (+), and trehalose (+).

ANALYTICAL METHOD FOR <u>VIBRIO PARAHAEMOLYTICUS</u>

<u>PHASE</u>	<u>SEAFOODS</u>	<u>EPIDEMIC SPECIMENS</u>
DAY 1 (Enrichment)	Fish, Shellfish, Crustaceans 50gm + 450ml 3% NaCl $(10^0 \ldots \ldots 10^{-4}$ dilns.$)$ G S T B (triplicate tubes)	Stools, Rectal swabs Cary-Blair transport medium T C B S
DAY 2 (Isolation)	T C B S	
DAY 3 (Screening)	T S I motility Gram stain	
	TSA TSB	
DAY 4 (Identification)	Biochemistry Serology	
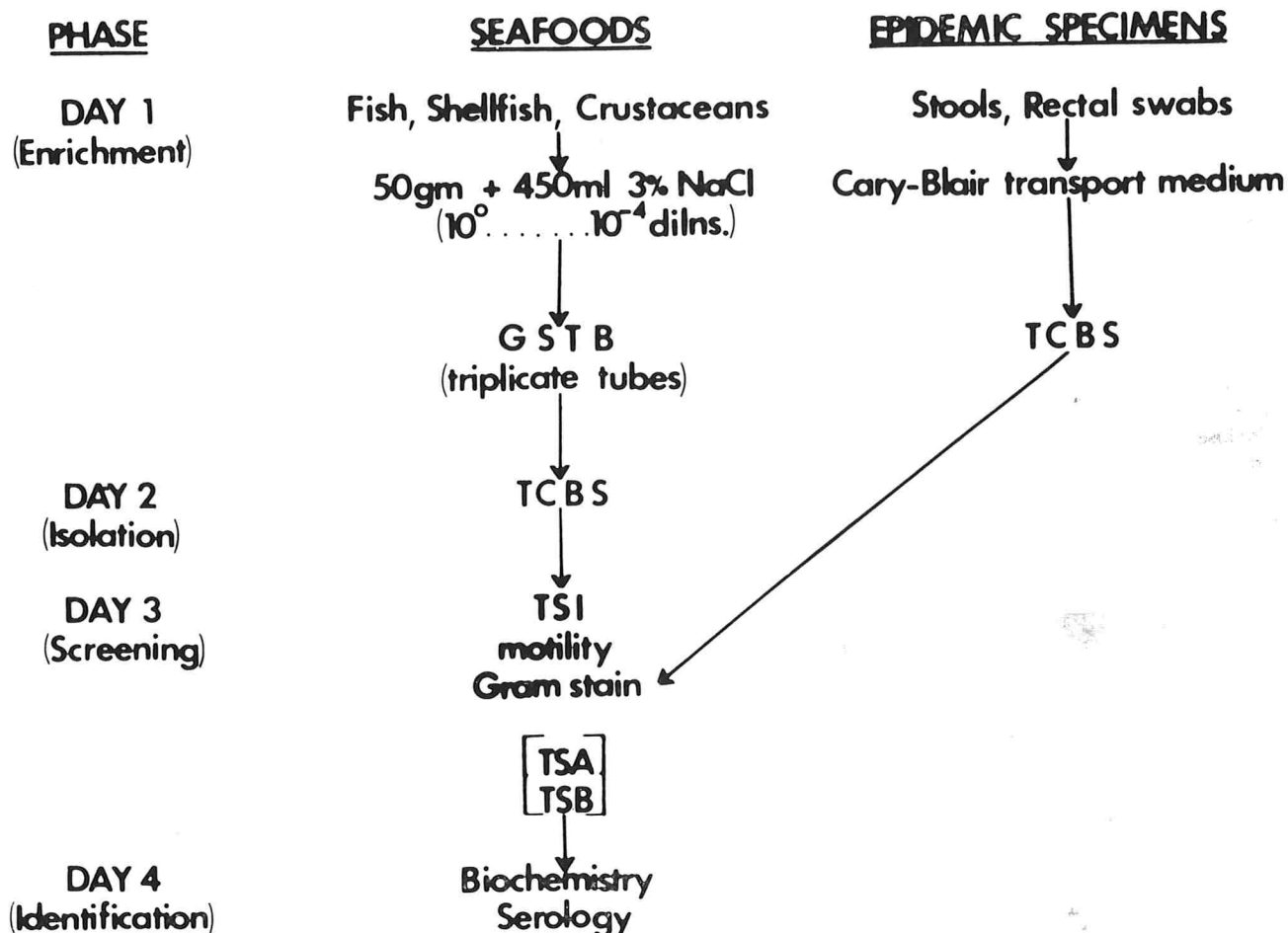
Figure 1.   Analytical method for V. *parahaemolyticus*.

In the marine environment V. *parahaemolyticus* is in constant competition with other marine vibrios which closely resemble it both biochemically and serologically.   These competitors are present in enrichment broths and on selection or isolation plates. Table 2 indicates how four interfering species of vibrio may be separated from V. *parahaemolyticus*. The data also illustrate the necessity for including these tests in the characterization of an isolate if these species are to be separated.   This separation key supersedes an earlier one published by Sakazaki. Many other interfering vibrio types in addition to these four are to be found in the marine environment.

