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Title: EVALUATION OF METHODS TO ENUMERATE PSYCHROTROPHIC BACTERIA

   IN FLUID MILK.

Abstract approved: _____
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Three rapid methods for the estimation of psychrotrophic bacterial
counts in fluid pasteurized milk were studied in this investigation.
Methods included the Limulus amoebocyte lysate test (LAL), the B-D-
glucose absorption test, and the carbon-14 labeled 2-deoxyglucose
affixative test.

The LAL test involved the addition of a lysate prepared from the
amoebocytes of horseshoe crabs (Limulus polyphemus) to concentrated
fluid milk samples. The Limulus lysate is highly specific and sensitive
for endotoxins produced by Gram-negative bacteria. The LAL method
attempted to predict the number of psychrotrophic bacteria in concen-
trated milk samples by comparing the extent of gelation to the bacterial
cell numbers. Subsequent gel and modified spectrophotometric test
methods were compared to conventional procedures used for the evaluation
of bacterial quality of fluid milk.

The B-D-glucose absorption test evaluated the ability of pure cul-
tures of representative milk spoilage organisms (Pseudomonas putrefaciens,
P. fluorescens, and Escherichia coli) to absorb B-D-glucose. Specific
concentrations of B-D-glucose (25 mg/100 ml to 300 mg/100 ml range) were
added to serial dilutions of bacteria. The subsequent absorption of

B-D-glucose was determined by an enzymatic colorimetric procedure. The corresponding optical density measurements were compared to control concentrations of B-D-glucose equivalent to the amounts added initially. The differences (range of 25-300 mg/ml) were compared to a standard curve for B-D-glucose concentration versus optical density. The concentrations of B-D-glucose were extrapolated from the plotted comparable optical densities. Absorbed concentrations of B-D-glucose were compared with the corresponding bacterial populations for individual serial dilutions.

The carbon-14 labeled 2-deoxyglucose affixative test involved the application of a radioactively labeled analog of glucose to pure cultures of P. putrefaciens, P. fluorescens, and E. coli. The analog was absorbed by bacterial populations of the test organisms and radioactivity (disintegrations per minute, DPM) was counted by liquid scintillation. The purpose was to correlate absorbed radioactivity (2-deoxyglucose absorption) to bacterial numbers. This method was also applied to fluid milk samples of varied age and bacterial quality, in an attempt to estimate psychrotrophic bacterial numbers.

EVALUATION OF METHODS TO ENUMERATE PSYCHROTROPHIC
BACTERIA IN FLUID MILK

by

MAX GREGORY CLARK

A THESIS

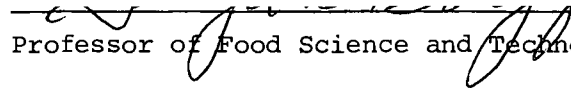submitted to

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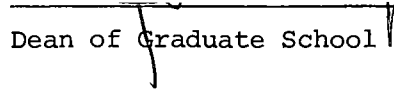Completed June 6, 1980

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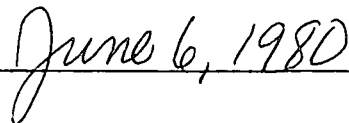Professor of Food Science and Technology

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To my mother and father, for
their stallworth support and
steadfast love.

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INTRODUCTION

The problem of maintaining good shelf life or achieving extended keeping quality has always been one of great concern to milk processors. In addition to significant product losses due to spoilage, customer dissatisfaction may occur in consumers who quickly lose confidence in a specific brand of milk that spoils or becomes unpalatable.

Research in microbiology and chemistry has resulted in the development of many new tools for more efficient cleaning and sanitizing of dairy equipment. This has resulted in an improvement of quality within the past two decades. On the other hand, changes in merchandizing methods have created a need for milk with extended shelf life compared to what was considered necessary only a few years ago. The practices of every-other-day processing and less frequent delivery, the trend towards larger consumer packages and greater volume of sales through stores and supermarkets, long distance hauling and other innovations have all served to extend the time period between processing and consumption. Futhermore, the conditions under which the milk is handled and stored during transfer from processor to consumer are often favorable for deterioration.

Keeping quality or shelf life of perishable dairy products, including fluid milk, also depends on the initial quality of the products, on care and techniques employed in processing and distribution, and on storage temperatures (21).

Temperature control is generally considered the key to extended shelf life for dairy products. A common rule-of-thumb followed by the

dairy industry is "for each 5 F (2.8 C) increase in temperature, the expected shelf life can be reduced by one-half." (21) Stated another way, a storage temperature of 35 F (1.7 C) should provide 20 days shelf life, 40 F (4.4 C) should provide 10 days, but 45 F (7.2 C) would provide only 5 days of shelf life (21).

Most psychrotrophic or spoilage type bacteria are capable of comparatively rapid growth at temperatures above 50 F (10 C), moderate growth at 46-50 F (7.7 to 10 C), slow growth at 41-45 F (5 to 7.2 C), and slower growth at temperatures below 40 F (4.4 C) (21).

Many tests have been applied to pasteurized milk to determine its sanitary quality. Among these are Standard Plate Counts (SPC), coliform counts, psychrophilic (or psychrotrophic) counts, tests using chemicals to measure microbial activity (reazurin and methylene blue reduction tests), and shelf life tests conducted at various temperatures, (Moseley, etc.). All of these methods have some merit but none can be considered the ideal test. There is need for a test which will quickly and accurately measure the degree of post-pasteurization contamination and predict the shelf life. The Standard Plate Count and coliform count are used extensively to determine compliance with local health requirements but they are of limited value in measuring shelf life.

In studies evaluating the relationship between microbial counts and keeping quality, Hankin et al. (77) found that microbial counts made on the day of bottling (initial counts) of fluid milk were generally low and poorly correlated with the length of time for the sample to become unacceptable at any storage temperature. The lower the initial count, the longer it took for the sample to become un-

acceptable. But initial bacterial counts have little apparent value by themselves for predicting keeping quality of milk at any storage temperature, since shelf life, as suggested by Patel and Blankenagel (149), is affected primarily by the type of organism and not by the total number present.

Hankin and others (18,76,79,191) reported that total bacterial counts bear little relation to keeping quality of milk. Hankin et al. also noted (77) that when samples were stratified for type of flavor defect, there was no relationship of flavor to microbial count, but there was statistical significance between specific microbial groups and keeping quality. The above study and other investigations (149, 192) prompts one to question the actual value of determining keeping quality based solely on bacterial tests. Microbial tests may not correlate with sensory analyses.

Thus, more discriminative tests for predicting keeping quality need to be developed; such tests should be rapid and reliable, and non-complex to facilitate routine laboratory analysis.

The three methods investigated in this study attempted to evaluate rapid approaches to measurement of relatively low numbers of psychrotrophic bacteria in fluid milk products.

The ability to accurately detect and enumerate milk spoilage bacteria in recently-processed milk could provide important information on the sanitary and temperature control conditions of the processing operation and possibly enable the processor to significantly extend product shelf life.

REVIEW OF LITERATURE

At the beginning of the post World War II era, the introduction of alternate day delivery of pasteurized fluid milk in some areas of the USA proved economically worthwhile. However, success depended on distribution and consumer handling of the product. Dahlberg (46) and Weese (193) in similar studies concluded that the keeping quality was good enough at all seasons of the year to permit every other day delivery without impairment of quality. Burgwald and Josephson (33) found that milk of good quality could be expected to retain excellent bacterial and flavor qualities for at least four days in summer and six to seven days in winter, if refrigeration temperatures were maintained near 4.4 C.

Alternate day delivery of market milk to retail food stores for sale contributes to an increase in the typical age of milk at the time of consumption. Prolonged storage of milk at low temperature permits significant increases in the population of psychrotrophic bacteria. Psychrotrophic organisms have been associated with the deterioration of flavor in both raw and pasteurized milk (24,42,83).

Definition of psychrotrophs

The term "psychrophilic" has been used for an extended period of time to describe organisms that grow at low temperatures. In 1902, Schmidt-Nelson (159) defined psychrophiles as bacteria which are

not only able to survive at refrigerator temperatures but also have the ability to multiply at 0 C. This definition was immediately challenged by Mueller (133) on the grounds that many such bacteria cannot correctly be termed "psychrophilic" since they have optimum temperatures between 20 and 30 C. Olson et al. (143) described psychrophiles as those bacterial species which are capable of relatively rapid growth at low temperatures, generally within the range of 35 F (1.7 C) to 45 F (7.2 C). Ingraham and Stokes (91) described them as organisms that "grow best" below a certain temperature, usually 20 C or 12 C to 18 C and sometimes as low as 5 C to 10 C. There are only a few reports of organisms with temperature optima below 20 C, and three are represented in Bergey's Manual of Determinative Bacteriology (31). The authors suggested that psychrophiles should not be defined by maximum or optimum temperature, but as bacteria that grow relatively rapidly at 0 C.

Later Ingraham and Stokes (91) considered that the term "psychrotrophic," (to increase or thrive at cold temperature) suggested by Mossel, and Eddy (53) should be substituted for the term "psychrophilic," because it more adequately described the group of bacteria able to grow at these low temperatures. Witter (196) defined psychrotrophs as bacteria that grow at a relatively rapid rate at refrigeration temperatures (7.2 C or less).

In a review, Morita (131) described psychrophilic bacteria as organisms having an optimal temperature for growth at 15 C or lower,

a maximal temperature at 20 C, and a minimal temperature for growth
at 0 C or below. Morita proceeded further to describe psychrotrophic
bacteria as organisms that do not meet the definition as stated above.
He hoped to stimulate others to use the terms psychrophile and psychro-
troph as defined in his review. Although there may never be a complete
agreement on these terms, a start must be made to end the confusion
about the lower end of the thermal spectrum of microbial life.

In the October 1976 meeting of the International Dairy
Federation, psychrotrophs were defined as microorganisms that grow
at 7 C or less, irrespective of optimal temperature (40).

In application to the dairy industry and for technological
reasons, psychrotrophs are considered to be those microorganisms which
can multiply relatively rapidly in milk and milk products at re-
frigeration temperatures of 3 C to 7 C. It has been generally accepted
that most psychrotrophic organisms are destroyed by pasteurization
(149). Flavor defects of microbial origin in pasteurized and properly
stored milk, therefore, are usually considered to be the result of post-
pasteurization contamination (149). Johns (95) has suggested that
certain compounds produced by microorganisms in raw milk might be
responsible for off-flavor development in pasteurized milk.

RAW MILK

In order to appreciate the effects of psychrotrophs on pasteurized
milk it is necessary to first look at the raw product. There have
been many studies on the representative microflora of raw milk, Rogick

and Burgwald (157) found that mesophilic bacteria outnumbered psychrotrophic bacteria in a 3:1 ratio in initial counts of raw milk samples. However, as storage progressed at 3 C to 7 C refrigeration temperatures, invariably the psychrotrophic bacterial count became higher after one week of storage. Characterization studies indicated that they were not thermoduric and probably were facultative, rather than true psychrotrophs. They were primarily cocci or nonspore-forming bacilli, inert or acid-forming bacterial types (157).

Sherman et al. (161) and Thomas and Chandra (177), reported that psychrotrophic bacteria able to grow in raw milk (held at refrigeration temperatures), were primarily Gram-negative, nonspore-forming rods.

Psychrotrophic microflora of refrigerated raw milk

Thomas and Druce (180) summarized studies of psychrotrophic microflora of refrigerated raw milk. Milk which has been stored at low temperature (0 C to 5 C) for two to three days differed both bacteriologically and physiochemically from milk held at a higher temperature, or at a lower temperature for a short time (171).

Thomas and Druce (180) further summarized the different types of microorganisms associated with raw milk spoilage. They indicated that the predominant psychrotrophic organisms include Acineto-bacter (Achromobacter-Alcaligenes), Flavobacterium, Coli-aerogenes, and Enterobacteria with the latter two groups appearing less frequently. Other bacterial genera that function as psychrotrophs

are <u>Staphylococcus</u>, <u>Leuconostoc</u>, <u>Micrococcus</u>, <u>Streptococcus</u>, and some

from the Diptheroid group (20,63).

This latter group of five genera is relatively unimportant in

considering effects on market milk and dairy products. The total

bacterial flora composed of psychrotrophs gradually increases in milk

held at 4 C to 7 C for 24-72 hours (180). This increase can be more

marked in farm bulk tank milk held at higher temperatures, compared to

milk in five or ten gallon cans. Thus Moller-Madsen (130) found that

psychrotrophs constituted 27% of the total bacterial content in fresh

raw milk, compared with 59% in 24 hours and 95% in 48 hours in the same

milk when held at 13 C; while Thomas et al. (181) reported that pre-

liminary incubation (PI) of farm tank milk samples at 18.5 C for 18

hours resulted in a marked proportional increase in Gram-negative rods

(36% initially, 64% after PI).

<u>Influence of poor quality raw milk on bacteriological numbers after</u>

<u>pasteurization</u>

Several investigators have shown that when production conditions

are optimal, the number of psychrotrophic bacteria in farm milk

supplies, irrespective of whether collection is daily or on alternate

days, in cans or farm bulk tanks, remains below a few thousand/ml

(97,120,175). Raw milk produced under hygienic conditions has a

satisfactory keeping quality, yet addition of 1% of an unsatisfactory

raw supply to the bulk before commercial pasteurization causes

deterioration in the keeping quality of the milk as illustrated by

Anderson and Meanwell (6).

Storgards (169) did not find any direct connection between the total colony count of raw milk samples and the colony count, methylene blue reduction, and the keeping quality of the pasteurized milk. Glenn (64) obtained results which indicated that the numbers and kinds of bacteria in raw milk may affect the initial flavor score of processed milk. Overcast and Adams (146) investigated the effect of excessive psychrotrophic bacteria growth in raw milk with the reintroduction of specific psychrotrophs into the milk after pasteurization. Results showed a slight stimulatory effect on the growth of Pseudomonas fluorescens in the pasteurized milk, but the effect was inhibitory on the growth of Pseudomonas fragi.

Franklin (59) found that cold storage at 5 C for up to four days before processing resulted in varying changes in the keeping quality after processing, which depended more on the types of bacteria than the numbers present in milk. This concurred with the earlier study by Glenn (64), thus demonstrating the relative importance of the kinds versus the number of bacteria present.

Adaptation of microorganisms to low temperatures

In observations by Chistyahov and Naskova (39), of the original number of psychrotrophic organisms killed, 80-90% gradually recovered and began multiplying, provided the temperature wasn't below -8 C. The cold adapted strains possessed stationary phases 14-36 times shorter at the same temperature. However, generation time in the log phase of growth proved slightly less for adapted strains than for

unadapted ones. Adapted strains less prone to dying on exposure to cold, developed better at -2 C than at 20 C. The reverse held true for unadapted strains as well (39).

Brown (29) compared psychrotrophs with mesophilic Pseudomonas regarding the effect of temperature on metabolism. Mesophilic cells grew at lower temperatures and behaved more like psychrotrophs. Brown doubted that the ability of psychrotrophs to grow at lower temperatures was related to differences in the nature of glucose oxidation. He found that cells of the psychrotrophic strain of Pseudomonas, grown at the same temperature as the mesophilic strain, continued to exhibit a lower temperature coefficient of glucose oxidation. Ingraham and Bailey (90) in a similar comparative study of psychrotrophs and mesophiles found temperature coefficient differences paralleling those found in Brown's study. These two studies indicated that the temperature response differences between psychrotrophs and mesophiles for growth and catabolism were probably due to some aspect of cellular organization rather than to enzymatic differences.

The genetic constitution of the cell determines its cardinal temperatures for growth. Although this premise is generally accepted, the data in the literature are completely lacking in order to postulate the exact genetic basis of psychrophily. Most of the research emphasis has been placed on physiological studies. Nevertheless, there is genetic evidence for the conversion of a mesophile to a psychrotroph by transduction (140), ultraviolet light (9), and

the mutation by ultraviolet light from a psychrotroph to a mesophile
(172). Since phage have been isolated from psychrotrophic bacteria
(49,50,108), transduction effect between cold-loving organisms is
possible (131). Unfortunately none of the host cells for the phage
are psychrophiles (131). It appears unlikely that either mutagenesis
by ultraviolet irradiation or transduction would change the total
enzymic make-up of cells sufficiently to promote psychrophily, since
there appears to be more than one enzyme in a psychrophilic cell that
is abnormally thermolabile (131).

One hypothesis of microbial evolution is that thermophiles were
the first to evolve (27), followed by the mesophiles and then the
psychrophiles. The evolution of psychrophilic bacteria is probably due
to many genetic events since a psychrophile has more than one enzyme
that is abnormally thermolabile and has differences in the membrane.
According to Allen (4) organisms that have lived in a cold environment
for generations may have experienced mutation that caused some of their
critical proteins or protein complexes to become relatively heat labile.
There are probably many pathways leading to psychrophily, since it
has been found that a specific abnormally thermolabile enzyme in one
psychrophile may not be abnormally thermolabile in another psychrophile
(131).

In the eighth edition of Bergey's Manual (31) V. marinus MP-1
is classified as V. fisherii. Biochemical characteristics used in
taxonomy do not characterize the thermal properties of enzymes or
membranes (131). There is at present no data in the literature that
indicate how the code (probably sequence of codons) for a "mesophilic"

enzyme differs from the code for a "psychrophilic" enzyme. The thermal properties of psychrophiles should be taken into consideration in bacterial classification, if we accept the hypothesis that the psychrophiles evolved from mesophiles (131).

Krajewska and Szer (107) conducted comparative studies of amino acid incorporation in a cell-free system of a psychrotrophic *Pseudomonas* and *Escherichia coli* and found that ribosomes and supernatant fractions of the psychrotrophic *Pseudomonas* and *E. coli* were interchangeable at 30 to 45 C during poly U-promoted phenylalamine incorporation. At 2 C to 9 C only the psychrotrophic *Pseudomonas* ribosomes were active with both supernatant fractions, and therefore the ability to perform protein synthesis at low temperature was related to ribosomal specificity. These authors also point out that the psychrotrophic system operates at a relatively high fidelity compared to the mesophilic and thermophilic subcellular systems. Does this indicate that through evolution the psychrophiles are more stable in terms of their genetic fidelity compared to the mesophiles and thermophiles (131)?