While the salt tolerance test is reasonably reliable when performed in the test tube, it is not as sensitive as an alternative halophilism shake culture method when greater accuracy is required.   Failure to recognize this fact has led to conflicting reports in the literature.   To perform this test, a medium containing 0.1% trypticase is supplemented with 0, 6, 8 and 10%

sodium chloride contained in 25-ml portions in 50-ml Erlenmeyer flasks.   These are inoculated with 0.1-ml portions of a 1:10 dilution of a 24-hr trypticase soy broth culture.   The cultures are incubated for
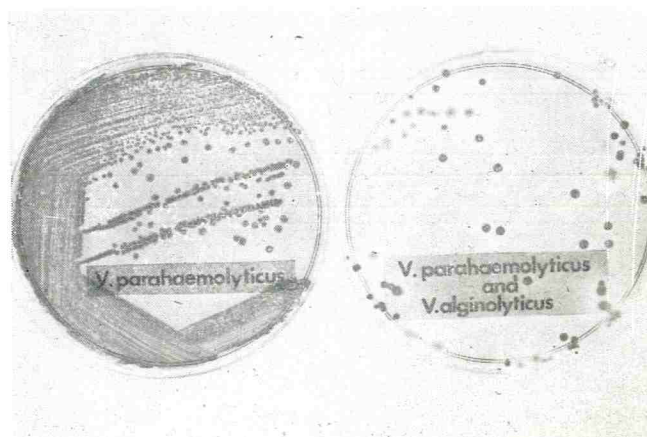
Figure 2.   Appearance of V. *parahaemolyticus* on TCBS agar.

TABLE 1. *V. parahaemolyticus* IDENTIFYING CHARACTERISTICS
(AFTER SAKAZAKI)

Tests	Response
Gram stain	Gram-negative
Morphology	Curved/straight rods
Motility	+
TSI	K/A, H_2S (−), gas (−)
Hugh-Liefson glucose	Fermentation (+), gas (−)
Cytochrome oxidase	+
Arginine dihydrolase	−
Lysine decarboxylase	+
Gelatin	+
Halophilism (NaCl)	6%, 8% (+)
	0%, 10% (−)
Growth at 42 C	+
Voges-Proskauer	−
Indole	+
Cellobiose	−
Sucrose	−
Maltose	+
Mannitol	+
Trehalose	+
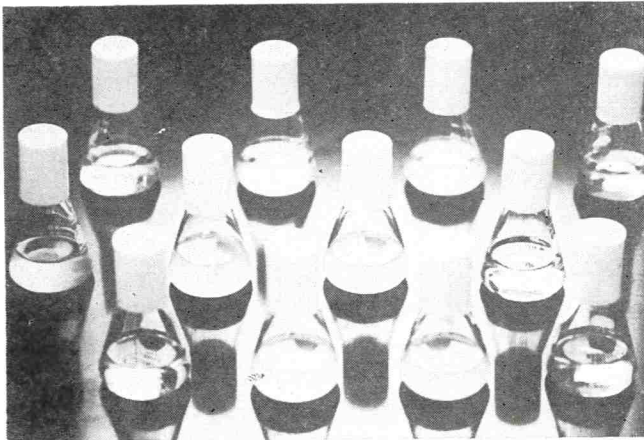
Figure 3. Halophilism shake cultures. *V. parahaemolyticus* reactions in front row. Unidentified vibrios in rear rows.
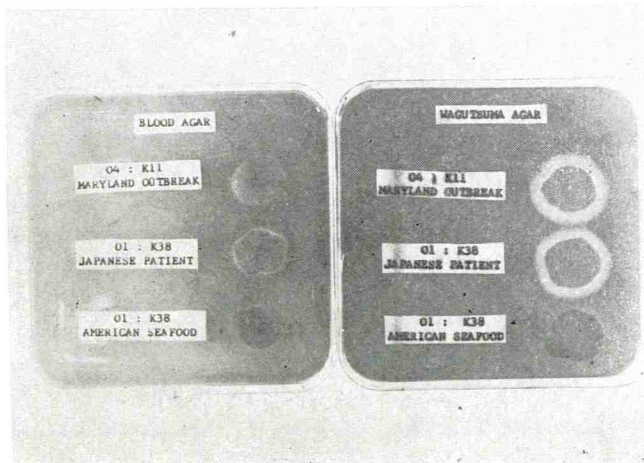
Figure 4a. The Kanagawa reaction shown on Wagatsuma agar (right) as contrasted with conventional hemolysis (left), 24 hr incubation at 35 C.

24 hr at 35 C in a shaker.

Figure 3 demonstrates the results of such a test. From left to right, the salt concentrations contained in the rows of flasks are 0, 6, 8 and 10%, respectively. In the front row, the test organism is *V. parahaemolyticus*. In the remaining rows, unknown *Vibrio* species are used.

Perhaps the most important laboratory test for *V. parahaemolyticus* is the Kanagawa test, named for the Japanese prefecture where it was devised. Its purpose is to detect a specific hemolysin which is associated in some fashion with the enteropathogenicity of *V. parahaemolyticus*. The Japanese literature suggests that isolates causing illness in humans are always Kanagawa-positive, but isolates recovered from seafoods are almost always Kanagawa-negative.

This specific hemolysin test is performed on special Wagatsuma agar. The composition of the medium is shown in Table 3. It is essential that the blood suspension be carefully prepared. Only freshly drawn human blood citrated in a final 0.5% concentration is employed. The blood is centrifuged, washed three times in physiological saline and made up to a final suspension of 20%. One hundred milliliters of this 20% suspension of fresh human red cells is added to 1 liter of the unautoclaved basal medium, which has been previously melted and tempered to 50 C. After the plates have been poured and allowed to harden, the surfaces are thoroughly dried before use.

To perform the Kanagawa test, a loopful of an 18-hr trypticase soy broth culture is spotted near the periphery of the plate. Several spottings may be made in a circular pattern on a single plate. The plate is incubated at 35 C for 24 hr. A positive test consists of a beta hemolysis, i.e., a zone of transparent clearing of the blood cells around the colony. No Kanagawa results are valid beyond 24 hr of observation. The Wagatsuma plates must be freshly prepared before the test, as they will not keep indefinitely.

Figure 4a shows the results of a 24-hr positive Kanagawa test. The plate on the right is Wagatsuma agar. From the top down the typical beta hemolysis produced by two enteropathogenic cultures is illustrated. The first, 04:K11, was recovered from the Maryland outbreak, and the second, 01:K38, came from a Japanese outbreak. The third, 01:K38, which is non-enteropathogenic, was isolated from a seafood in our laboratory and is Kanagawa-negative. The plate on the left is a conventional blood agar plate and the three cultures exhibit some degree of hemolysis, which is easily distinguishable from the beta hemolysis seen on the right.

Figure 4b shows the same plates after 48 hr of incubation. On the Wagatsuma plate beta hemolytic

TABLE 2. SEPARATION OF V. *parahaemolyticus* FROM RELATED MARINE VIBRIOS

Species	TCBS	43 C	NaCl 8%	10%	Lysine decar-boxylase	VP	Sucrose
V. *parahaemolyticus*	+	+	+	–	+	–	–
Vibrio sp. 6330	±	±	–	–	+	–	–
Vibrio sp. 6267	±	±	–	–	–	–	d
V. *alginolyticus*	+·	+	+	+	+	+	+
V. *anquillarum*	–	–	–	–	–	+	+

zones have increased in diameter and have fused, but the seafood culture is still Kanagawa-negative, although it has produced a hemolytic zone. On the conventional blood agar, all three cultures appear to have produced a similar type of hemolysin which has increased in width. Once again these are easily distinguishable from the Kanagawa hemolysis seen on the right.

Once all the biochemical tests have been performed, the identity of the unknown seafood isolate is established. However, the serotyping of the unknown organism can be of considerable value especially in epidemiological studies in parahaemolyticus food outbreaks.

In Table 4 is outlined the antigenic schema of V. *parahaemolyticus* as described by Sakazaki (5) and also by Zen-Yoji. At the present time 11 "O" groups and 52 "K" types are represented. Since types K4 and K30 appear in duplicate 0 groups, a total of 54 serotypes are established at the present time. The H antigens are not employed diagnostically because all the parahaemolyticus H antigens are serologically identical. Because many other marine vibrios are agglutinated with V. *parahaemolyticus* O and K antisera, it is realized that the agglutination test by
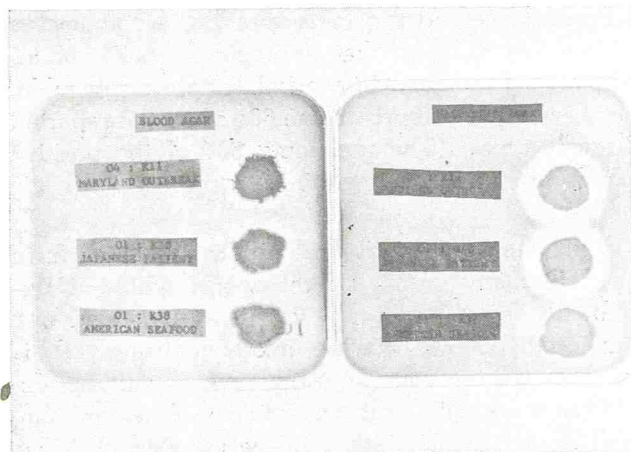
Figure 4b. The Kanagawa reaction after 48 hr incubation at 35 C.
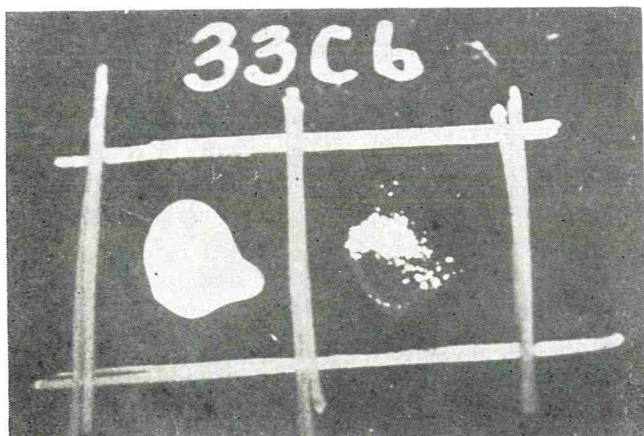
Figure 5. V. *parahaemolyticus* K antiserum agglutination (right), and unagglutinated control (left).

TABLE 3. COMPOSITION OF WAGATSUMA AGAR

Yeast extract	3 g
Bacto-peptone	10
Sodium chloride	70
Dipotassium phosphate	5
Mannitol	10
Crystal violet	0.0001
Bacto-agar	15
Distilled water	1 liter

Adjust to pH 8. Do not autoclave. Steam 30 minutes, temper to 50 C. Add 100 ml of 20% suspension of washed, human red cells.
Mix, plate and dry before use.
Inoculate—Loopful 18 hr STB culture.
Incubate—35 C for 24 HOURS ONLY.
Positive test—Narrow zone of beta hemolysis.