Numerous reasons have been put forth to explain why microorganisms expire when exposed to temperatures above their maximal growth temperature, but most of the research has been done with mesophiles. These explanations include the accelerated use of the intracellular amino acid pool (74), enzyme inactivation by elevated temperature (54,111), changes in extent of cellular lipid saturation (103), disruption of the intracellular organization (89), inactivation of an enzyme-forming system (133,182), the loss of permeability control (132), and thermally

induced leakage of ribonucleotides, ammonia, ninhydrin-positive

material, potassium, and phosphate (167).

Temperature-time relationships in growth and storage

Optimum growth temperature - The optimum growth temperature in milk for

most psychrotrophs is 30 C, for some 30-40 C, and for a few about 15

C or below (180). Lawton and Nelson (113) described the optimum growth

as 21-32 C for most of the isolates they investigated. Of the

Pseudomonas and Flavobacterium spp. studied, eight out of nine isolates

were facultative psychrotrophs rather than obligate. This indicated

the ability to grow at higher temperatures, thus contributing to a

total count grown at 32 C.

Optimum growth temperature is usually defined as the temperature at

which the rate of growth is most rapid, and has usually been determined

approximately by visual assessment of the rate of appearance and in-

crease of turbidity in liquid cultures, or the rate of appearance of

colonies or masses of growth on solid media (180). Thomas and Druce

(179) reported that the optimum growth temperature of growth in glucose

phosphate-peptone water for 235 psychrotrophs isolated at 3 C to 5 C

from refrigerated milk was about 22 C for 27%, 22-30 C for 8%, and

approximately 30 C for 61% of the strains, only 4% grew best at

37 C.

However the optimum growth temperature can be established more

accurately by determining the generation time during the exponential

growth phase of the culture; the temperature at which the generation

time is shortest being considered the optimum growth temperature
(180). Many investigations have been made since 1903 and a critical
review has been made by Ingraham and Stokes (91). Recently Morita
(131) compiled information on generation times for specific psychro-
trophic bacteria in his review of psychrophilic bacteria.

Storage temperature and time - Holding milk at 7 C as compared with
4.4 C, results in a much more rapid increase of psychrotrophs (180).
Thomas and Druce (180) in their review article summarized the effects
of refrigeration on the psychrotrophic bacteria count of raw milk.

The survival of psychrotrophs in raw milk appears to depend on
the initial number of organisms (117), along with the (162) species of
the contaminating microflora and the temperature of refrigeration
(180). Thomas and Druce (178) and Marth (119) found that with heavy
initial contamination and storage at 7 C or above, a relatively rapid
build-up of psychrotrophs and the production of off-flavors may
occur.

Biochemical changes occurring in raw milk

Due to the predominance and subsequent growth of psychrotrophs
in raw milk, many off-flavors and odors can occur such as: fruity,
unclean, rancid, putrid, discoloration, cheesy, soapy, bitter, and
winey (196). Lipolysis and proteolysis are considered some of
the most important activities of deteriorating psychrotrophs
(199,106). Sandvik and Fossum (158) studied accumulation of
proteinases in 4 C stored farm bulk tank milk. The predominant

Pseudomonas produced some proteinases at this temperature, but greater

amounts at 10 C and 20 C.

Flavobacterium, Aerobacter, and Pseudomonas species showed in-

creased proteolytic activity of >50% in a study by Kiuru et al. (106)

of 300 psychrotrophic bacterial isolates. Flavor defects appeared

when the bacterial count was approximately 10^8 ml, or higher.

Significant changes in casein composition were found when these flavor

defects first appeared.

Ohri and Slatter (137) found that after storage of bulk tank

milk for an additional 48 hours at 4 C, the incidence of rancid,

oxidized, and "unclean" flavors attributable to psychrotrophic

activity, increased considerably. Storgards (170) found that if

rapid cooling to below 4 C is not initiated, lipolytic activity could

go unchecked, rendering milk less suitable for beverage purposes.

McCaskey (124) studied microbial and enzymatic activity in raw milk

held at low temperatures (4.4 C and 1.1 C), with reference to

Pseudomonas fluorescens and Achromobacter lipolyticum. At 4.4 C,

the bacterial content increased sharply after three to five days.

Bacterial testing of refrigerated raw milk intended for processing

The bacterial flora of raw milk stored at 3 C to 5 C for two to

three days is usually dominated by psychrotrophs and the results of

numerous studies indicate that these bacteria exhibit very slow dye

reduction at 37 C in milk (7,8,38,85). The use of methylene blue,

resazurin and nitrate reduction tests are quite unsuitable for routine

grading of farm bulk tank milk, as reduction time may be unduly extended because the bacterial population is in a dormant state following refrigeration (7,42,94,171).

More recently, preliminary incubation of farm bulk tank milk was completed before bacteriological examination by means of dye reduction tests and colony counts (SPC). Druce and Thomas (52) presented details and results of 35 of these investigations in eight countries. PI seems to furnish more information regarding the care taken in production handling, and storage temperature than can be obtained from analysis of freshly taken samples of milk, regardless of which of several current testing methods is employed (94).

Swartling (171) demonstrated the relationship between total bacterial content and methylene blue reduction applied directly at 37 C and after PI for 18 hours at different temperatures. He found correlation coefficients (r) of -.42, (at 37 C), and -.60, -.63, and -.64 for PIs at temperatures of 12, 15, and 17 C, respectively. Other tests in evaluating raw milk include the microscopic colony count (MCC) used by Juffs (102), to rapidly enumerate psychrotrophic bacteria. This test requires 48 to 72 hours at 7 C or 16.5 hours at 21 C (102).

PASTEURIZED MILK

Pasteurization effect

There is ample evidence to show that psychrotrophic bacteria do not survive laboratory or commercial pasteurization. Witter (196) lists fifteen references to support this contention, and the results

of nine additional investigations are quoted which demonstrated that the prominent psychrotrophic pseudomonads are quite sensitive to heat. Thomas and Druce (180) reviewed and tabulated the results of investigations dealing with the effect of laboratory pasteurization of farm milk supplies. The results of laboratory pasteurization of psychrotrophic organisms are influenced by many factors, such as the age and number of the bacterial cells, pH of the milk, and growth temperatures before heating. Thomas and Druce (180) reviewed and tabulated the results of investigations dealing with the laboratory pasteurization of specific cultures of psychrotrophs: Pseudomonas, Alcaligenes, Achromobacter, and Klebsiella. None of the cultures isolated at 7 C or below survived, whereas some of the cultures isolated at 8 C to 10 C were partially heat resistant.

Heat resistant enzymes

Since optimum temperatures for organisms to produce enzymes such as lipases, proteases, and carbohydrases are usually lower than the optimum temperature for cell production, there is opportunity for milk held for several days at refrigeration temperature to develop off-flavors induced by microbial enzymes, even though the bacterial population remains below that normally associated with microbial caused defects (180), (ie. rancidity, oxidation, and the like). Many investigations into thermostable lipases, proteases, and to a lesser extent carbohydrases have been conducted (180).

Table 1. Investigations of heat stable enzymes and the organisms
         associated with them in pasteurized milk.

Investigators	Organisms	Heat stable enzymes
Sohngen (163)	Ps. fluorescens	Lipase
Virtanen (187)	Ps. fluorescens Ps. punctatum	Protease
Nashif & Nelson (134)	Ps. fragi	Lipase
Stadhouders, Mulder & DeVries (164)	Pseudomonas cultures	Lipase
Stadhouders & Mulder (166)	Pseudomonas Achromobacter Serratia Flavobacterium Alcaligenes	Lipase Lipase not heat resistant
Pinheiro, Liska, & Parmelee (152)	Ps. fragi Ps. mucidolens Ps. fluorescens	Lipase more heat resistant than Ps. fluorescens
McCaskey (124)	Pseudomonas cultures	Proteinases resist pasteurization temperatures
Sandvik (158)	Pseudomonas cultures	

Heat resistant psychrotrophs

Alcaligenes tolerans was first described and named by Abd-El

Malek and Gibson (1), who found that it was the only Gram-negative rod

occurring in milk after laboratory pasteurization at 63 C for 30

minutes. Gyllenberg et al. (70) confirmed the results of a later study

by Gibson and Abd-El Malek (63) stating that Alcaligenes tolerans was

in no instance detected in farm milk supplies immediately after

pasteurization (63 C for 30 minutes) on plates incubated at 30 C

for five to six days. However, signs of proliferation were detected

in some samples within 24 hours at 10 C to 22 C. Since there hadn't

been any definite evidence that Alcaligenes tolerans could multiply

at 5 C, it was doubtful that this strain could be considered a psychro-

troph (70). Gyllenberg and Eklund (69) also questioned whether this

organism was a true psychrotroph as it failed to grow at 2 C and at 5

C the growth was very slow.

The incidence of thermoduric spore-forming psychrotrophs has

recently come into prominence. As early as 1957 Bacillus cereus

and Bacillus mycoides were found to produce rapid deterioration of

milk (63). Psychrotrophic strains of Bacillus were isolated from soil,

mud, and water, were enriched at 0 C, grew optimally at 20 C to 28 C,

but failed to grow at 30 or 35 C, according to Larkin and Stokes (112).

Both spore formation and cell germination occurred at 0 C. Bacillus

coagulans grew out in 13-17 days at 2 C with a generation time of 24

to 30 hours, but failed to grow at 32 C. The same organism produced

typical psychrotrophic spoilage in sterile milk stored at 2 C.

Experiments using Bacillus species (lentus, licheniformis, and cereus)

were conducted similar to that of B. coagulans. During this study by

Grosskopf and Harper (67) initially all of the psychrotrophic spore-

formers would survive pasteurization. Shehata and Collins (160) con-

sidered these sporeforming bacteria to be variant strains of mesophilic

bacilli adapted to low temperatures. They found species of Bacillus

capable of growing at 7 C or less to be present in 25 to 35% of raw

milk samples. Martin (121) reported that Bacillus species accounted

for about 95% of the total sporeforming bacteria in milk while Clostridium species comprised the remainder. In the United States approximately 43% of the Bacillus organisms in raw milk are B. licheniformis and 37% are B. cereus (121). Reports from other countries indicate the reverse with a predominance of Bacillus cereus (121). Spores of both these organisms survive low temperature pasteurization, some persisting through even the lower temperatures in the UHT range, as reported previously by Martin (121).

Credit et al. (45) found that 84% of the bacteria, isolated from commercial pasteurized milk held at 4 C to 5 C for 30 days, belonged to the genus Bacillus, confirming earlier reports (121, 112). The predominance of sporeforming psychrotrophic bacilli is probably attributable to: 1) the rather high level of raw milk commonly positive for psychrotrophic sporeformers, 2) trends toward increased pasteurization time and temperatures, thereby eliminating many sporeforming species, 3) conditions caused by extended refrigeration storage which select for a heat resistant psychrotrophic organism such as Bacillus. Langeveld (110) found that homogenization increases the rate of spoilage at 5 C of non-contaminated milk which has been pasteurized until it just gives a negative phosphatase reaction. This increased rate of spoilage is also the result of a smaller doubling time in Bacillus circulans (ie. increased spoilage in a shorter time period). In homogenized milk the generation time is 25 hours versus approximately 45 hours in unhomogenized milk. In contrast, the doubling times of two

psychrotrophic Pseudomonas strains were about 6.5 hours and

7 hours in homogenized and unhomogenized milk, respectively.

Spoilage by psychrotrophic B. circulans is a common occurrence in

much of the commercially pasteurized milk produced in the Netherlands,

when stored for two weeks or more at refrigerator temperatures (110).

In a study by Washam et al. (189), psychrotrophic bacteria were

isolated from 227 pasteurized milk samples which had a shelf life in

excess of 20 days at 7.2 C. Of the 700 cultures isolated, 135 were

resistant to heating at 72 C for 16 seconds and were able to re-

establish growth at 7.2 C. Mikolajcik and Simon (129) found no relation-

ship between Gram-negative counts of raw milk or initial mesophilic

spore counts of heated milk and bacterial numbers in heated stored milk.

Collins in a presentation at the annual meeting of the American

Dairy Science Association (1979), emphasized the control of heat

resistant psychrotrophic bacteria, especially Bacillus species in

milk and milk products and their impact on shelf life. He stated that

even if post pasteurization contamination was controlled absolutely and

all milk was stored at <1 C, from the cow to the consumer, the typical

shelf life of milk would still be limited to three to four weeks, due

to the eventual spoilage caused by psychrotrophic, heat resistant,

sporeforming bacteria (ie. Bacillus strains).

Psychrotrophic microflora

The relative frequencies of occurrence of the predominant taxonomic

groups of psychrotrophic bacteria in refrigerated commercially pasteur-

ized milk are summarized by Thomas and Druce (180). These investigators, who isolated their cultures at 4 C to 7 C agree that Pseudomonas, Achromobacter, Alcaligenes, Flavobacterium, and some Coli-Aerogenes group bacteria predominate and are in decreasing order of proportion. The composition of pasteurized milk is very similar to that recorded for raw milk samples (180). Dempster (51) demonstrated a greater frequency of Pseuodomonas and Achromobacter in his study. He also demonstrated a lesser frequency of Coli-Aerogenes organisms in pasteurized samples versus raw samples. Van Der Zant (186) found that certain species of Pseudomonas inhibit the growth of Achromobacter. This may explain the frequent dominance of Pseudomonas species in milk products refrigerated for extended storage times. The relative changes in the composition of the total microflora of milk (mainly pasteurized) during refrigeration at 7 C have been studied by Gyllenberg et al. (73).

During sampling, psychrotrophic bacteria (Pseudomonas fluorescens, Pseudomonas fragi, psychrotrophic Pseudomonas aeruginosa, Alcaligenes viscolactis, and Enterobacter) only constituted 6.1% compared with 57.2% after 48 hours at 7 C, while mesophilic and thermoduric bacteria (Alcaligenes tolerans, Corynebacteria, and Micrococci) decreased from 93.8% initially to 43.0% after refrigeration (73).

Source of post pasteurization contamination

The psychrotrophic organisms found in dairy products are indigenous to soil and water sources (66,104,168). Many investigators have demonstrated that the presence of psychrotrophs in commercially pasteurized milk is the result of organisms introduced at one or more points during the period after pasteurization or post pasteurization contamination (56,58,145,182).

Many of these investigations have shown that no psychrotrophs could be detected in milk samples drawn from the holding tank of HTST pasteurization units, whereas the bottled or cartoned milk invariably contained psychrotrophs.

Rogick and Burgewald (156) found no psychrotrophs in 4.1 ml of low temperature and HTST pasteurized milk samples drawn aseptically from equipment, but average psychrotrophic colony counts 5 x 10^4/ml were recorded for the bottled milk after a few days of cold storage. Psychrotrophs are widely distributed in dairy plants and dairy farm equipment, as demonstrated by an Overcast study (45). Wide variation in the number and type of psychrotrophs is found in market milk processed by different pasteurization times and temperatures (182). In the examination of in-line samples, the extent of recontamination from the equipment and containers has been demonstrated by Olson et al. (144) and Ford and Babel (58).

Overcast (145) and Thomas et al. (182) examined washed half pint milk bottles and found psychrotrophic counts of 10-16/bottle,

with maximum counts of 10^3/bottle. Dempster (51) examined rinses of washed milk bottles and found that 35% of the psychrotrophic microflora were Flavobacterium with Achromobacter forming 26%, Pseudomonas 17%, and yeast 13%; all cultures were isolated at 6 C. This suggested that the primary source of contamination may have been the final rinse step of the mechanical washer.

Overcast (145) observed that the environment of one dairy processing plant contained a mean of 14 psychrotrophic microorganisms/foot^3 of air. On a volumetric basis this would be equivalent to approximately one psychrotroph/1/2 gallon milk container and one psychrotroph in a quart container of milk. At 7.2 C, psychrotrophs could readily multiply to a cell count of 10^6/ml within eight days, thus demonstrating the significance of airborne contamination. This is the case in milk plants employing non-aseptic conditions for filling containers. Thomas and Druce (180) summarized the incidence of psychrotrophs in bottles of pasteurized milk.

Effect of psychrotrophs on refrigerated pasteurized milk

Thomas and Druce (180) reviewed studies of the bacterial numbers associated with the onset of off-flavors and other defects in refrigerated pasteurized milk conducted by Baumann and Reinbold (14) and Crawford and Mabbit (43). A comprehensive study of psychrotrophic population levels associated with the flavor or physical changes in pasteurized milk has been reported by Punch et al. (154). While

psychrotrophic counts are still relatively low, spoilage could be the result of unusually high enzyme production by bacteria at low temperatures. Whether heat resistant or not, the enzymes produced by these organisms are more numerous at these lower temperatures (127). As psychrotrophs multiply in refrigerated milk, subtle changes take place during the early growth period, with no obvious physical changes in the product. On tasting, the sampler may first perceive a "lack of freshness," followed by some degree of "stale" flavor. After this flavor deterioration phase, more distinctive off-flavors and odors may develop including rancid, bitter, putrid, cheesey, fruity, or fermented (145, 196).

Procedures for monitoring keeping quality

Both the growth rate and biochemical activity of bacteria are decreased by a reduction in temperature. Logically, the keeping quality of milk and dairy products is increased by a reduction in storage temperature; this logic is consistently supported by available literature (7,22,26,46). However, at lower temperature storage, psychrotrophs may increase markedly (10,32), overgrow the normal flora (3,156,196), and ultimately cause product spoilage (23,32,122). These observations have led to the general assumption that psychrotrophic contamination and keeping quality are closely related (22,28).

Generally, chemical test methods, which include protein stability, tyrosine and tryptophan production, pH and acidity have proven unsuccessful for use in reliably predicting the keeping quality of milk

and milk products. Nutting (136) reported reasonable success in correlating the lactate ion concentration of milk to the keeping quality of the raw product, but this could not be extended to pasteurized milk. Methylene blue (62,109) and resazurin reduction tests (62,109) as normally performed have not provided good indices for keeping quality. These tests are more closely correlated to the total bacterial population than to the psychrotrophic bacterial count.

Replacing the SPC in raw and pasteurized milk

A comprehensive review (81,83), indicates that the commonly used tests, such as Standard Plate Counts (5), have rarely been closely correlated with production sanitation. With the adoption of farm bulk tanks, industry-wide and scientific criticism of the relevance of SPC results continued (13). Several factors seemed to be responsible for this lack of correlation: 1) storage at lower temperatures as has been emphasized; 2) the larger dilution of contaminating bacteria from unclean milk contact surfaces, as the volume of milk per farm increased; 3) the limited growth which takes place, even on dirty equipment (183); 4) the incubation temperature of 32 C, which is too high for growth of many psychrotrophs, and some thermodurics (82). At 32 C, a significant percentage of organisms, representative of unsanitary conditions, may not be recovered. This may help explain why there are many instances of unsatisfactory conditions and low SPC results. A lower incubation temperature, even if it involves a longer period of time, should improve most "total" viable count procedures.

There is widespread concern by food microbiologists and dairy industry quality control personnel as to the adequacy of 32 C incubation. Most countries outside North America have followed the suggestion of the International Dairy Federation, which in 1958 recommended an incubation temperature of 30 C for 72 hours (92). In Britain the value of this combination of temperature and time for advisory purposes was recognized as early as 1945 (175), although routine dye reduction tests are still conducted at 37 C. Collaborative studies by a committee of the American Dairy Science Association, chaired by M. L. Speck, reported in favor of a lower incubation temperature in 1955 (11). Huntanen (87) found higher total bacterial counts at 27 C after 48 hours incubation than at 30 C for the same period, and still higher counts than those found at 33 C. Iowa investigators (82) on the other hand recommended 28 C for 48 hours. Crawley and Twomey (44) found 30 C more effective for plate, thermoduric, and coliform counts as well as for nitrate and methylene blue reduction tests.

Several investigators have reviewed the limitations of the Standard Plate Count as a laboratory tool for predicting milk product keeping quality (22,32,34), however, other researchers report that the psychrotrophic count is not effective either (20,25,142). This is probably due to low levels of psychrotrophs which are biochemically active, grow rapidly in the product, and finally result in reduced keeping quality, as compared to a relatively significant number of biochemically inert microorganisms. Olson et al. (142) emphasized that

the type of contaminating microorganism was more important than the numbers of bacteria.

In the development of various keeping quality tests, most investigators still recognize the importance of psychrotrophic bacteria. In their test for keeping quality Broitman et al. (28) measured triphenyltetrazolium chloride reduction in milk to which Nacconal NR ST was added to inhibit Gram-positive psychrotrophic bacteria. However, statistical analysis of their data, would have shown that within the 95% confidence limits for predicting keeping quality, it varied ± 2 days to ± 6 days, depending on the reduction time. Day and Doan (47) measured neotetrazolium reduction in evacuated tubes and concluded that a positive reduction test occurred an average of 3.75 days prior to spoilage of the sample. Statistical treatment of their data would have shown a 95% confidence limit for predicting the keeping quality of ± 3 days. Gyllenberg et al. (71) developed a keeping quality test based on the ability of ammonium lactate-crystal violet agar to detect _Pseudomonas_ in milk. While not actually predicting keeping quality, the Mosley test described by Johns (96) is valuable for quality control and gives an indication of psychrotrophic growth by comparing the increase in SPC at 32 C before and after storage of the sample at 7.2 C for five days. The recognized method for psychrotrophic cells is for incubation at 7 C for ten days on SPC plates (96). Johns (98,99) also indicated that psychrotrophs were largely responsible for the marked increase in counts observed in many samples subjected to preliminary incubation (PI) for 18 hours

at 12.8 C. Application of taxometrics to an experimental material

including 41 samples of market milk indicated the occurrence of only

two distinct groups of milk samples, viz. samples of good keeping

quality and samples of poor quality, respectively (72). The

Nacconol-TTC test of Broitman et al. (28) when carried out immediately

upon sampling, a comparison of colony counts from plain ammonium lactate

crystal violet agar, respectively, also performed immediately upon

sampling, and tests for the occurrence of pseudomonads and/or _Alcali-_

genes _viscolactis_ in the milk at the time of sampling were found to

be the most reliable tests in order to distinguish between both groups

of milk samples.

Olson (141) used 2 ppm crystal violet to inhibit the growth of

Gram-positive types of bacteria and 50 ppm of 2, 3, 5 triphenyl-

tetrazaolium chloride to make the colonies of Gram-negative types

distinctive, in studies to detect spoilage organisms in milk and cottage

cheese. The test was called the "crystal violet tetrazolium" test

but later shortened to "CVT". Line counts on milk with crystal violet

showed a general correlation with shelf life. High counts on

this medium were correlated with short shelf life of products stored

at 7.2 C. The "CVT", however showed little correlation with the

Mosley test in evaluating plant sanitation (162).

Since most psychrotrophs of practical importance to the dairy

industry are Gram-negative organisms (1,75), Freeman et al. (61)

tested 58 chemicals as selective inhibitors of Gram-positive organisms

in an attempt to enumerate predominant psychrotrophic bacteria within

72 hours. Olson's and Broitman's work prepared the way for Freeman's et al. studies of selective inhibitors of Gram-positive organisms. Freeman et al. (61) found five effective chemicals, including sodium desoxycholate, that aided in enumerating these predominant psychrotrophic bacteria. Blankenagel and Okell-Uma (19) following up the work of Freeman, developed a test for these organisms, Gram-negative rods, based upon their ability to tolerate 0.5% sodium desoxycholate (SDC). This desoxycholate test or DCT used a standard incubation temperature of 32 C, even though subsequent studies showed maximum counts at 25 C. While in most instances there was close agreement between the DCT count (1000/ml limit) and other tests; in further studies, test comparisons varied considerably. Blankenagel (16) reported that only a small percentage of the organisms revealed in the DCT test, were oxidase-positive.

Blankenagel and Humbert (17) developed a modified CVT test using disks for indicators. Their results were obtained in 48 hours. This test demonstrated three advantages: 1) nine ml of sample were used instead of one ml, 2) gelatin agar showed proteolytic off-flavors such as, unclean, bitter, fruity, putrid, etc. which are the result of microbial breakdown of milk proteins, 3) the test is easy to administer and requires no special equipment. Taylor (173) used a modified agar (water agar) that provided an equally simple and quantitative test with results in 18-20 hours, showing proteolytic activity of organisms and acid production by colonies.

More recently the cytochrome oxidase test was shown to be specific for oxidase-positive organisms. Hankin et al. (78) used this test to

determine types of organisms in raw milk. He found it an effective tool for the evaluation of cleaning and sanitizing procedures on the farm. A level of 2% oxidase-positive organisms, which either survive pasteurization or are post pasteurization contaminants, are considered "potentially"psychrotrophic in bulk milk supplies. In pooled plant samples of pasteurized milk, this test correlates positively with counts derived by the standard methods technique for determining psychrotrophic organisms (PBC). Applying the same oxidase test to plates prepared for the SPC, Hankin (78) measured these "potential" psychrotrophs in dairy products. This simple test afforded a rapid measure of "potential" psychrotrophic spoilage organisms, predominantly the oxidase-positive Pseudomonas.

Later testing includes work on analyzing the pyruvate content in milk. Von Tolle et al. (188) developed a continuous flow method of analysis of pyruvate in fluid milk products. Measurable pyruvate, while not being changed by pasteurization, was associated with bacterial counts of 10^5-10^6/ml or greater. They considered it possible to record a close picture of the hygienic quality of milk from production to consumption. The automated system developed could process 80-120 samples/hour. Marshall and Harmon (118) used the Von Tolle automated pyruvate method as a quality test for grade A milk. Simpler than conventional counts (SPC), keeping quality of raw or pasteurized milk may be characterized by two determinations prior to and after incubation. Using Pseudomonas fragi, Pseudomonas fluorescens, and Escherichia coli as test organisms, a pyruvate difference test (ΔP), distinguished

between concentrations of 10^2-10^3/ml, 10^3-10^4/ml, and 10^3-10^4/ml, respectively (118). However, the initial pyruvate (IP) and (ΔP) correlated poorly with SPC counts of raw and pasteurized milk. Only in raw milk was a significant correlation of IP and ΔP with PBC observed.

Rate of growth and pyruvate production which correlate in pure and mixed cultures, suggest the applicability of the test in maintaining quality control of pasteurized milk and in receiving bulk raw milk. This test is biased towards detecting the most inocuous contaminants of milk (lower numbers of Gram-negative organisms detected than Gram-positive organisms). Daily monitoring of keeping quality by running a large number of samples (120 samples/hour) is one advantage, yet initial investment in equipment is expensive. Abrecht and Tatini (2) developed a quicker manual method for measuring pyruvate in milk, which may offset cost with simplicity yet sacrifice sample processing speed.

Though Witter (196) demonstrated the low correlation of the Psychrotrophic Bacteria Count and keeping quality in his review, he also maintained the importance of enumerating psychrotrophs. Oliviera and Parmelee (139) developed a plating method for the rapid enumeration of psychrotrophic bacteria in raw and pasteurized milk. SPC plates were incubated at 21 C for 25 hours. Counts obtained were in good agreement with the standard Psychrotrophic Bacteria Count (PBC). Correlation coefficients between methods of counts of 132 raw and 190 pasteurized milk samples were .992 and .996, respectively.

Parmelee (148) also modified the conventional resazurin test to serve as a method for early detection of psychrotrophs in

pasteurized milk. Two variations of a method employed by Parmelee used resazurin, sodium desoxycholate and trypticase soy broth in specific quantities. According to Parmelee, the first approach provided an accuracy of 80% in detecting samples which would have a psychrotrophic count of over one million after. ten days at 7 C. This method used a 16 hour incubation period at 32 C. The second approach provided an accuracy of 95% in detecting samples which would have a psychrotrophic count of over one million after ten days at 7 C. This approach used a 15 hour incubation period initially at 32 C, then further incubation of 16 hours at 21 C. The first approach was useful in determining the level of post pasteurization contamination by the next day with an accuracy of approximately 80%. It was also useful as a test for line samples to determine the source of contamination of the pasteurized milk with psychrotrophs. The second approach accomplishes the same results as the first approach, but with an accuracy of approximately 95% and a time requirement of 31 hours. Both approaches attempt to measure Gram-negative psychrotrophs. However this test does not allow for possible Gram-positive cocci and sporeformers that affect shelf life.

Methods of enumerating bacteria applicable to dairy products

The millipore filter technique (12) was developed for the concentration, removal, and enumeration of bacteria in liquids and air. This method still relies on several days of incubation to determine viable cell counts. Application of this technique in

conjunction with other methods will be discussed in Materials and Methods.

Deland et al. (48) used the evolution of $^{14}C-CO_2$ as an index of bacterial growth. Previte (153) applied this radiometric detection of food borne bacteria, analyzing for Salmonella, Staphylococcus, and Clostridium within three to four hours.

Winter et al. (195) developed a rapid method for estimating the extent of microbial contamination on food and food processing equipment. Preparations were examined microscopcially and data compared to the SPC method. Over sixty comparisons resulted in a correlation coefficient of .906.

Goff et al. (65) described a membrane transfer procedure in studying the possible role of sublethally injured bacterial cells in the keeping quality of retail pasteurized milk. Generally the organisms presumed injured and subsequently recovered, were the same types as those considered uninjured (65). The organisms found were predominantly Streptococci and Micrococci that produced only slow changes in litmus milk at 5 C, so they were of doubtful significance in the shelf life of retail milk at refrigeration temperatures. Cerny (36) describes a method for the distinction of Gram-negative from Gram-positive organisms. The test can be performed by laying L-alanine-4-nitroanilide impregnated materials upon impregnated test materials; and by adding the bacteria to the test solution, for example, in microtiter plates. Cerny achieved good results with the described methods during examination of contaminated pasteurized milk.

Cerny (37) in a later paper confirmed that all Gram-negative bacteria tested gave a positive test reaction with L-alanine-4-nitroanilide. This test can easily be performed by laying the substrate impregnated filter papers on agar plates of colonies; after a short incubation time (five minutes), Gram-negative bacteria can be recognized by the appearance of a yellow stain due to liberated p-nitraniline. This biochemical test is based on the fact that typical Gram-negative bacteria possess an aminopeptidase, which reacts with specific substrates even if intact cells are used. This enzyme was primarily described by Cerny (35) and Teuber and Cerny (174). The majority of Gram-negative bacteria were able to split the substrate used (L-alanine-4-nitroanilide), whereas the majority of Gram-positive bacteria did not react at all or showed only weak peptidase activity under similar conditions. In 1975, an aminoendopeptidase of E. coli was described by Lazdunski et al. (114), which seemed to be identical to the previously described aminopeptidase of E. coli from Cerny's and Teuber and Cerny's studies. This test shows applicability to the enumeration of Gram-negative organisms involved in the shelf life of milk.

Hardy et al. (80) used automated impedance measurements to rapidly assess whether a sample of frozen vegetables contained more than 10^5 organisms/gram. The time for analysis was approximately five hours compared to the 48 to 72 hours required for Standard Plate Count methods. This method is currently being applied to milk products in some laboratories across the nation. Owens (147) carried out a study to determine if certain types of lecithinase-producing bacteria were present in pasteurized milk, either as pre- or post pasteurization

contaminants and if they are capable of causing the formation of "bitty cream" or a sweet curdle effect. The numbers of lecithinase producing organisms in pasteurized milk increased from less than one/.01 ml at zero hour to greater than 1×10^4/ml in 48 hours. When incubated at 22 C, all samples showed a marked degree of the "bitty" defect.

In a recent study, Pettipher et al. (150) using a modified filtration technique of Cousins et al. (41), produced optimum conditions for rapid filtration of raw milk. The procedure, using a two-stage protease surfactant treatment, was successful in minimizing filter clogging. Studies examining samples contaminated with mammalian cells, e. g., infected urines, with a protease from Aspergillus oryzae and a surfactant were reported to minimize the same type of filter clogging (151) in a procedure for determining bacterial adenosine triphosphate. This procedure was modified to permit filtration of milk by Cousins' group (41) and Pettipher et al. (150) used membrane filtration and epifluorescent microscopy for the direct enumeration of bacteria in raw milk. Somatic cells were lysed by treatment with trypsin and Triton X-100 so that two ml of milk containing up to 5×10^6 somatic cells/ml could be filtered. The majority of the bacteria (ca. 80%) remained intact and were concentrated on the membrane. After being stained with acridine orange, the bacteria fluoresced under ultraviolet light and could easily be counted. The clump count of orange fluorescing cells on the membrane correlated well (r = 0.91) with the corresponding plate count for farm, tanker, and silo milks. The technique

is rapid, taking less than 25 minutes, inexpensive, costing less than 50 cents per sample, and is suitable for milks containing 5×10^3 to 5×10^8 bacteria per ml.(150). This procedure would seem to be applicable to testing pasteurized fluid milk to a certain extent.

In a study by Stadhouders et al. (165), a 1% sodium citrate concentration was added to Streptococcus sp. inoculated skim vats after neutralization with 33% NaOH. Two liter volumes were separated into six tubes and centrifuged (10,000 RPM), the sediment (ie. cells) recovered and resuspended in a volume of HP-milk equal to that of the original starter. The addition of citrate served to facilitate the clearing of the milk and these particular bacteria were easily separated and recovery was good after neutralization (pH 7.0), compared to non-neutralization of identical vats. This method was applied to milk samples of 180 ml volumes, as outlined in the Experimental section.

Since many psychrotrophs are proteolytic Gram-negative rods (173) and they have been associated with spoilage of milk and dairy products in the past, tests to detect these types of organisms in a short period of time were examined. The Limulus amoebocyte lysate test (LAL) has been shown to be specific and highly sensitive to bacterial endotoxins associated with Gram-negative bacteria (101, 100,116). The LAL method for estimating bacterial populations evolved from an observation by Bang in 1956, where a vibrio-caused infection of the horseshoe crab (Limulus polyphemus), resulted in the clotting of Limulus blood (Levin and Bang).

Jay (93) used this technique in developing a simple, rapid, and reliable method for the assessment of microbial quality of refrigerated ground beef. A LAL Gel test (Difco Pyrotest reagent) and later a spectrophotometric method (86), were used to assess the bacterial quality of pasteurized fluid milk samples. Comparison tests were utilized to evaluate the LLT for its efficacy of measuring Gram-negative bacteria.

The B-D-glucose absorption test was developed to further measure Gram-negative bacterial counts. It has been established that glucose is readily adsorbed by many psychrotrophic bacteria (68,198). The method outlined in the experimental section was adapted from a procedure described by Raabo and Terkildsen (155), with minor changes in the quantity of chromogen to increase the sensitivity. Enzymatic methods of this kind have provided a high degree of specificity in estimating blood glucose (84) levels. Keston (105) proposed the simultaneous use of glucose oxidase and peroxidase coupled with a chromogenic oxygen acceptor, such as o-dianisidine or o-toluidine, for the colorimetric determination of glucose in biological fluids.

Numerous investigators have adapted Keston's early work in glucose determination to various fluids including blood, urine and cerebriospinal fluid. Keston's original procedure has undergone numerous modifications (190,57,125). In further experimentation, McComb et al. (125), subsequently demonstrated that 2-deoxyglucose, D mannose, and D fructose were also substrates for glucose oxidase but at much lower rates.

In a final attempt to measure Gram-negative bacterial counts in a short period of time the carbon-14 labeled 2-deoxyglucose affixiate test was developed.