TABLE 4. ANTIGENIC SCHEMA OF V. *parahaemolyticus*

O group	K type
1	1, 25, 26, 32, 38, 41, 56
2	3, 28
3	4*, 5, 6, 7, 29, 30*, 31, 33, 37, 43, 45, 48, 54, 57
4	4*, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55
5	15, 17, 30*, 47
6	18, 46
7	19
8	20, 21, 22, 39
9	23, 44
10	24, 52
11	36, 40, 50, 51
Total 11	52 K types/54 serotypes

*Indicates occurrence in duplicate O groups.

itself is not conclusive for the recognition of this organism. Complete morphological, physiological and biochemical characterizations are required for the identification of *V. parahaemolyticus*.

Commercial Japanese parahaemolyticus antisera are available from the Nichimen Co. in New York City. These consist of 7 polyvalent K antisera groups and also the monovalent K antisera for the constituent members of these groups. The routine serological identification of *V. parahaemolyticus* is therefore based solely on the K antigenic analysis at the present time. In practice, an unknown isolate is first tested in polyvalent K group antisera. A positive test is then followed by testing in the monovalent K antisera to determine the specific serotype. Heretofore, K typing was sufficient to identify the 0 group of the unknown, since each K type occurred in only the 0 group. This rule still holds fairly well with the exception of the K4 antigen, which may occur in groups 03 and 04, and K30, which may occur in groups 03 and 05. In these instances 0 group antisera would be required to effect a precise identification.

Figure 5 presents an example of K serology. On the left is the unagglutinated control. On the right is a positive agglutination reaction.

Discussion

The preparation of a suitable 50-g sample from a fish requires the thin slicing of the skin surface and the securing of the gills and viscera of the fish. Stool samples from victims of foodborne outbreaks should be speedily obtained, treated as previously indicated and analyzed in the laboratory. The carrier state is short-lived, lasting perhaps only one week, and procrastination may result in negative findings. In epidemics, laboratory reports should include a complete biochemistry, serology, and Kanagawa test of suspected isolates. Although it is a difficult fact to accept, the serotype of patient isolates frequently will not match the serotypes of isolates recovered from the incriminated seafood.

We have employed a glucose salt Teepol broth as an enrichment medium with much success. It is easily prepared, quickly inoculated, and enables the organism to be enumerated in very low count foods by means of the Most Probable Number technic. Further, its use permits the employment of two separate selective agents, Teepol and bile salts, against competitive flora in the analytical sequence. *V. parahaemolyticus* counts in seafoods range from 1 to 10,000 per gram, with maximal numbers occurring in the summer months.

The thiosulfate-citrate-bilesalts-sucrose plating agar is a productive medium. *V. parahaemolyticus* colonies are readily selected on the basis of color.

This agar is commercially available and easily prepared for use. Accuracy of selection from this plate is of a high order; perhaps 80 to 90% of all picks are *V. parahaemolyticus*-positive. This compares favorably with the selection of coliform group members from eosin-methylene-blue agar.

Triple-sugar-iron agar in the procedure is an efficient screening tool. In addition to screening isolates, it indicates how effectively the enrichment and selective mechanisms have operated in the analytical sequence. If the method is poor in selecting the correct organism, the performance of TSI will likewise be poor *(7)*.

Some authors employ starch hydrolysis to separate the vibrios on starch agar media. However, most marine vibrios and related forms produce amylase, resulting in the mimicking of *V. parahaemolyticus* by many marine species *(7)*. Further, some analytical procedures utilize minimal biochemical criteria, resulting in an inability to speciate *V. parahaemolyticus* properly, or to separate it from closely related forms *(7)*.

In our laboratory we have examined hundreds of seafood samples, comprising several dozens of species. The records indicate that approximately 40% of all positive *V. parahaemolyticus* isolates are serotypable. These serotypes comprise some 60% of all the reported antigenic types. The remaining 60% of the positive isolates are untypable within the present antigenic schema.

V. parahaemolyticus is evidently a free living form found in many oceans of the world where it undoubtedly enjoys a saprophytic type of nutrition along with other similar marine microorganisms. It is found in marine waters and sediments and on the outer surfaces, gills, fins, and viscera of healthy fish, shellfish, and crustaceans. On the other hand, it has also indulged in a fortuitous type of parasitic nutrition by causing a disease in the blue crab in the Chesapeake Bay. Likewise it may be the cause of disease in shellfish and shrimp. In man it is responsible for epidemic gastroenteritis, and secondarily it has been associated with local and systemic infections resulting from minor skin cuts or abrasions occurring in the marine environment. Evidently the presence of *V. parahaemolyticus* in much of our seafood supplies must be accepted as an ecological reality.

Conclusions

The methodologist is faced with difficulties in the analytical laboratory. He must analyze maximum numbers of samples in minimal time with great accuracy. The realities of regulatory work compel him to balance these conflicting goals as best he can. In

any event, the methodology must be based on sound determinative bacteriology.

Any methodology may be subject to errors such as the misapplication of correct determinative data or the application of incorrect determinative data. The methodology is also dependent upon the efficiency of the enrichment, selection, and isolation media and their sequential employment in any particular analytical system. Their failures will likewise result in a deficient analytical system.

Examples of some methodological and determinative difficulties may be observed in some of the recently published *Vibrio* literature. Some authors will accept without confirmation cultures which are "considered to be" or "labeled as" a specific organism *1, 4)*. The stated identity may be incorrect and may result in determinative errors. Other workers will identify an organism solely on the phenotypic analysis of only three or five cultures *(3, 4)*.