Radioactive labeled glucose has been used in transport studies and carbon 14 in biological fluids analysis. Bruno and Christian (30), applied carbon 14 (^{14}C) to aqueous bicarbonate solutions and determined the levels with liquid scintillation counting techniques. Using these techniques, Mickelson (126) studied the inhibition of sugar transport into Streptococci cells by a specific complex. From his studies, D-glucose was readily adsorbed, yet demonstrated little or no respiratory effects on Streptococcus agalacitiae (126). Sugar transport in intact cells of S. agalactiae were comparably equivalent for the two substrates. Using an activity of 0.9 uCuries, Mickelson showed an approximate 1:1 relationship of nanomoles of 2-deoxyglucose transported/mg dry cells/ time. In approximately 2.5 minutes, 24 nanomoles of substrate were transported into the S. agalactiae cells. From these adsorptions, the use of this analog of glucose for cell adsorption studies with psychrotrophic bacteria was entertained. The technique lends itself to measurement of cell adsorption of uptake of this substrate by pre-scribed cell types and the subsequent correlation of this uptake with cell numbers. Since E. coli actively adsorbs D-glucose and presumably the 2-deoxyglucose analog, it was used as the preliminary test organism (68).

It has been shown that many psychrotrophic organisms, especially Pseudomonas sp. are rapid adsorbers of D-glucose and this technique

may be applicable to pure cultures and mixed culture combinations in milk samples (68,29).

EXPERIMENTAL

Media

Standard Plate Count agar (Difco laboratories), was used to enumerate bacterial populations of commercial samples of milk and laboratory stock cultures.

Nonfat dry milk (Galloway West Co., Fond du Lac, WI), reconstituted to 11% solids in sterilized distilled water was used to maintain cultures taken from lyophilized stocks. Bacterial strains included Pseudomonas fragi, P. viscosa, P. nigricans, P. fluorescens, P. putrefaciens, Alcaligenes metalcaligenes, and Chromobacterium lividums. Cultures were transferred every 7 to 14 days and stored at 4 C between transfers.

In the B-D-glucose absorption test, yeast extract, tryptone, glucose lactose broth was used to maintain Pseudomonas cultures (putrefaciens and fluorescens) in proportions of 1.0%, 2.0%, 2.5%, 2.5%, respectively. Reconstituted sterilized nonfat dry milk (NDM) was used to grow Escherichia coli and violet red bile agar was used to enumerate colonies.

In the carbon-14 labeled 2-deoxyglucose affixative test, E. coli, P. putrefaciens, and P. fluorescens were grown in nutrient broth (Difco laboratories).

Dilutions blanks of 1.0% KH_2PO_4 in double-distilled water, 0.1% peptone water (194), and phosphate buffered double distilled water (5), were used in 10 and 100-fold dilutions.

Limulus ameobocyte lysate test

Gel test - Difco Pyrotest^{TM} (Difco laboratories) consisted of small
volume tubes of lyophilized lysate of the amoebocytes from the blood
of horseshoe crabs (Limulus polyphemus). The protein in the Limulus
lysate preparation coagulates in the presence of Gram-negative bacterial
endotoxins. These tubes were completely sealed and only pyrogen-free
plastic syringes were used for sample preparations. Milk samples
(commercial) were spun down and the pellet of organisms reconstituted
in pyrogen-free water (Sterile water for injection, Cutter Labs.,
Inc., USP). Dilution blanks also consisted of pyrogen-free water in
100-ml quantities. The lyophilized lysate coagulates and gives the
appearance of a positive coagulase tube test. The extent of gelation
is a function of the amount of unbound endotoxin, which approximates
the number of Gram-negative bacterial cells. The grading of the lysate
gelation reaction was scored according to the following scheme:

Reaction

4+	Firm gel with considerable opacity
3+	Soft gel with moderate to considerable opacity
2+	Weak gel with slight to moderate opacity and adhesion of starch-like flocules to sides of tube when tube is slanted
1+	Very weak gel with slight opacity and with some starch-like folocules adhering to sides of tube
negative	No visible increase in viscosity or opacity

Jorgensen and Smith (101)

<u>Assay</u> - Milk samples, pelletized and reconstituted in pyrogen-free water, were added in 0.2 ml quantities to the lysate tubes (10 mm x 75 mm). Negative controls were obtained through addition of 0.2 ml of pyrogen-free water; positive controls were obtained by addition of 0.2 ml of Pyrotol 5 ng and 0.5 ng quantities of <u>Escherichia coli</u> endotoxin (Difco laboratories). The reaction mixtures were incubated for 60 minutes at 37 C (water bath). The resultant reaction was observed and graded for degree and quality of gelation (previous grading scheme).

<u>Spectrophotometric modification (86)</u>

<u>Pyrostat assay</u> - The kit (Worthington Biochemical Corporation) included five vials (ten ml each) containing <u>Limulus</u> amoebocyte reagent in the lyophilized form and one vial of reference endotoxin containing 100 ug of <u>E. coli</u> endotoxin. The methodology of this assay is as follows:

endotoxin	<u>LAL* proenzyme(s)</u>	activated enzyme(s)
coagulogen	<u>activated enzyme(s)</u>	aggregated protein
turbidity	$\dfrac{360 \text{ nm}}{\text{spectrophotometer}}$	apparent absorbance

*LAL = <u>Limulus</u> amoebocyte lysate

<u>Performance of assay</u> - The following steps were followed:

1) Labeling was as follows: a) Test sample-reconstituted pellets of milk organisms

  b) LAL control (sterile water for injection)

c) Reference endotoxin standards (RES) dilution tubes 4,5,6,7

d) The diluted sample controls (if turbidity of the samples contribute significantly to the optical density at 360 nm)

2) Prepare sample control by adding 0.1 ml of sample to 1.0 ml of pyrogen-free water.

3) Add 1.0 ml of chilled LAL reagent to each assay tube (except the sample control) and place on ice. Clean glassware was depyrogenated by baking in dry-heat at 180 C for four hours.

4) At conveniently spaced time intervals the reaction was initiated by aseptically adding to the respective tubes 0.1 ml, using a sterile pyrogen-free micropipetter of the following:

a) each test sample (dilutions of 10^1 to 10^5)

b) sterile, pyrogen free water (LAL control)

c) The RES dilutions (agitated briefly on a vortex mixer before sampling). After each addition, contents of the tube were mixed thoroughly but gently and transferred to a 37 C water bath before proceeding to the next tube.

5) The spectrophotometer (Beckman DU model 2400) was set at 360 nm and zeroed against water. The LAL control was not used as a blank for zeroing the spectrophotometer because reconstituted LAL may not be free of trace amounts of contaminating endotoxin which, if present, would result in a continuously increasing turbidity.

6)   When the first tube in the assay series had incubated for 60 ±

1 minutes at 37 C (water bath), it was briefly agitated on a vortex

mixer, the contents transferred to a cuvette (need not be pyrogen-free),

and the optical density read.

7)   Step number six was repeated for the remaining reaction tubes

(remaining dilutions), including the LAL control. The cuvette was

drained and rinsed with sterile water for injection before subsequent

readings were taken. Sample controls were run and their optical

densities were substracted from the optical densities of the

corresponding reaction mixtures (dilutions). The resultant values were

then corrected from observed optical densities. The net optical densities

for the RES and sample reaction mixtures were calculated according to

the following equation:

net optical density = observed optical density
                      - LAL control optical density

A standard curve was constructed by plotting each RES net optical

density versus its respective endotoxin concentration (ng/ml). From

this standard curve the endotoxin concentration and subsequent cell

concentration of each test sample were compared (plotting cell number

versus optical density).

Comparison tests used to evaluate efficiency of LAL test

SPC - Standard Plate Count (5), was used for the enumeration of the

total bacterial count in examinating dairy products. Incubation

conditions used were 32 C ± 1 C for 48 hours ± 3 hours.

RPC- Rapid psychrotroph count (139), was used for the rapid enumeration of psychrotrophs present in dairy products. Plates were prepared using the same method for the Standard Plate Count but incubation conditions of 21 C for 25 hours were utilized instead.

Moseley keeping quality test- This test (96) measured the change in total bacterial count (SPC) during incubation of a sample at 7 C to 7.2 C for at least seven days. Samples of commercially pasteurized milk were stored at 7 C to 7.2 C for one to two days then a SPC performed. After five days, a sample SPC was performed giving the Moseley count.

CVT- Crystal violet is inhibitory to Gram-positive and 2, 3, 5 triphenyltetrazolium chloride colors Gram-negative bacteria red , thus selecting for Gram-negative bacteria under specified conditions. Sterile crystal violet solution (0.02 g in 100 ml H_2O) was added to 100 ml of SPC agar. One ml of 0.05% alcoholic TTC (0.5 grams of 2, 3, 5 triphenyltetrazolium chloride in 100 ml of 95% Ethanol) was added to the same 100 ml quantities of sterile SPC agar. Milk samples were diluted in 2.0% sodium citrate solutions or 0.1% peptone water, shaken and mixed with prepared agar in pour plate fashion. Plates were incubated at room temperature, 22 C to 24 C for three to four days. The latter was normally used. Red colored colonies were counted only, ie., Gram-negative bacteria.

Pyruvate test- To a five ml sample of milk, 0.05 ml of rennet solution (three parts commercial rennet and five parts Trizma buffer (pH 7.0)

(Sigma Chemical Co.), were added, mixed, and incubated at 45 C (water bath) for five minutes. This sample was centrifuged at 8000-10000 RPM for ten minutes, removed, the supernatant decanted into another tube, steamed for five minutes, and recentrifuged for an additional ten minutes.

The sample was removed and the supernatant filtered through 0.8 um filter paper and two ml of this placed in a quartz cuvette. To this two ml volume, NADH (dehydrated, reduced form of nicotinamide adenine dinucleotide, 0.45 mg in one ml Trizma buffer) was added to make three ml total. After mixing for two minutes, the absorbance was read at 340 nm on a Beckman DU model 2400 spectrophotometer. After this measurement, LDH (lactic dehydrogenase enzyme, activity 6000-12000 units/ml Trizma buffer), was added to initiate the reaction. After two minutes at 37 C, the final absorbance was read at 340 nm. The NADH and LDH solutions were kept on ice until needed. The following reaction takes place:

pyruvate $NADH_2$

LDH + H^+

lactate NAD^+
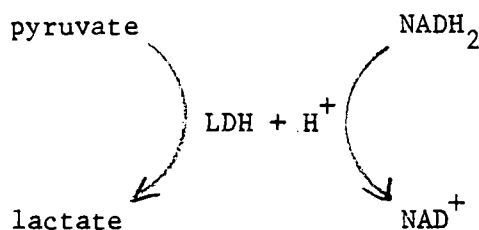
Figure 1. Transformation of sodium pyruvate to sodium lactate through the reduced NAD mediated lactic dehydrogenase enzyme system.

The decrease in concentration of $NADH_2$ is measured colorimetrically at 340 nm and the concentration of pyruvate determined as follows:

$$\text{micromoles (um) pyruvate/ml of sample} = \frac{\text{absorbance}}{E_{340 \text{ nm}}^{\text{mm}} \text{NADH} \times \text{dil. factor}}$$

$$E_{340 \text{ nm}}^{\text{mm}} = 6.2$$

dilution factor $= 0.62$

one um pyruvate/ml $= 100.0$ ppm

According to Von Tolle (188), pyruvate values vary between 0.5 to 30 ug/ml. Amounts greater than 1.5 ppm are believed to be due to bacterial counts ranging from 10^5 to 10^6/ml.

Parmelee tube test for psychrotrophs

Parmelee tube test (PTT) (148) evaluation of samples was conducted by two different procedures:

Method one: One ml of 5.5% sodium desoxycholate and one ml of 6.0% Trypticase Soy Broth were added to a test tube containing nine ml of commercial milk sample. To this mixture one ml of 0.005% resazurin was added and mixed by inversion. This mixture was incubated at 32 C for 16 hours.

Method two: One ml of 5.5% sodium desoxycholate and one ml of 6.0% Trypticase Soy Broth were added to a test tube containing nine ml of commercial milk sample. This mixture was incubated for 15 hours at 21 C, then one ml of 0.005% resazurin was added and incubated for 16 hours at 32 C. At the end of this period, observations were made as the tubes were inverted and read.

Observation of color changes provided interpretation as to the relative population of psychrotrophic bacteria as follows:

- purple- low psychrotrophic contamination, good keeping quality

+(-) pink - medium psychrotrophic contamination and fair to low keeping quality

+ white- heavy psychrotrophic contamination and poor keeping quality

Controls were either sterile milk (permanent pink color) or sterile distilled water (purple color).

Concentration of cells from milk samples

Milk clarification - One percent sodium citrate concentration was attained by the addition of 20 ml of a 10% sodium citrate solution (weight/volume) to 180 ml quantities of commercial milk samples and NDM samples inoculated with strains of: P. putrefaciens, P. fragi, P. viscosa, P. nigrificans, P. fluorescens, Chromobacterium lividums, and Alcaligenes metalcaligenes. These cultures were grown in re- constituted sterile NDM for one to two days, the cells recovered by centrifugation of the clarified milk, and the percentage of recovery determined.

B-D-glucose adsorption test

The procedure was essentially that of Raabo and Terkildsen (155), with a minor change in the quantity of chromogen used to increase the sensitivity. The sample was added to a mixture containing glucose oxidase, peroxidase, and o-dianisidine. The reaction was allowed to proceed to completion in approximately 30 minutes at 37 C. The final color intensity was proportional to the glucose concentration. A

distinct advantage of this procedure was that precise timing was not
mandatory.

A Bausch and Lomb Spectronic 20 spectrophotometer was calibrated
to detect concentrations of glucose in the range of 25 to 300 mg/100 ml.
The relationship between concentration and optical density was linear
over this concentration range. Serial dilutions from a glucose
standard solution of 1.0 mg/ml (5.56 mmol/liter), in benzoic acid, 0.1%,
were made of 25, 50, 100, 150, 200, and 300 mg/100 ml concentrations
of the glucose to construct the standardized graph.

Detectable quantities of glucose in sample volumes of 0.5 ml in
the presence of five ml of glucose oxidase, peroxidase and o-dianisidine
solution were measured. The reaction proceeded to completion in
approximately 45 minutes at room temperature. The final color
intensity was proportional to the B-D-glucose concentration. The
procedure is based upon the following coupled enzymatic reactions:

1)   Glucose + 2 H_2O + O_2 $\xrightarrow[\text{oxidase}]{\text{glucose}}$ gluconic acid + 2 H_2O_2

2) H_2O_2 + o-dianisidine $\xrightarrow{\text{peroxidase}}$ oxidized o-dianisidine

    (colorless)   (brown)

The intensity of the brown color measured at 425-475 nm is pro-
portional to the original glucose concentration (105).

Sample procedures

a)   Cultures of Pseudomonas putrefaciens and P. fluorescens were
utilized as test organisms. Samples were grown unshaken overnight

at room temperature; bacterial cell populations approached 10^8/ml.

Serial dilutions (ten fold) were used and one ml or 100 mg/100 ml of glucose standard solution was added to one ml portions of each dilution. These were initially incubated at 1, 3, 6, 6.5, and 7 hours and 0.5 ml aliquots taken and mixed with five ml of chromogen enzyme standard solution. This mixture was incubated at room temperature for 45 minutes and the optical density determined at 450 nm.

Dilution blanks of 0.1% peptone water were used. Controls included 0.1% peptone water in 5.5 ml volumes and a standard solution of 100 mg/ml (0.5 ml) in five ml of chromogen enzyme solution.

Bacterial counts were taken initially before addition of glucose at 1, 3, 6, 6.5, and 7 hours.

b)   Pseudomonas putrefaciens in concentrations ranging from 0.1, 1.0, 5.0, and 8.0 ml in a total volume of 180 ml of reconstituted NDM were treated with sodium citrate and the cells recovered in pelletized form. This pellet was resuspended in 0.1% peptone water (ten ml) after 15 hours of growth at room temperature. The same procedure was used for the glucose addition, where samples were incubated for one and six hours to facilitate glucose adsorption.

c)   This study involved concentration steps but 1, 3, and 7.5 hours of incubation were used after glucose addition.

d)   These experiments utilized P. putrefaciens grown overnight (15 hours) and stored at 10 C before cell recovery, with a six hour incubation period for adsorption.

e)   Glucose was added to <u>Escherichia</u> <u>coli</u> and cultures were shaken

during incubation: <u>method</u>-glucose was added in one ml quantities (100

mg/100 ml) to one ml volumes of <u>E</u>. <u>coli</u>.   Incubation was at 27 C for one

hour on a temperature controlled shaker. <u>Escherichia</u> <u>coli</u> was grown

for 15 hours in NDM, the previous concentration and cell recovery

procedure used.   For standard bacterial counting, 0.1% peptone water

was used for dilution blanks (99 ml), while pelletized cells were

reconstituted in 0.1% peptone water as well and one mg/ml of glucose

was added to nine ml of cells in each dilution.   Controls for procedure

included 0.1% peptone water for zeroing the spectrophotometer and one

mg/ml glucose was added for the initial concentration.

The amount of adsorption of glucose in the <u>Pseudomonas</u> and <u>E</u>.

<u>coli</u> experiments were measured by extrapolating the concentration of

glucose from the standard curve constructed to calibrate the spectro-

photometer.   Cell concentrations were derived from the graphing of

glucose adsorption versus cell numbers.

Carbon 14 labeled 2-deoxyglucose affixative test (analog of glucose)

D-glucose is readily transported across the cell membrane of

bacteria that utilize this compound as a carbon source (126).

Test procedure-

a)   <u>Escherichia</u> <u>coli</u> grown over a 48 hour period at room temperature

(shaker unit) was used as the test organisms for 2-deoxyglucose ad-

sorption.   Initially grown in 2% brilliant green lactose bile broth,

the cell adsorption was minimal and nutrient broth was substituted to

delete any interference that growth in BGLB broth might produce. Cell concentrations in one ml volumes were subjected to radioactive 2-deoxyglucose (five ul of 0.5 uCuries activity) for at least five minutes of incubation at 37 C (no shaking). Dilution blanks consisted of phosphate buffered water (5) in 99 ml volumes for pure cultures of E. coli.

Samples of E. coli impregnated with 2-deoxyglucose were filtered onto 0.45 um filters by the millipore filtration technique (12) into 250 ml collection flasks. The filters were washed twice with volumes of four ml of 0.05 M phosphate (37 C). The filters were dried and placed in scintillation vials containing ten ml of fluor (4 grams 2,5-diphenyloxazole:PPO + 0.1 ml gram 2.2'-n-phenylenobis (5-phenyloxazole):POPOP).

Samples were analyzed on a Beckman 8000 LS Counter. Quenching was corrected by using a ^{14}C program containing the added parameter of H#, a new method of external standardization. Controls of 0.5 uCuries/5 ul activity on filters (0.45 um) were treated the same as the sample vials. Percent adsorption was calculated (DPM absorbed/average total DPM x 100). Samples analyzed were as follows:

1) Initial sample set involved pure culture adsorption of concentrations in the 10^7 cell/ml range, using previous method.

2) These samples involved the use of serial dilutions ranging from 10^3-10^7 cells/ml by addition of 0.5 uCuries of 2-deoxyglucose to one ml of each dilution (no shaking).

3) This sample set involved serial dilutions ranging from 10^6-10^{10} cells/ml by addition of the same quantity of 2-deoxyglucose. Lower limits of zero and upper limits of 1000 were used for counting purposes. Counting efficiency for the ^{14}C isotope approached 95%.

4) This sample set involved use of pure cultures of psychrotrophic Pseudomonas putrefaciens and P. fluorescens. Similar procedures for 2-deoxyglucose adsorption in the cell concentration range of 10^6-10^{10} were applied except for incubation temperature changes. P. putrefaciens was incubated at 32 C for five to fifteen minutes with shaking following the incubation at room temperature (five minutes at least). P. fluorescens was shaken at room temperature during the duration of adsorption (five minutes to fifteen minutes). Controls were similar to the previous techniques with a wide open window of lower limits of zero to upper limits of 1000. Quenching was corrected for by use of H#.

5) The final sample set involved the application of the previous technique in #3 to commercial milk samples, first stored for one day at 4 C and then stored for seven days at 7 C. Each sample was pelletized and concentrated according to the previous method. A standard plate count was run for comparison. A control experiment to evaluate the adsorption of 2-deoxyglucose in normal milk dilutions (no concentration steps) was performed.

RESULTS AND DISCUSSION

Concentration of milk sample

In this study, the initial milk concentration experiments indicated highly variable cell recovery. Table 2 shows the mean of 20 experiments using 2% sodium citrate in ten ml samples of milk reconstituted to original volumes (with 0.1% peptone). This bacterial cell concentration procedure was employed for the Limulus amoebocyte lysate test. Due to the extreme variation in cell recovery, this specific method of milk sample/cell concentration was discontinued in favor of the procedure of Stadhouders (165). This procedure provided more consistent recovery of bacterial cells (Table 3).

For the initial cell recovery method, the range was from 0.00% to 550.0% cells recovered (Table 2). The Stadhouder procedure had a range of 5.00% to 144.0% cell recovery with a mean of 27.3%. These results showed more consistent recovery using 180.0 ml samples as opposed to 10.0 ml samples.

To evaluate the efficiency of the procedure, cell recovery data for the supernatant and reconstituted pellet were studied and compared. From the data presented in Table 3, the reconstituted pellet procedure proved more consistent and efficient and was used in subsequent experiments. The increased variability in both supernatant and reconstituted pellet recovery parts of the procedure could be a function of milk lipid retention of bacterial cells. The supernatant was a mixture of whey and milk lipid and retention by this lipid fraction could have

Table 2. Bacterial cell recovery using 10% sodium citrate solution
(1.0 ml in 9.0 ml of milk) and centrifugation at 12000 RPM.

| Sample | Log total numbers 32 C | | pH | | % Recovery |
	Control	Sample	Control	Sample	
1	3.71	3.18	6.75	7.00	29.6
2	6.81	4.34	6.75	7.00	0.3
3	6.10	5.86	6.75	7.00	56.6
4	3.15	3.40	6.75	7.00	178.0
5	4.85	4.88	6.75	7.00	108.0
6	2.78	3.52	6.75	7.05	550.0
7	<2.00	<2.00	6.78	6.97	0.0
8	5.81	3.51	6.79	6.97	0.5
9	2.00	2.00	6.81	6.99	100.0
10	<2.00	<2.00	6.80	6.97	0.0
Cell recovery of above samples after 5 days of storage					
1a	8.11	8.32	-	-	161.0
2a	7.67	7.20	-	-	34.0
3a	8.84	8.40	-	-	126.0
4a	8.36	8.28	-	-	83.0
5a	8.34	8.15	-	-	64.0
6a	7.87	7.04	-	-	15.0
7a	4.04	4.68	-	-	436.0
8a	7.81	5.93	-	-	0.4
9a	6.90	6.75	-	-	71.0
10a	6.90	6.75	-	-	71.0
Mean	5.50	5.14	6.77	7.00	104.2

Table 3. Bacterial cell recovery using 20 ml of 10% sodium citrate solution in 180 ml of milk (1% final concentration) with centrifugation at 10,000 RPM; log cells per ml.

| Sample | Log cell numbers 32 C | | | % Recovery | |
	Control	Supernatant	Pellet	Supernatant	Pellet
1	2.70	2.00	2.15	20.0	28.0
2	2.70	2.00	2.30	20.0	40.0
3	6.96	4.32	4.77	16.2	44.5
4	5.30	4.20	4.68	8.0	23.9
5	9.53	8.75	7.64	16.4	5.6
6	9.56	7.75	8.90	16.0	5.6
7	7.52	6.73	6.94	16.4	26.4
8	3.11	2.64	3.27	33.8	144.0
9	7.60	6.58	6.68	9.5	12.0
12	2.30	2.30	1.52	100.0	16.7
13	4.20	2.95	3.68	5.6	29.9
14	4.15	2.48	3.67	2.1	33.7
15	4.18	2.60	3.51	2.0	21.5
16	4.26	<2.00	3.37	0.6	12.9
17	8.11	6.75	7.37	4.3	17.8
18	8.20	7.43	7.39	16.9	15.4
19	8.58	7.92	7.50	22.1	8.3
20	8.56	7.49	7.22	8.6	4.6
Mean	5.97	4.46	5.14	17.7	27.3

Table 4. Determination of pH on microbial cells recovered from milk, using the equivalent of 1.0% sodium citrate (v/v basis).

Sample	Control		Supernatant		Pellet	
	Initial[a]	Final[b]	Initial[a]	Final[c]	Initial[a]	Final[c]
1	6.60 pH	7.02 pH	6.60 pH	7.02 pH	6.60 pH	7.02 pH
2	6.70	7.00	6.70	7.00	6.70	7.00
3	6.88	7.00	6.88	7.00	6.88	7.02
4	6.88	7.02	6.88	7.02	6.88	7.02
7	6.70	7.01	6.51	7.75	6.51	7.19
8	6.70	6.99	6.67	7.75	6.67	7.19
9	6.85	6.99	6.67	7.75	6.67	7.19
10	6.85	7.03	6.70	7.70	6.70	7.15
11	6.70	6.99	6.70	7.70	6.70	6.85
12	6.81	7.03	6.81	7.80	6.81	7.30
13	6.79	7.00	6.79	7.80	6.79	7.18
14	6.77	7.02	6.77	7.70	6.77	7.01
15	6.76	7.00	6.76	7.69	6.76	7.22
16	6.77	7.00	6.77	7.80	6.77	7.20
17	6.68	7.00	6.68	7.58	6.68	6.75
18	6.70	7.01	6.70	7.47	6.70	6.67
19	6.25	7.00	6.25	7.57	6.25	6.41
20	6.33	7.05	6.33	7.42	6.33	6.36
Mean	6.71	7.01	6.68	7.52	6.68	6.97

a Prior to neutralization
b Neutralization with 33% NaOH before citrate addition
c After addition of citrate and centrifugation

increased the percent recovery of the supernatant to a higher value.

Table 5 shows the application of this cell recovery procedure to selected individual strains of psychrotrophs responsible for spoilage of fluid milk products. Bacterial cell recoveries ranged from 32.1% to 168.4% (reconstituted pellet). Supernatant and reconstituted pellet sections of the procedure were again compared. Supernatant recoveries range from 9.97% to 38.9%. The highly variable recoveries could be explained by differences in specific milk lipid retention of individual bacterial cells. Among the Pseudomonas genera, individual bacterial cell recovery was extremely variable. Yet these individual strains were grown in reconstituted nonfat dry milk where the milk lipid content was at a minimum. Increased cell recoveries for total bacterial counts could be explained by this lack of interfering milk lipid. Stadhouders in his study (165) used skim milk for growth and reconstitution of pelleted lactic streptococcal starter bacteria. He minimized any effect milk lipid might have had on cell recoveries. Variability in the psychrotrophic bacterial cell recoveries could also be explained by size and shape differences between the specific strains. These Gram-negative rods vary in size and shape and may not concentrate as well as lactic Streptococcus bacteria (as well as individual differences), which are coccal in shape.

Due to the variability of the bacterial cell recovery procedure, its usefulness in sample preparation may be limited.

Table 5. Microbial cell recovery using the equivalent of 1.0% sodium citrate (v/v basis) and growth in reconstituted nonfat dry milk.

| Organism | Number of Samples | Log cells per ml at 32 C | | | % Recovery | |
		Control	Supernatant	Pellet	Supernatant	Pellet
Pseudomonas fragi	7	5.88	4.84	5.93	36.30	168.4
Pseudomonas putrefaciens	10	6.78	5.38	6.29	10.10	51.9
Pseudomonas nigrificans	4	6.76	6.26	6.30	38.90	23.2
Pseudomonas viscosa	6	5.67	4.34	5.47	16.35	132.7
Chromobacterium lividum	5	6.21	4.76	4.47	20.92	32.1
Alcaligenes metalcaligenes	6	6.31	5.36	5.85	9.97	39.8
Escherichia coli	4	8.48	6.84	8.18	10.78	71.5

Limulus amoebocyte lysate test (LAL)

Gel test - Two experiments were conducted on a commercial homogenized milk (local dairy). One quart of this milk was stored for one day at 7 C prior to conducting the LAL gel test in addition to the following microbial quality tests: 1) SPC for total bacterial enumeration, 2) rapid psychrotrophic bacteria count, 3) Moseley keeping quality test, 4) Parmelee tube test, 5) crystal violet triphenyltetrazolium test, 6) pyruvate test. After the elapse of five days of storage at 7 C, the same milk quality tests were performed.

Table 6 shows comparative results of bacterial quality tests performed on above mentioned milk samples after one and five days at 7 C storage. Low initial bacterial counts were observed in the sample, as indicated by the SPC and RPC results. These counts represented the bacterial numbers in the original milk sample. The LAL gel test was performed on the concentrated milk sample or the reconstituted pellet which contained even fewer bacterial cells. The Moseley test exhibited a bacterial count in the 10^6 range. This showed a potentially low or limited potential keeping quality not indicated by the initial total or psychrotrophic bacterial counts. The Parmelee tube test, conducted in this experiment, showed modest to substantial psychrotrophic contamination (method I), and substantial psychrotrophic contamination (method II). Any color change indicates the sample will contain over 10^6 psychrotrophs after 10 days at 7 C (45 F) (148). The CVT test indicated low initial Gram-negative contamination while the pyruvate test showed 3.22-1.50 ppm/ml of initial pyruvate (IP) or 1.72 ppm/ml

associated with <10^3 cells/ml. By comparison, there was a positive LAL gel test at the 10^2 dilution and a "barely" positive test at the 10^4 dilution. Positive and negative control test results were as expected.

In comparison to Jay's work (93) on hamburger quality, the results of this experiment exhibited a positive titre (inverse of dilution where test was positive) at the 10^2 dilution. The E. coli endotoxin concentration associated with a positive titre was 5 ng/ml. The gelation reaction was scored and assigned a value according to the grading scheme previously described in the Experimental section.

Table 6 shows the fifth day performance of the gel test on a higher bacterial cell concentration. The SPC and RPC bacterial counts were significantly increased over the storage period, in the 10^6 range. The bacterial cells recovered in the reconstituted pellet were in the same approximate log range as the original samples but only constituted 13% recovery. The Moseley test reflected higher bacterial counts, though this was due to contaminating overgrowth. However the results should be similar to the first days' Moseley test in showing a proportional increase in bacterial numbers. The Parmelee tube test (method I) indicated substantial psychrotrophic contamination with potential poor keeping quality, while the more accurate test method-II, showed very little increase in psychrotrophic contamination. The CVT test revealed little increase in contamination. The pyruvate test value of 4.35 ppm/ml (IP) corresponded with 10^6 cells/ml, for both total and psychrotrophic bacterial counts.

Comparatively, the LAL titre was +4 for the 10^2 dilution and +2 for the 10^4 dilution, which approached a barely positive clot reaction. A 10^6 dilution of the pelletized milk sample bacterial cells was considered negative for the LAL gel test. The 10^2 dilution contained 10^3 total and psychrotrophic bacterial cells while only containing 10^1 cells/ml from the CVT test. This dilution exhibited a positive clot reaction (ie. a titre of 10^2), while the 10^4 dilution contained 10^1 to 10^2 total and psychrotrophic bacterial cells yet no detectable cells from the CVT count. This dilution exhibited a barely positive clot reaction. The lower sensitivity for total and psychrotrophic bacterial counts was 10^1 to 10^2 cells/ml, while distinct positive sensitivity was observed at the 10^3 cells/ml level. Sensitivity to the Gram-negative cell count (CVT), appeared at the 10^1 cell/ml level (10^2 dilution) yet showed false positive at the 10^4 dilution. The sensitivity to E. coli bacterial endotoxin was 5 ng/ml of endotoxin. This was associated with 10^3 cells, total and psychrotrophic bacterial counts and 10^1 for the Gram-negative cell count (CVT).

Jay (93) applied the LAL gel test to ground beef samples, and suggested that an LAL titre of 10^3 had about the same degree of significance as an extract-release volume (ERV) of 25 ml relative to fresh gound beef microbial quality. Briefly the ERV is the volume of extract that passes through filter paper and is collected in a graduated cylinder in 15 minutes. By this method, beef of good microbial quality yields extracts of 25 ml or more while micro-biologically spoiled beef yields volumes between zero and 25 ml (93).

The ERV test can be performed in 15 minutes; the LAL test requires one hour, but the ERV method gives no indication of the relative numbers of nonviable Gram-negative cells, while the latter method does.

Jay (93) reported that apparently most, if not all, Gram-negative bacteria in ground beef can produce a positive LAL test result. The *Limulus* lysate literature reveals that most investigators have worked with either purified endotoxins or with one or more of the following organisms: Escherichia coli, Klebsiella pneumoniae, Neisseria meningiditis, Pseudomonas aeruginosa, Salmonella minnesota, S. typhi, S. enteriditis, Serratia marcesans, Shigella dysenteriae, and Vibrio sp. Jay (93) showed positive responses with genera that can be found in milk products such as: Alcaligenes metacaligenes, Flavobacterium sp., Proteus mirabilis, Pseudomonas geniculata, Enterobacter sp., Pseudomonas sp. (2 strains), Achromobacter sp. (2 strains), and 5 miscellaneous isolates. Among the lysate-negative organisms reported were Staphylococcus aureus, Streptococcus hemolyticus, Pneumococcus, and yeasts.

As in Jay's study (93), the titres showed results of the cell counts, SPC and RPC (rapid psychrotroph count), taken on milk concentrates. Using the grading scheme of Jorgensen and Smith (101), the initial titre was 10^2 or +2 score, which approached a barely positive response. This reflected a viable count of $<10^2$ cells/ml; the 10^4 dilution was negative.

The LAL procedure offers several advantages (93), as a rapid test or projection test for evaluating the microbial quality of perishable foods (predominant Gram-negative spoilage organisms):

1) it is simple to perform and read.

2) it responds to significant spoilage organisms.

3) it detects viable and non-viable Gram-negative bacteria.

4) it correlates closely with the ERV test for meat freshness. The two methods, conducted simultaneously, provide an excellent rapid microbial profile.

5) it readily serves as a simple screening test.

The application of the LAL test to milk samples provides similar advantages, with the exception that sample evaluation extends to three hours or more including a one hour LAL test incubation period. This is minimal compared to 48 hours for the SPC, 25 hours for the RPC, or 16 hours for the Parmelee tube test. The LAL test could be used as a simple shelf life projection test for fluid milk. The titre count (inverse of the dilution with a positive coagulation, +3 to +4 score) can aid in evaluating the microbial profile of the sample.

However, milk spoilage is not determined by microbial load alone. There is no set microbial level that clearly dictates spoiled samples or ones that are approaching poor keeping quality (79). Any sample with a population of greater than 10^6 cells/ml (total or psychrotrophic bacterial count) can be considered approaching an unpalatable condition (185).

Watrous (192) has shown that initial bacterial counts via SPC, coliform, and psychrotrophic counts on commercially pasteurized samples were of little value in predicting keeping quality. Patel and Blankenagel (149) clearly pointed out that milk with low bacterial counts does not necessarily achieve a long shelf life.

Table 6. Microbial quality test results of homogenized milk for total and psychrotrophic bacteria.