The purposes of numerical taxonomy are vitiated if by its use *V. parahaemolyticus* appears to be urease-positive or *V. alginolyticus* to be Voges-Proskauer-negative, or if there is disagreement in the halophilism tests *(4)*. The best interests of all would be served if the multiplicity of the tests employed in this vital technic by various workers were unified, and if the interpretation of data and application of the methodology could be reconciled.

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EVALUATION OF A SURVEY OFFICER

a. To evaluate the program as a whole in terms of compliance with the Ordinance and Code.

b. To evaluate how well local sanitarians interpret the Ordinance and Code.

c. To evaluate and determine how well the local sanitarian can communicate with his clientele.

d. To evaluate the ability of the sanitarian to enforce the regulations as required by Ordinance and Code.

e. To evaluate the records of the sanitarian.

f. To evaluate the weak points in the program and to establish, with the sanitarian, proper approaches of correction.

g. To create a *sense* of *appreciation* in the mind of the sanitarian being surveyed.

h. To evaluate the sensitivity of the person being surveyed.

12. Solicit the help, aid, and suggestions voiced throughout the survey.

13. Any criticism offered is of a constructive nature and should be accepted and appreciated as such.

14. Any program that tries to justify every wrong with an excuse has to reestablish why the item was marked. Then use the deficient item as an approach for correction rather than a point of argument. Thus, the survey can become a tool to improve the status of the programs.

15. Figures, as such, in any given survey mean very little if the Survey Officer has not been honest in debiting all violations noted. The Survey Officer must be concerned with conducting a survey that will not only point out deficiencies in the programs, but also, to establish the strong points noted on the survey.

The Survey Officer should only be concerned with reporting the facts, as they were found, to whom it may concern. Then, and only then, will he be performing a duty and service as intended by the Ordinance and Code. True, sometimes the truth does hurt, but examine your program, put the best construction on it, and proceed to make the improvements noted. Somebody once said, it isn't so bad to have bed bugs, but it is really bad to keep them.

FLAVOR CHARACTERISTICS OF EGG YOLK AS AFFECTED BY DIFFERENT AMOUNTS OF SODIUM SULFATE IN THE LAYING HEN DIET[1]

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Abstract

Eggs from Single Comb White Leghorn hens (DeKalb) fed a standard laying hen diet supplemented with 0, 0.4, 0.8 or 1.2% sodium sulfate were stored at 13 C for six weeks and hard-cooked at weekly intervals for flavor evaluation of the yolk. Triangular taste tests indicated that egg yolk from hens fed a diet containing 1.2% sodium sulfate were different in flavor when compared to control eggs stored the same length of time. Panelists indicated that hard-cooked yolks produced by hens on the 1.2% sodium sulfate diet were drier, of coarser texture, and generally less flavorful than were yolks produced by hens on the control diet. There were no significant flavor differences noted between yolk from fresh control eggs and those from a 1.2% sodium sulfate supplemented diet. Total sulfur analyses of whole eggs indicated no significant differences in the sulfur content of whole eggs from hens fed the control or sodium sulfate supplemented diets.

Research concerning precursors of egg flavor has been extremely limited. A flavor profile of yolk developed by Koehler and Jackson (7) indicated sulfur to be an important flavor component of the low density and lipovitellin fractions. They also reported a loss in "sulfuriness" in the flavor of storage eggs. Lowe (8) reported that large quantities of sulfides in eggs give a characteristic odor and flavor to cooked eggs. Many other reports concerning egg flavor have been concerned with development of foreign odors during storage (5), and some have indicated slight flavor and odor differences caused by dietary influence (2, 3, 6, 10).

Machlin et al. (9) found sulfate sulfur to be incorporated into cystine in egg albumen and egg yolk when radioactive sulfur was administered orally to laying hens. Research (4) with purified and practical-type poultry diets supplemented with sodium sulfate has indicated that the chicken can utilize inorganic sulfate for growth with a concomitant effect on the dietary sulfur amino acid requirement.

Objectives of this study were to determine the flavor and textural quality of yolk from hard-cooked eggs (previously stored for up to 6 weeks) from hens fed diets containing various supplemental levels of sodium sulfate.

A preliminary study (11) comparing whole, hard-cooked eggs from hens on a control diet and a diet supplemented with 0.2% sodium sulfate had indicated no significant differences in the flavor of fresh (within 36 hr of production) hard-cooked eggs. However, significant flavor differences were noted when the eggs were stored for 1 to 6 weeks and then hard-cooked and evaluated for flavor. Flavor differences observed were confined to the yolk only; therefore, the albumen portion was removed from each hard-cooked egg slice for all subsequent experiments.

Materials and Methods

Individually caged Single Comb White Leghorn hens (De-Kalb), 34 weeks of age, were fed a standard laying hen diet (Table 1) supplemented with either 0, 0.4, 0.8 or 1.2% sodium sulfate.

Fresh eggs from hens on the control (0%) and 1.2% sodium sulfate supplemented diets were collected, washed, and stored at 13 C for no longer than 48 hr before hard-cooking. Flavor evaluation of stored eggs included control eggs and eggs from hens receiving the three levels of sulfate supplementation that were collected, washed, and stored at 13 C for up to 6 weeks. A total of 48 eggs per level of sodium sulfate were evaluated, and 144 control eggs were required for the 6-week storage studies.

Triangular taste tests as described and statistically analyzed by Roessler et al. (12) were employed for egg flavor evaluation. Eggs were hard-cooked for each taste panel analysis by placing 8 eggs from hens on both control and sulfate supplemented diets in cold tap water which was then heated rapidly to a boil. The container of eggs was then immediately removed from the heat, allowed to stand for 15 min and eggs were cooled rapidly under cold running tap water. All eggs were peeled and cross-sectioned with an egg slicing device to insure uniform egg slices, with only the 4 center slices being used for taste panel evaluations. Each panelist received 2 egg slices that were alike (of the same treatment but not of the same egg) and one egg slice from an opposing treatment.

The order of serving the yolk slices was based on the six possible orders that could be presented, as indicated by Roessler et al. (12); each panel session utilized a different order of presentation in regard to the experimental treatments. Evaluation of egg yolk slices was conducted at 7-day intervals at either 10:00 a.m. or 3:00 p.m. The storage periods for each treatment level were initiated on different days, thus, panel sessions were conducted at least twice weekly over a 15-week period (including preliminary and fresh yolk studies). Panelists were served the samples to be tasted in a food products laboratory with fluorescent lighting (isolation booths were not used), and asked to evaluate their samples without comment. Panelists were requested to choose the

TABLE 1. DIET COMPOSITION

Ingredient	Percent
Yellow corn	69.95
Soybean meal (50% protein)	19.00
Alfalfa meal (20% protein)	2.50
Iodized salt	0.25
Defluorinated phosphate (18% P and 32% Ca)	1.38
Ground limestone	6.42
Micro-ingredients[1]	0.50

[1]Supplied per kilogram of diet: 6,600 I.U. vitamin A, 2,200 I.C.U. vitamin D_3, 500 mg. choline chloride, 40 mg. niacin, 4.4 mg. riboflavin, 13 mg. pantothenic acid, 22 mcg. vitamin B_{12}, 125 mg. ethoxyquin, 20 mg. iron, 2 mg. copper, 198 mcg. cobalt, 1.1 mg. iodine, 100 mcg. zinc, 84 mg. manganese and 2.2 mg. menadione dimethylpyrimidinol bisulfite.

TABLE 2. TRIANGULAR TASTE TEST ANALYSES OF EGG YOLK FROM LAYING HEN DIETS SUPPLEMENTED WITH EITHER 0.4, 0.8, OR 1.2% Na_2SO_4

Storage time (weeks)	1.2% Na_2SO_4		0.8% Na_2SO_4		0.4% Na_2SO_4	
	No.[1] correct	Total[2] no.	No. correct	Total no.	No. correct	Total no.
1	5	9	3	9	3	9
2	6*	9	3	9	3	9
3	4	9	6*	8	2	8
4	5	8	2	8	5	8
5	6*	9	5	9	3	8
6	2	9	1	8	2	8
Totals:	28*	53	20	51	18	50

[1]"No. correct" refers to the number of panelists that correctly identified the two egg samples that were alike.

[2]"Total no." refers to the total number of panelists tasting at that particular panel session.

*Significant at the 5% level of probability.

two yolk samples most alike in flavor, or the different tasting sample. Eight to nine panelists were selected for their sensory perception exhibited in other related product palatability analyses and also the previous preliminary triangular taste test study of whole hard-cooked eggs.

Forty-four whole egg samples from hens on the control diet and the 0.4, 0.8, and 1.2% sodium sulfate supplemented diets were analyzed for total sulfur content by a commercial laboratory[2].

RESULTS AND DISCUSSION

The triangular taste test results comparing fresh control egg yolk with fresh egg yolk from hens on a standard laying diet supplemented with 1.2% sodium sulfate were non-significant. These data confirmed the previous preliminary triangular taste test results for fresh control eggs and fresh eggs from hens on a diet supplemented with 0.2% sodium sulfate (11). Panel performances indicated there were differences in the flavor of egg yolks stored 1 through 5 weeks for the 1.2% sodium sulfate panel sessions (Table 2). Statistical significance was noted for the second

and fifth week panel sessions and for the total number of correct choices out of the total number of tastings. Non-significant results occurred for detection of flavor differences with egg yolk from hens on the 0.8% and 0.4% sodium sulfate supplemented diets. A trend seemed apparent, however, in that most panelists detected flavor differences between the second and fifth weeks of storage and in every instance no more than two panelists could correctly choose two alike samples by the sixth week of the egg storage (Table 2).

The percentages of total sulfur in whole eggs from the control diet, and 0.4, 0.8, and 1.2% sodium sulfate supplemented diets were 0.084, 0.082, 0.081, and 0.086%, respectively. Although the total sulfur values of eggs from hens on the 0.4% and 0.8% sodium sulfate diets were numerically lower than the other values, there were no statistically significant differences.

Comments of panelists correctly choosing the alike samples, whether in fresh or storage evaluation, included the predominant mention of "dryness," "lack of flavor," "blandness," or "coarse texture" for yolks from hens on the sulfate supplemented diets. These flavor and textural characteristics were noted at each level tested and for each week of storage except the sixth, when all egg yolks were of apparent equal blandness and dryness.

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CHARLES GAYLE SHRADER
DIES IN VERSAILLES

Mr. Charles Gayle Shrader, age 53, of 4507 Statton
Road, Louisville, passed away unexpectedly of an ap-
parent heart attack at 11:30 a.m. Saturday, January
6, at Versailles, Ky.

Mr. Shrader was Chief Sanitarian of the Milk Di-
vision for the Louisville-Jefferson County Department
of Health. Gayle was employed by the Louisville
and Jefferson County Health Department in October,
1952. He was made supervisor in March, 1955 and
Milk Control Chief Public Health Sanitarian in De-
cember 1967. He was a native of Oldham County,
the son of the late Robert F. Shrader, and was a resi-
dent of LaGrange and Oldham County prior to mov-
ing to Louisville a few years ago. Mr. Shrader was
a member of the Fern Creek Church of Christ, Ken-
tucky and International Association of Milk, Food and
Environmental Sanitarians.