Microbial Methods		Day 1[a]		Day 5[a]	
SPC		2.90 CFU/ml		6.48 CFU/ml	
RPC		2.30		6.43	
Moseley test		6.48		----[b]	
CVT		2.00[b]		2.30	
Pelletized SPC		----		5.60	
Pelletized SPC		2.00		5.58	
Pyruvate test		1.72 ppm/ml		4.35 ppm/ml	
Parmelee tube test	Method[c]	I	II	I	II
Negative control		–	±	±	–
Positive control		+	+	+	+
Sample		+	+	+	–
LAL		Extent of Clotting[d]			
5 x 10^2 dilution		+3		+4	
5 x 10^4		+1		+2	
5 x 10^6		–		–	
Positive control (5 ng/ml)		+4		+4	
Negative control		–		–	

a Cell counts are logarithmic
b Laboratory accident
c Parmelee tube test grading scheme: (–) purple, (±) pink, (+) white
d LAL gel test grading scheme: (–) no clotting, (+4) highest degree of clotting
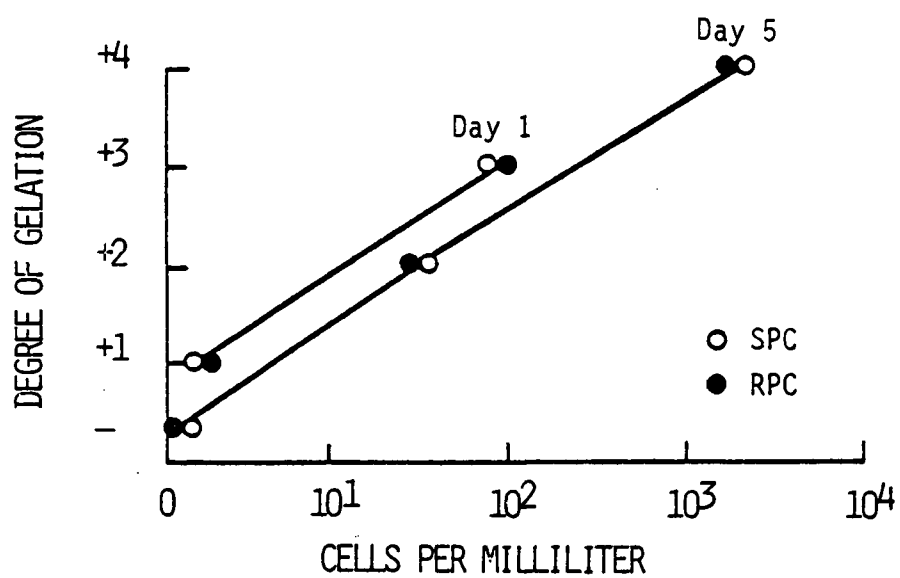
Figure 2. Relationship between LAL gel test gelation and microbial cell counts, SPC and RPC.

The sensitivity of the LAL test was encouraging; it was able to detect the presence of between 10^1 to 10^3 cells/ml based on total and/or psychrotrophic bacterial counts. The LAL test results also correlated with the Gram-negative bacterial count (CVT) within the range of 10^1 cells/ml or a positive titre of 10^2, though occasionally showed false positive results at the 10^4 dilution (titre) (Table 6).

The LAL gel test also has specific disadvantages or limitations when applied to fluid milk:

1)   the reagent is relatively expensive. The gel test with

      endotoxin standards and negative controls costs $80.00

      ($4.00 per vial). This represents a cost of approximately

      $12.00-$20.00 per test.

2)   the results require subjective judgement. The determination

      of coagulation endpoint is made by visual observations.

3)   equipment has to be completely sterile and pyrogen free.

4)   pyrogen free water is expensive. This also includes any

      other required pyrogen free compounds.

Spectrophotometric modification of the LAL gel test

To improve the endpoint elucidation of the LAL gel test, a modified spectrophotometric method was examined. The spectrophotometric modification was approximately half as expensive as the LAL gel test. Table 7 summarizes the spectrophotometric test results and the results of the other microbial milk quality tests.

The initial cell counts were low for the reconstituted pellet based on the total bacterial count and RPC. The Moseley test revealed cell growth up to the 10^7 range after five days storage at 7 C. The Parmelee tube test (methods I and II) exhibited substantial psychrotrophic contamination. The CVT test reflected $<10^2$ cells/ml growth and the pyruvate (IP) content of the milk showed 2.00 ppm/ml associated with $<10^3$ cells/ml.

This procedure was more sensitive and more quantitative than the gel test. The improved sensitivity of the test was demonstrated where the lowest dilution (or titre) was associated with 1 cell/ml and a distinct optical density reading (uncorrected), total and psychrotrophic counts, the CVT displayed no growth.

Table 7 details the comparison between the spectrophotometric test and the previously described bacterial quality tests for milk. Figure 3 shows the standard curve illustrating the relationship between bacterial endotoxin and optical density.

The LAL spectrophotometric test was performed on the milk sample dilutions and the resulting optical densities were determined. These optical densities were compared to the standard endotoxin curve and the corresponding endotoxin concentrations extrapolated. Figure 4 shows the relationship between endotoxin concentration and bacterial cell number. However, these optical density values were uncorrected for background interference from the milk suspensions. Table 7 illustrates the net optical density values determined after substracting this correction factor; negative net optical densities corresponding to the

Table 7. Comparison of microbial milk quality tests with the
          LAL spectrophotometric method.

Microbial Methods   Day 1

Pelletized SPC   3.86[a]
Pelletized RPC   2.30
Moseley test   7.77
CVT test   no growth

Pyruvate test   2.00 ppm/ml
Parmelee tube test   Method   I   II
              Negative control   –   –
              Positive control   +   +
                        Sample   +   +

| LAL Dilutions | Optical densities | | | SPC[a] | RPC[a] |
	Background	Sample	Net		
5×10^2	1.057	0.301	−0.756	1.15	0.30
5×10^3	0.248	0.129	−0.119	0.15	<0.00
5×10^4	0.189	0.087	−0.102	<0.00	<0.00
5×10^5	0.240	0.096	−0.144	<0.00	<0.00

[a]Log of CFU/ml
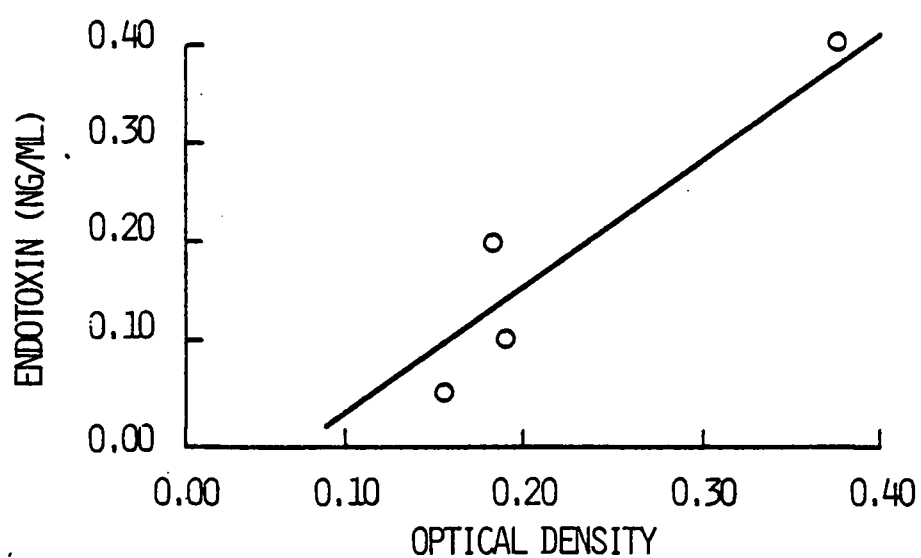
Figure 3. Standard endotoxin curve of E. coli endotoxin
(ng/ml) versus optical density.
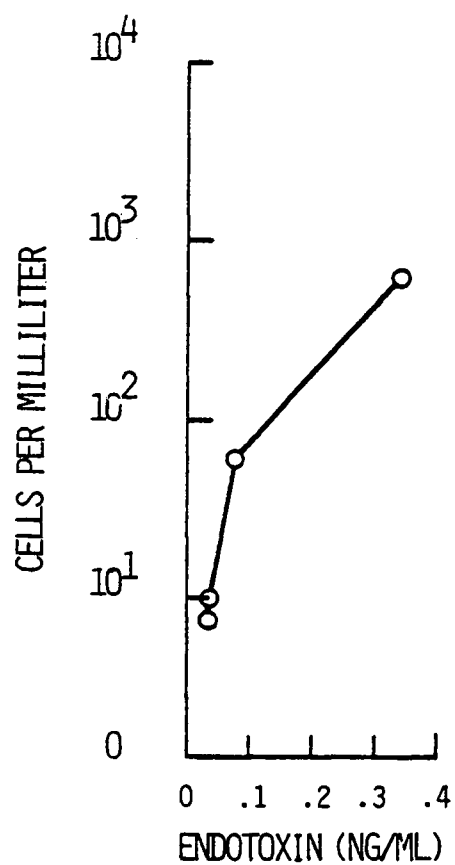
Figure 4. Extrapolated E. coli
endotoxin (ng/ml) versus
cells/ml, SPC and RPC.

milk sample dilutions may be seen. Since the net optical densities were negative, the procedure required revision. One standard curve was prepared for endotoxin levels versus optical densities, when it was concluded the test required modification. After several additional unsuccessful trials the method was abandoned due to this discrepancy in background optical density, plus the disadvantage of perishability of the lysate within hours of reconstitution in bulk. The complexity of the procedure and elevated cost per test prompted termination in favor of alternate methods of detecting Gram-negative and/or psychrotrophic bacteria.

An alternate method, may be the improved microslide method for the Limulus amoebocyte lysate gel test. Frauch's microslide method (60) was improved to facilitate the endpoint determination of the LAL gel test. Frauch added microliter amounts of sample bacterial preparation to microamounts of lysate reagent onto a slide (glass plate used for the Latex test with a black backside (55)), and used calibrated glass capillary tubes (pyrogen free). The slide was then placed in a "moist chamber." This chamber consisted, for example, of a flat plastic box, the bottom of which was covered with a piece of moistened cellulose. Two glass rods served as support of the slide. The entire assembly was incubated at 37 C for 30 minutes and the slide then observed against a bright background. A solid gel or an increase in viscosity of the combined bacterial preparation and LAL gel was easily detected. Differences in degree of gelation indicated differences in amount of endotoxin present (ie. bacterial cells). Two newly performed additions to the slide test by Okuguchi (138) were the inverted phase

contrast microscope and a staining procedure. The staining procedure was proposed as an improvement in the LAL gel test. Combined with the inverted phase contrast microscope, the staining procedure aided in endpoint determination of gelation for the LAL test. The sensitivity of the method was equal to or higher than that of other methods. The volume of the lysate used in this method was a little as one tenth of that used in the gel test.

Okuguchi (138) stated that the LAL gel test may not be suitable for determination of large numbers of samples, due to the high cost of the lysate obtained from commercial sources.

A few improved techniques such as the clot protein method (135) and the spectrophotometric method (86), were reported for the LAL test (138). These methods have many advantages in quantitative studies of the gelation reaction but require complicated procedures and the simplicity of the LAL test is lost (138). Frauch's slide test is one of the modified LAL tests that is simple and convenient in dealing with many samples (15,60), but the reaction is difficult to determine.

Through application of the improved microslide method to concentrated milk samples, it is believed that a shelf life projection test could be developed from the old gel test method at a fraction of the cost, with equal or increased sensitivity, plus convenience and simplicity in endpoint determination. This would also serve to reduce the subjectivity of the test.

B-D-Glucose adsorption test

B-D-Glucose was added to P. putrefaciens and P. fluorescens

cultures in one ml volumes. A Bausch and Lomb spectronic 20 spectro-

photometer was calibrated with B-D-glucose standards of concentrations

ranging from 25 to 300 mg/100 ml. In this range the relationship

between B-D-glucose concentration and optical density is linear while

on either side of these limits, inaccuracies are observed in optical

measurements (155).

Figure 5 summarizes the relationship between glucose concentration

and optical density at 450 nm wavelength. Table 8 shows the results

of addition of glucose to cell concentrations ranging from 10^1 to 10^7

cells/ml. The incubation period was one hour at room temperature.

A control was prepared to compare the initial amount added and the

amount absorbed. From the standard curve of glucose concentration

(mg/100 ml) versus optical density, glucose concentrations were extra-

polated. Substracting the glucose concentrations (determined from

the standard glucose curve), the amount of absorbed glucose was

determined. Table 8 summarizes data for a similar study employing

a six hour incubation period at room temperature. Figure 6 shows

very little relationship between adsorbed glucose and the cells/ml

for both 1 and 6 hour period experiments. Table 9 summarizes data from

a study of the addition of a smaller quantity of glucose to cell popu-

lations of P. putrefaciens. This study included incubation periods of

one, three, and six hours for the same sample. By using a lower

glucose level, it was anticipated that the sensitivity might have been
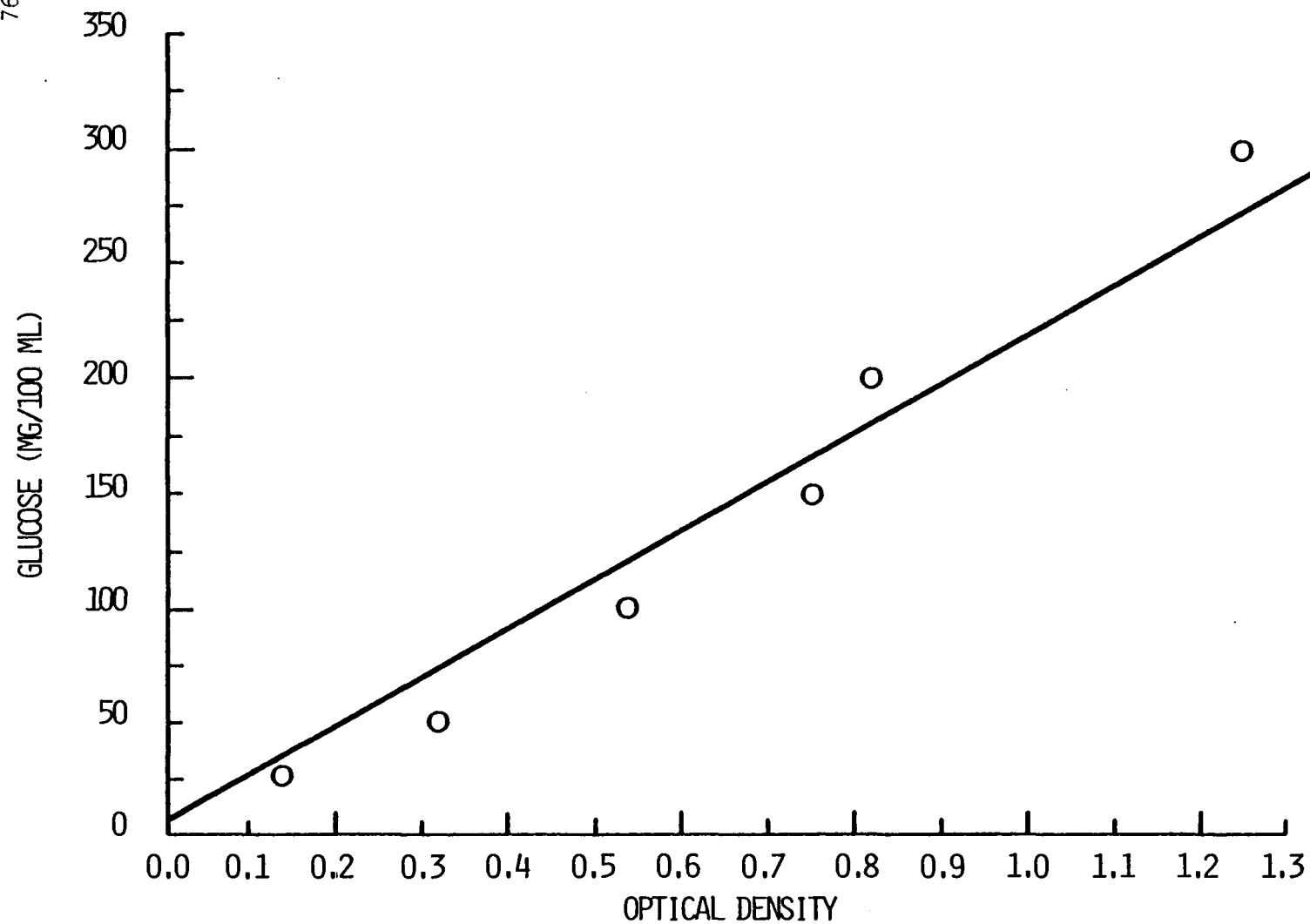
Figure 5.   Standard curve of glucose concentration (mg/100 ml) versus optical density (450 nm).

Table 8. Glucose absorption test results showing dilution, optical density, initial cells/ml, glucose concentration from graph, and net glucose absorption for _Pseudomonas_ _putrefaciens_.

	Study 1 (1 hour)					Study 2 (6 hours)			
Dilution	Optical density	Glucose present[a]	Glucose absorbed	Log cells per ml	Dilution	Optical density	Glucose present	Glucose absorbed	Log cells per ml
10^1	1.20	265	13	6.20	10^1	1.24	273	5	6.79
10^2	1.15	255	23	5.20	10^2	1.26	281	−3	5.79
10^3	1.10	244	34	4.20	10^3	1.24	273	5	4.79
10^4	1.00	223	55	3.20	10^4	1.25	278	0	3.79
10^5	0.99	221	57	2.20	10^5	1.24	273	5	2.79
10^6	0.98	220	58	1.21	10^6	1.22	270	8	1.77
10^7	0.98	220	58	1.11	10^7	1.25	278	0	0.65
10^8	1.00	223	55	0.70	10^8	1.23	271	6	0.15
Control	1.25	278	−−	0.00	Control	1.25	278	−	0.00

[a]Glucose values in mg/100 ml

Figure 6.   Absorbed glucose mg/100 ml versus
            P. putrefaciens cells/ml, Study 1:
            (1 hour) and Study 2: (6 hours).

Table 9. Glucose absorption test results showing dilution, optical density, initial cells/ml, glucose
concentration from graph, and net glucose absorption for _Pseudomonas_ _putrefaciens_. All
counts are logarithmic Study 3.

Dilution	Optical density			Glucose present[a]			Glucose absorbed[a]			Log cells per ml
	Hour 1	Hour 3	Hour 6	Hour 1	Hour 3	Hour 6	Hour 1	Hour 3	Hour 6	
10^1	0.605	0.571	0.550	134	125	124	-101	-90	-89	6.65
10^2	0.183	0.195	0.188	41	44	43	-6	-9	-8	5.65
10^3	0.140	0.150	0.140	34	35	33	1	0	2	4.65
10^4	0.120	0.165	0.132	28	37	31	7	-2	4	3.65
10^5	0.130	0.140	0.160	29	34	36	6	1	-1	2.65
10^6	0.125	0.150	0.150	29	35	35	6	0	0	1.65
10^7	0.133	0.138	0.132	29	34	29	6	1	6	0.86
10^8	0.138	0.175	0.135	30	41	30	5	-6	5	0.30
Control	0.146	0.146	0.146	35	35	35	–	–	–	0.00

[a]Glucose values in mg/100 ml

increased and thus provide more meaningful results (ie. glucose

uptake). Examination of Figure 7 shows no distinct relationship between

glucose uptake and cell numbers. Table 10 shows data from another study,

slightly modified, which used lower glucose concentrations for incubation

periods of one, three, and seven and one-half hours at room temperature

for P. putrefaciens. The plot of data in Figure 8 also shows no

distinct relationship between glucose absorption and cell numbers.