He is survived by his wife, the former Jean Hatfield;
a daughter, Miss Susan J. Shrader of Louisville; a son,
Mr. Robert G. Shrader of Louisville; his mother, Mrs.
Alma Shrader of LaGrange; three sisters, Mrs. Virgil
(Alberta) Edwards, Mrs. John M. (Virginia) Mc-

Combs and Mrs. Jay (Jean) Evans, all of Louisville;
three brothers, Vincent M. Shrader and Robert D.
Shrader, both of Louisville, and Norman E. Shrader
of LaGrange.

LUTHER A. BLACK
1904-1973

Dr. Luther A. Black widely known for his import-
ant contributions to the development and operation
of the U. S. Public Health Service Grade A Milk
Program died at the age of 69 on January 24, 1973
after a short illness. He leaves his wife Margaret,
3722 Pleasant Street, Cincinnati, Ohio, and two daugh-
ters, Mrs. Jean Hardin, of St. Louis and Mrs. Janice
Runyon, of Seattle.

Dr. Black received B.S., M.S., and Ph.D. degrees
in bacteriology from the University of Illinois. In
1926-1927, he was employed as Bacteriologist in the
State Health Department, Springfield, Illinois, and
from 1928 to 1930, as Assistant Professor of Bacter-
iology and Associate Dairy Bacteriologist at the State
College of Washington Agricultural Experiment Sta-
tion in Pullman, Washington. From 1930 to 1935, he
was Associate Professor of Bacteriology at the Uni-

versity of Maryland in College Park, and was Professor of Bacteriology at that institution from 1936 to 1941.

His career in the U. S. Public Health Service began in 1941 as a Bacteriologist in the Sanitation Section in Washington, D. C., and in 1943 he was transferred to Cincinnati, Ohio. From 1944 to 1948 he was Senior and Principal Bacteriologist in the Water and Sanitation Investigation Station. From 1949 to 1953 he served as Chief, Milk and Food Sanitation Laboratory of the Environmental Health Center, and from 1954 until he retired in 1966 as Chief, Milk Sanitation Research, Robert A. Taft Sanitary Engineering Center at Cincinnati, Ohio.

Dr. Black was the *author or coauthor of over 100 publications* in bacteriology, laboratory methodology, milk, and food sanitation. In 1960 he received the Citation Award of the International Association of Milk and Food Sanitarians for outstanding service to the Association in providing leadership in the development and unification of milk and food sanitation laboratory methods and standards, and in 1967 he received an Honorary Life Membership. He received the Superior Service Award of the Department of Health, Education and Welfare "For meritorious contributions to public health through introduction of new laboratory methods and development of a nationwide program of milk laboratory certification." Dr. Black served the American Public Health Association as a member of several committees and as Chairman of the Sub-committee on Standard Methods for the Examination of Dairy Products.

After retirement, Dr. Black pursued a wide variety of activities including lapidary and family geneology.

LETTER TO THE EDITOR

A request for information

DEAR SIR:

At the present time I am exploring a body of language known only to persons who have had long experience in various trades, crafts, or occupations.

As my particular field is that of Occupational Medicine—the specialty in medicine which relates the effects of work to health—I am interested in learning from any readers terms which have been given to specific diseases or injuries characteristic of their trade or occupation; such as, for example, brassfounders' ague, grocer's itch, milker's nodules, copperman's chest, or packer's itch. If there is knowledge of the condition which has been given the designation, some descriptive information would prove additionally helpful.

If any reader can recall names or terms of this type, I would appreciate hearing from him at the address shown below. For your assistance in this effort, my many thanks.

J. S. FELTON, M.D.
275 *Bellino Drive*
Pacific Palisades, California 90272

ANNOUNCEMENT

$1,000.00 Dairy Education and Industry Sanitarians Award sponsored by the Milking Machine Manufacturers Council will be awarded for the first time in 1973 at the Annual Meeting in August. Deadline for nominations is June 1, 1973. Please contact: Dick B. Whitehead, Chairman of the Recognition and Awards Committee, Division of Occupational Health, Mississippi State Board of Health, Box 1700, Jackson, Mississippi 39205, for details.

SEP-KO CHEMICAL MANAGER ELECTED VICE PRESIDENT OF H B FULLER

Everett H. Wostrel, general manager of Sep-KO Chemical Division, a subsidiary of H B Fuller Company, has been elected a vice president of H B Fuller.

Wostrel has been associated with Sep-KO, a Minneapolis-based manufacturer of chemical products for the dairy industry, for 21 years. The Sep-KO organization was acquired by Fuller in 1971.

H B Fuller Company is a leading manufacturer of adhesives, specialty chemicals and related equipment for broad range of industries and markets. Fuller has plants and technical service centers in 23 U. S. cities and facilities in a number of foreign countries.

UNIVERSITY VETERINARIAN RETIRES AFTER 20 YEARS

Dr. C. Wayne Burch, University of Wisconsin Extension veterinarian, will retire December 31, after more than 20 years of service.

Burch has been Extension veterinarian and professor of veterinary science at the UW-Madison since 1952. Earlier this year he was named "Extension Veterinarian of the Year" by the American Association of Extension Veterinarians.

For the past 10 years his chief interest has been in

the area of prevention, control and eradication of bovine mastitis. He has taught numerous dairy herd health short courses for dairymen, veterinary practitioners, vacational agriculture teachers and county agents in all the dairy counties of Wisconsin. His absence from the University will be deeply felt.