Table 11 shows the application of the B-D-glucose absorption to P.

putrefaciens recovered from reconstituted nonfat dry milk (NDM). This

study attempted to examine an improved medium growth for P. putrefaciens,

with B-D-glucose added directly. Little improvement in adsorption was

observed (Figure 9). Table 12 shows the application of the previous

experiment by the addition of different concentrations of P. putrefacens

to reconstituted NDM and the subsequent recovery in pellet form and

reconstitution in 0.1% peptone. Figure 10 shows the relationship

between adsorption and cell numbers for an incubation period of 6.25

hours at room temperature. The primary difference between the two

experiments was the initial glucose concentration. The latter experi-

ment used a lower concentration of B-D-glucose to examine the potential

for improved sensitivity. Both experiments (Figure 9 and Figure 10)

show evidence of substantial variation in glucose adsorption.

Certain species of P. putrefaciens do not utilize glucose; it is

possible that this subspecies did not possess the capability of ferment-

ing glucose (31).

Pseudomonas fluorescens was also used as a test organism to

evaluate the procedure. Following three test trials on pure cultures,

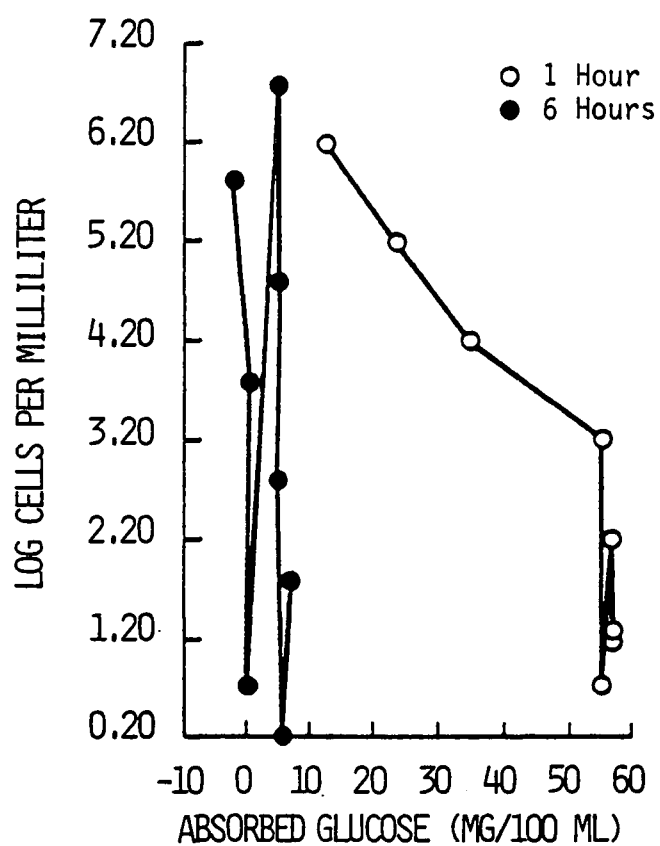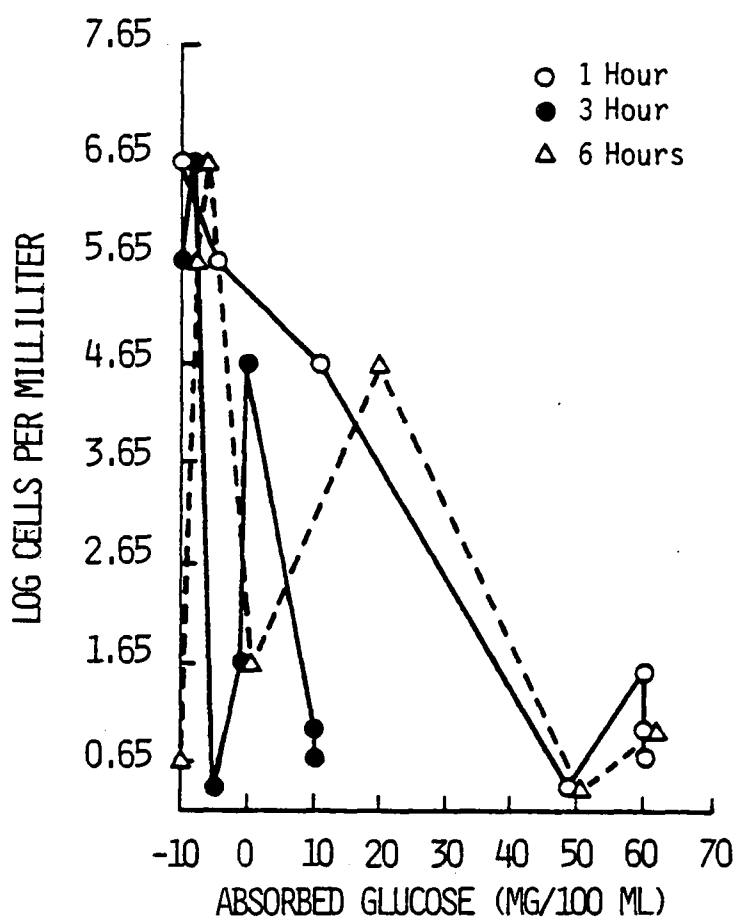Figure 7.   Absorbed glucose mg/100 ml versus P. putrefaciens
            cells/ml, Study 3: 1, 3, and 6 hours.

Table 10. Glucose absorption test results showing dilution, optical density, initial cells/ml, glucose concentration from graph, and net glucose absorption for Pseudomonas putrefaciens.

Dilution	Optical density			Glucose present[a]			Glucose absorbed[a]			Log cells per ml
	Hour 1	Hour 3	Hour 7.5	Hour 1	Hour 3	Hour 7.5	Hour 1	Hour 3	Hour 7.5	
10^4	0.100	0.088	0.101	24	21	24	1	4	1	3.43
10^5	0.098	0.095	0.103	24	24	24	1	1	1	2.43
10^6	0.107	0.108	0.111	25	25	26	0	0	-1	1.43
10^7	0.96	0.090	0.111	24	20	26	1	5	-1	0.00
10^8	0.105	0.113	0.106	25	24	26	0	1	-1	0.00
Control	0.107	0.107	0.107	25	25	25	-	-	-	0.00

[a]Glucose values in mg/100 ml
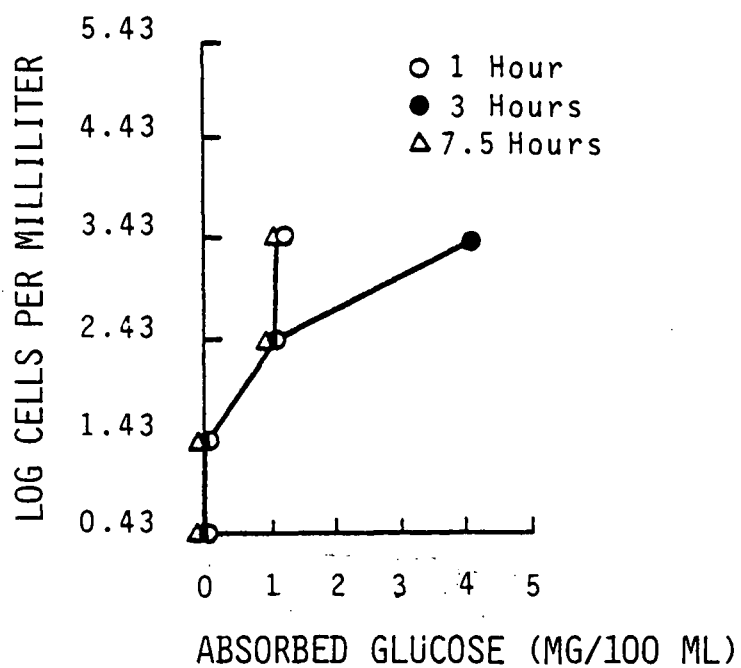
Figure 8.   Absorbed glucose mg/100 ml versus
             P. putrefaciens cells/ml, study 4:
             1, 3, and 7.5 hours.

Table 11. Glucose absorption test results showing dilution, optical
density, initial cells/ml, glucose concentration from
graph, and net glucose absorption for Pseudomonas
putrefaciens. All counts are logarithmic Study 5
(6 hours, recovered from milk).

Dilution	Optical density	Glucose present[a]	Glucose absorbed[a]	Log cells per ml
10^4	0.300	66	1	4.71
10^5	0.261	58	9	3.71
10^6	0.310	68	-1	2.71
10^7	0.292	65	2	1.71
10^8	0.323	71	-4	0.00
Control	0.305	67	-	0.00

[a]Glucose values in mg/100 ml
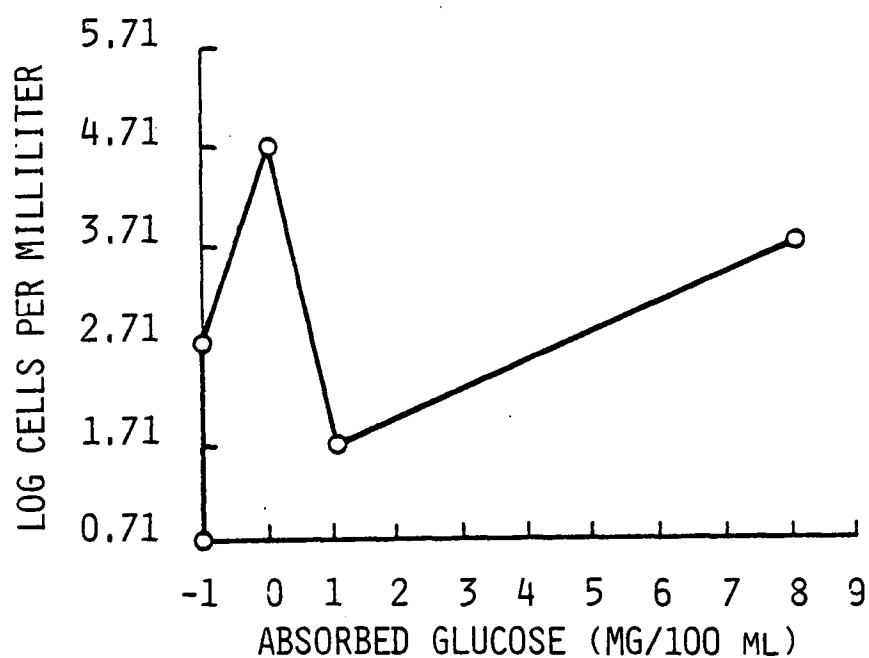
Figure 9.   Absorbed glucose mg/100 ml versus
            P. putrefaciens cells/ml, study 5:
            6 hours incubation and recovered from
            reconstituted NDM.

Table 12. Glucose absorption test results showing dilution, optical density, initial cells/ml, glucose concentration from graph, and net glucose absorption for Pseudomonas putrefaciens. 6.25 hours incubation. Study 6.

Dilution	Optical density	Glucose present[a]	Glucose Absorbed[a]	Log cells per ml[b]
10^4	0.143	33	3	4.15
10^5	0.149	33	3	3.15
10^6	0.136	31	1	2.15
10^7	0.144	34	4	1.10
10^8	0.153	34	4	0.45
Control	0.136	30	–	0.00

[a]Glucose values in mg/100 ml

[b]Cell recovery percentage was 53.3 when P. putrefaciens was recovered from nonfat dry milk
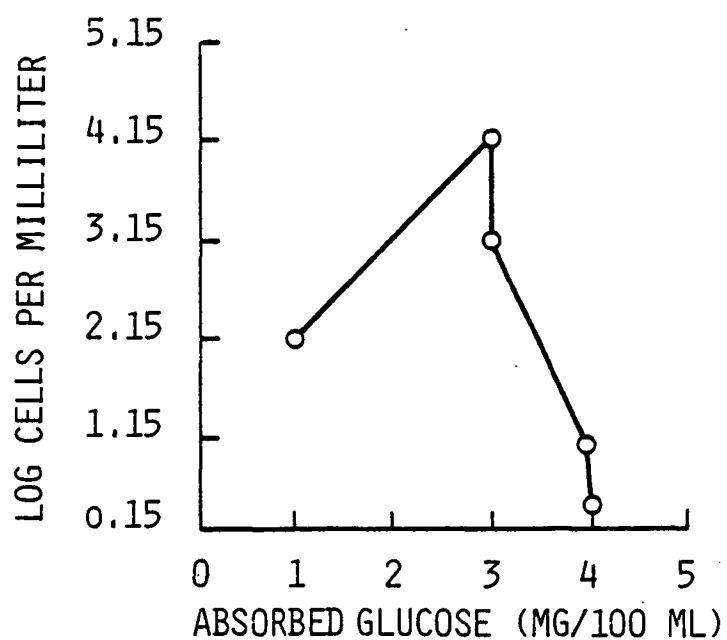
Figure 10. Absorbed glucose mg/100 ml versus
P. putrefaciens cells/ml, study 6:
6.25 hours incubation and average
of four cell recoveries from NDM.

the procedure proved insensitive in the ability to absorb glucose and differentiate between serial dilutions of cells (Figure 11, Table 13).

Previous growth media used for P. putrefaciens and P. fluorescens were tryptone, yeast extract, glucose lactose broth and reconstituted NDM. The oversaturation of cells with glucose and/or lactose may have adversely affected glucose adsorption.

In a further attempt, Escherichia coli cells were subjected to the same test as the two pseudomonads for comparison. This microorganism was grown in nutrient broth, to decrease possible oversaturation with carbohydrate. In modifying the methodology, glucose was added to nine ml of bacterial cell cultures and incubated at 27 C in a temperature controlled shaker to improve aeration and absorption. Table 14 summarizes the experimental results. There is no apparent relation-ship between glucose adsorption and cell numbers. The absorption first increases, then peaks, and finally exhibits an inconsistent pattern (Figure 12). This microorganism obviously lacked sensitivity for glucose absorption while using this particular procedure. The sensi-tivity of the test is apparently limited to measurement at the level of 25 mg/100 ml (0.25 mg/ml). Mickelson (126) in his glucose trans-port study with Streptococcus agalactiae found that 24 nanomoles of glucose were transported (or absorbed) per mg of dry cells in 2.5 minutes. This is equivalent to approximately 0.004 mg glucose/mg dry weight cells. Since the sensitivity of the test is basically 0.25 mg/ml, a discrepancy arises. It seems the lower sensitivity of the test may not be adequate to detect differences in absorption.

Table 13. Glucose absorption test results showing dilution, optical
density, initial cells/ml, glucose concentration from graph,
and net glucose absorption for <u>Pseudomonas</u> <u>fluorescens</u>.
6 hour incubation. Average of three studies.

Dilution	Optical density	Glucose present[a]	Glucose absorbed[a]	Log cells per ml
10^1	0.863	191	59	6.99
10^2	0.913	202	48	5.99
10^3	0.886	196	54	4.99
10^4	0.901	199	51	3.99
10^5	0.896	197	53	2.99
10^6	0.877	194	56	1.99
10^7	0.913	204	46	0.91
10^8	0.935	208	42	0.00
Control	1.180	250	--	0.00

[a]Glucose values in mg/100 ml
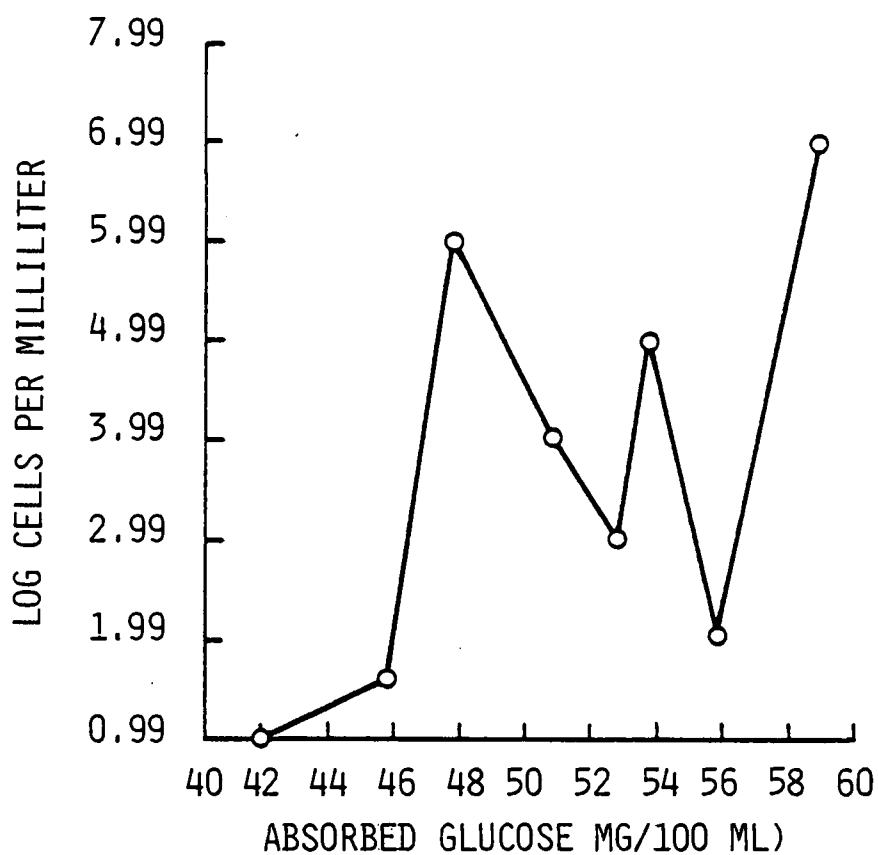
Figure 11. Absorbed glucose mg/100 ml versus
P. fluorescens cells/ml, study 7:
6 hours incubation and average of
three tests using pure cultures.

Table 14. Glucose absorption test results showing dilution, optical density, initial cells/ml, glucose concentration from graph, and net glucose absorption for _Escherichia coli_. 1 hour incubation. Cells shaken at 27 C, 1 mg/ml of glucose added to 9 ml of cells.