PROCEEDINGS OF ENVIRONMENTAL CONTAMINANTS IN FOODS SYMPOSIUM AVAILABLE

The Proceedings, "Environmental Contaminants in Foods," of the Sixth Annual Symposium, held November 18, 1971, sponsored by the Western New York State Institute of Food Technologists and Cornell University's New York State Agricultural Experiment Station, is available for distribution. Copies may be obtained by contacting D. L. Downing, Department of Food Science and Technology, New York State Agricultural Experiment Station, Geneva, New York 14456 and requesting Special Report No. 9.

TEXAS A&M SCHEDULES DAIRY INDUSTRIES CONFERENCE

The Sixth Annual Dairy Industries Conference, sponsored by the Department of Animal Science, Texas A&M University has been scheduled for April 11 and 12, 1973. All activities will be held at the Holiday Inn, Bryan, Texas. Further information may be obtained from Dr. H. E. Randolph, Dairy Section, Department of Animal Science, Texas A&M University, College Station, Texas 77843.

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POSITION WANTED

B. SC., DIP. Dairy Technology, 15 years quality control and sanitary laboratory experience both in private and government laboratories of Dairy/Food products, seeks immediate permanent employment in government or reputed private laboratory. Starting salary $10,000.00. For details, write: Chaudhuri, 155 Myrtle Dr., Augusta, Georgia 30904.

VI

Improving milk quality for increased profits

Dr. John H. Nicolai, Extension Dairy Specialist, University of Kentucky, Lexington, Kentucky

To prevent the possibility of being degraded because of high somatic cell counts, consider taking these steps:

Check your milking machine before the start of milking. Cover these points:

1. See that all rubber parts are clean and uncracked. Discard one-piece, molded rubber inflations after 1000 individual cow milkings. Discard multi-piece, ring-type inflations after 500 individual cow milkings.
2. Narrow bore inflations with an internal diameter measuring ¾" or less are recommended over liners with a wider bore. The interior neck of the liner (short milk tube) should be at least ⅜" in diameter.
3. The slope of the milk pipeline should be at least 1½" in 10 feet, without risers. Use a yardstick, a level and a 6-inch ruler to calculate this slope. In three feet the slope should be about 1½".

Have your serviceman do the following:

1. Measure the air flow at the vacuum pump and at the most distant end of the system with an air flow meter. Do not measure through stall cocks. Remove an elbow or end cap so the full capacity of the line can be determined. You should experience less than a 10 percent loss of air flow between the pump to the most distant end of the line. If the loss is more than 10 percent, the problem may be:
   a. *The line is plugged.*
   b. *The line is too small.*
   c. *Too many bends and elbows.*
   d. *Leaks in the system.*
   e. *The vacuum pump is too small.*
2. There should be 12 cubic feet per minute (CFM) of air flow per milking unit using the New Zealand Method, or 6 CFM using the American standard. This measurement should be made on the milk pipeline or pulsator line at the most distant end with no units operating and all vacuum controls closed. Remember, this is a minimum recommendation. Most systems have other components that require air flow and will need more total air flow as a result.
3. The vacuum level inside a rubber inflation should be maintained at 12½" of mercury when cows are being milked and all units running.
4. Check each pulsator with a vacuum recorder to determine milk-to-rest ratio (liner open—liner collapsed). A 50:50 ratio is adequate.

5. The rubber liner should open or close 50 to 60 times a minute.

During milking, check the following:

1. Record the milk-to-rest ratio of each pulsator. It should not vary from the measurement taken when the system was not under load. If it has a wider ratio under load, any of the following may help:
   a. *Shorten the milk hose.*
   b. *Lower the milk line.*
   c. *Increase the size of the claw.*
   d. *Install weigh jars.*
2. Record the vacuum fluctuation inside the inflation at the teat end of each unit of a fast milking, high producing cow for 30 seconds. Then, using the same procedure, record the vacuum fluctuation on the unit closest to the bulk tank when all units are being operated on the high producing fast milkers. This fluctuation should not exceed 2" of mercury and the vacuum at the teat end should not drop below 10½" of mercury.
3. See that all filtering mechanisms are outside the milking system.
4. If your system meets these recommendations, your pipe lines will be large enough.

Have your veterinarian do the following:

1. Observe your milking procedures such as washing udders, drying udders, using the strip cup, attaching machines, removing the units, and teat dipping.
2. Review your treatment programs for dry cow therapy and treatment of clinical mastitis during lactation.

After you have corrected management and milking system problems to your satisfaction, ask your veterinarian to return to the farm and do the following:

1. Composite sample each cow, dry and milking, and test for *Streptococcus agalactiae*. Treat each cow having *Strep. ag.* with penicillin and recheck the entire herd 21 days later by resampling. Re-treat those cows that are still infected. Any cow not responding to the second or third treatment should be culled.
2. Run monthly CMT on every lactating cow.
3. Keep herd health cards on every cow.
4. Treat clinical cases of mastitis promptly. Follow advice of your veterinarian.

In summary, every dairyman should follow these suggestions to improve milk quality, increase profits, and lower somatic cell counts:

1. Have your milking machines checked by a reputable serviceman at least twice a year.
2. Use approved milking practices at every milking.
3. Check for *Strep. ag.* twice a year.
4. Use dry cow therapy on every cow in every quarter at drying off.
5. Use a teat dip at every milking.
6. Keep an individual herd health record card on each cow.