Dilution	Optical density	Glucose present[a]	Glucose absorbed[a]	Log cells per ml[b]
10^1	0.491	109	29	4.78
10^2	0.480	106	32	3.78
10^3	0.482	103	35	2.78
10^4	0.472	104	34	1.78
10^5	0.476	105	33	0.78
Control	0.593	138	--	0.00

[a] Glucose values in mg/100 ml

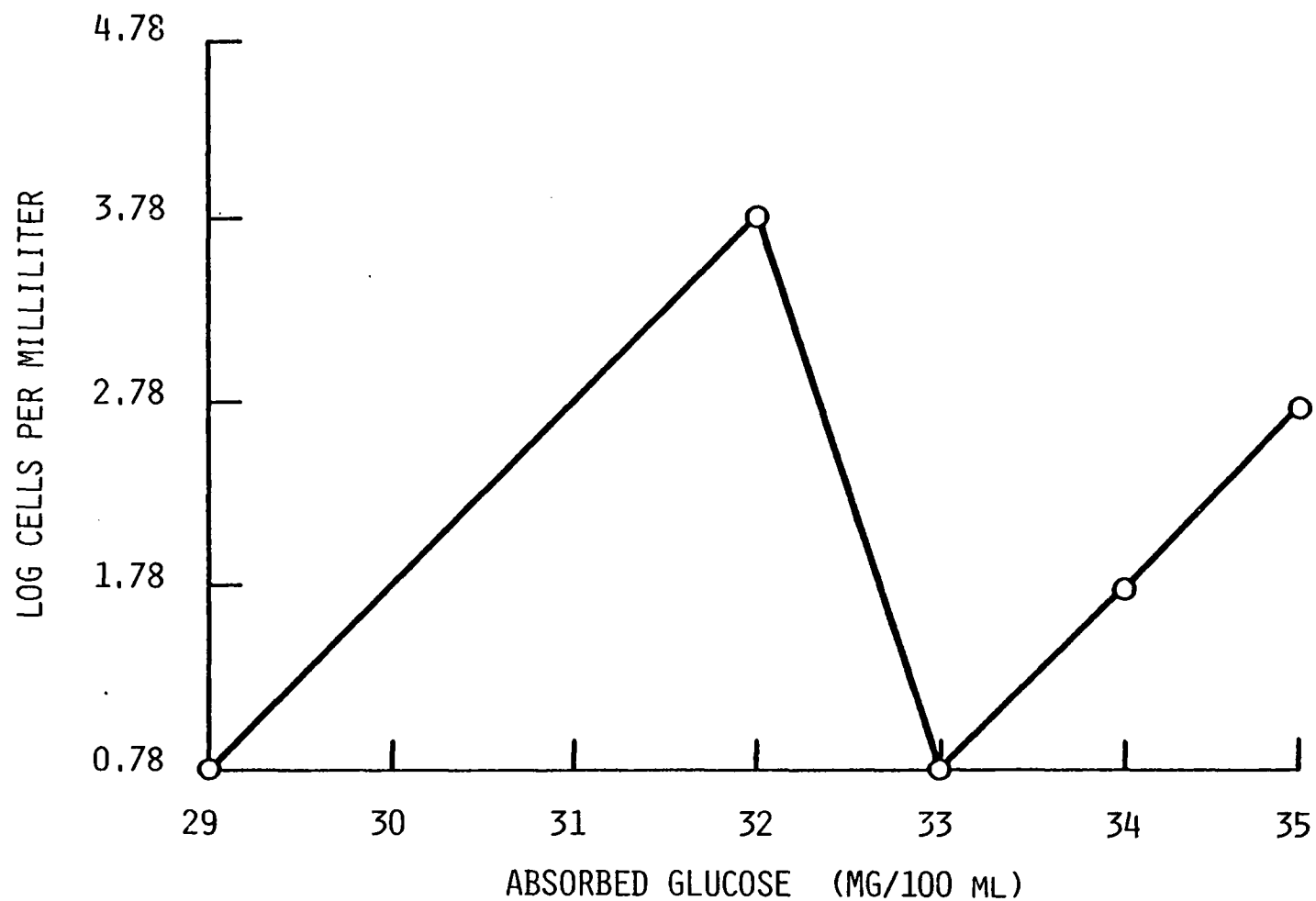[b] Cell recovery was 71.5% when _E. coli_ was recovered from nonfat dry milk (NDM)

Figure 12. Absorbed glucose mg/100 ml versus E. coli cells/ml, study 8:
1 hour incubation, cells shaken at 27 C and one mg/ml of glucose
added to nine ml of cells.

The data from this study show glucose absorption, yet there appears to be little difference in each serial dilution. According to Mickelson's study (126), there should be no absorption. Changes in glucose determination by the procedure may be governed by the influence of dilution. This contention may be supported by observation of an E. coli study. Addition of small concentrations of glucose were added in approximately 10-fold amounts compared to earlier studies. The optical densities were in the range of 0.500, similar to the control; nine ml of cell preparation were treated with glucose and the procedure performed. The samples were diluted and the optical densities observed. If glucose concentrations were added to only one ml cell preparations, then the optical density range would be outside the sensitivity of the procedure. The accuracy and sensitivity thus seemed to be governed by dilution effects.

E. coli has a slightly longer generation time than most typical psychrotrophic pseudomonads at room temperature (91). Even if the cells are not in the logarithmic growth phase, cell metabolism is active, and the microorganisms' cell size increase, without cell division occurring.

The cell populations of P. putrefaciens, P. fluorescens, and E. coli approximated a range of 10^1 to 10^7 cells/ml. This range of bacterial cells may not have been sensitive enough to absorb B-D-glucose in quantities detectable by this procedure. This procedure performed on cell populations exceeding 10^7 cells/ml may demonstrate improved sensitivity to B-D-glucose absorption and differentiation between serial dilutions of bacterial cells.

Carbon-14 labeled 2-deoxyglucose affixative test

Carbon-14 labeled 2-deoxyglucose is a nonmetabolizable analog glucose (126). Mickelson (126) demonstrated inhibited and unhibited transport of this analog across cellular membranes of Streptococcus agalactiae. In excess of 24 nanomoles of 2-deoxyglucose (within 2.5 minutes) were transported across the membrane per mg dry weight of cells (126).

In this study, Mickelson's technique (126) was adapted in an attempt to relate the amount of detectable adsorption of 2-deoxyglucose to cell numbers.

Table 15 displays results of applying 0.5 uCuries/5 microliters (ul) quantities of 2-deoxyglucose (1.58 nanomoles or 0.26 ug), to pure cultures of E. coli. Figure 13 shows the relationship of disintegrations per minute (DPM) or radioactivity absorbed per ml of cell numbers (approaching 10^8 cells/ml). Absorption of 2-deoxyglucose increased as cells numbers increased (Figure 14). The data curve typified a negative logarithmic relationship. Decreased adsorption was observed for initial cell populations, followed by a substantial increase in absorption and eventual leveling-off effect.

In the case of gram-positive Streptococcus agalactiae, absorption was in the nanomole range while E. coli appears to absorb lower amounts of 2-deoxyglucose in the picomole range.

Studies on amino acid and galactoside uptake were reviewed by Leder (115). These studies show that when E. coli bacteria were incubated with radioactive substrate at 25 to 37 C, the subsequent

Table 15. Carbon-14 labeled 2-deoxyglucose affixative test results showing the number of DPM absorbed, original DPM added, percent DPM absorbed, and cells/ml for Escherichia coli. Picomoles are calculated.

Picomoles	DPM absorbed	DPM added[a]	Percent absorbed[b]	Log cells per ml
179.5	5.01	5.95	11.43	8.34
171.4	4.99	5.95	11.09	8.23
156.5	4.95	5.95	9.97	8.04
72.4	4.65	5.95	4.61	7.95
6.3	3.55	5.95	0.40	7.46
117.2	4.83	5.95	7.61	8.00 Mean

[a]Average DPM values calculated for one mean; log count

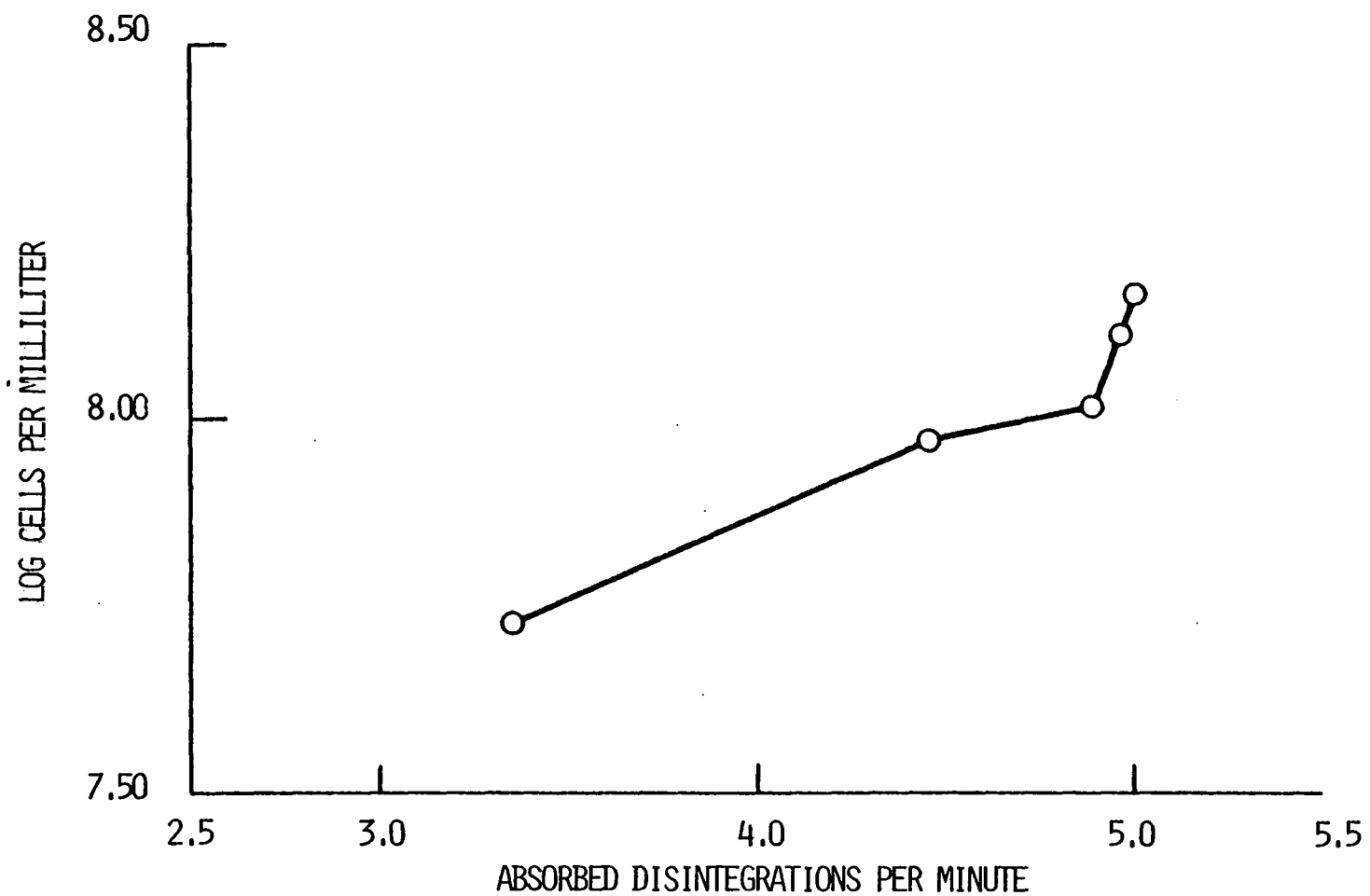[b]Percentages corrected using corrected mean DPM value

Figure 13.   Absorption of carbon-14 labeled 2-deoxyglucose versus log cells/ml for pure cultures of E. coli.

Figure 14. Absorption of carbon-14 labeled 2-deoxyglucose (picomoles) versus log cells/ml for pure cultures of E. coli.

reaction or absorption could be stopped by chilling to 0 C. The effect of wash temperature on retention of substrate pools was significant. Wash temperatures of 10 to 25 C revealed no significant deviation from 100% retentions of radioactivity counts observed. However, from 0 to 10 C wash temperature, changes in observed count retentions were profound. Radioactive counts (DPM) decreased from 100% to <5% retention (115).

Mickelson (126) found similar results in his study. Cells washed with 0.05 M phosphate (pH 7.0), at 25 to 35 C, retained at least twice as much radioactivity as when washed with buffer 4 C (126). The ice-cold wash caused excessive leakage of the labeled substrate that had been absorbed by the cells (115). The importance of wash temperature could explain some variations in absorption if careful temperature control was not maintained.

Table 16 shows results of an experiment conducted to investigate dilution effect and sensitivity in absorption of E. coli. Figure 15 reflects the relationship of cell numbers ranging from 10^1 to 10^7 cells/ml versus absorbed counts (DPM).

Referring to the lower limit of the first study, where cell numbers were 2.9×10^7 cells/ml, the absorption was larger for approximately the same amount of cells/ml, 1.8×10^7 cells/ml at the upper limit observed in this study. This could be explained by temperature of wash solutions or the sensitivity in dilution accuracy.

The lower limit of sensitivity seems to be approximately 2 counts per 10^4 cells/ml. As figure 14 indicates, the inconsistencies in counting become evident as the lower limits of sensitivity are

Table 16. Carbon-14 labeled 2-deoxyglucose affixative test results showing the number of DPM absorbed, original DPM added, percent DPM absorbed, and serial dilutions of Escherichia coli in cells/ml. All counts and DPM are logarithmic.

Dilution	DPM absorbed	DPM added[a]	Percent absorbed[b]	Log cells per ml
10^1	3.95	5.96	0.970	7.26
10^2	3.10	5.96	0.140	6.26
10^3	3.46	5.96	0.320	5.65
10^4	3.08	5.96	0.130	4.65
10^5	3.09	5.96	0.135	3.65
10^6	2.80	5.96	0.070	2.63
10^7	2.95	5.96	0.100	1.15

[a]Average DPM values calculated for one mean; log count

[b]Percentages corrected using corrected mean DPM value

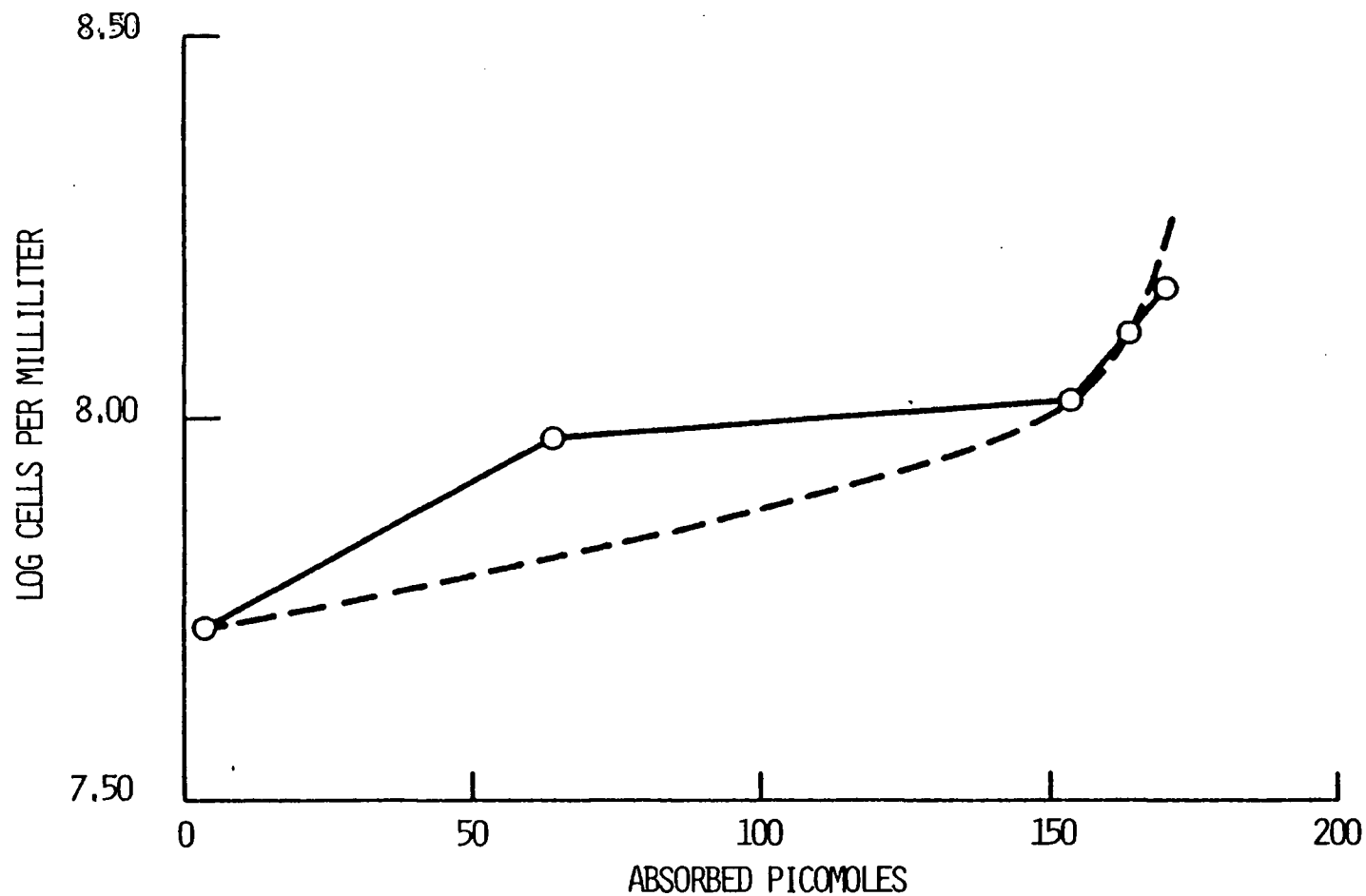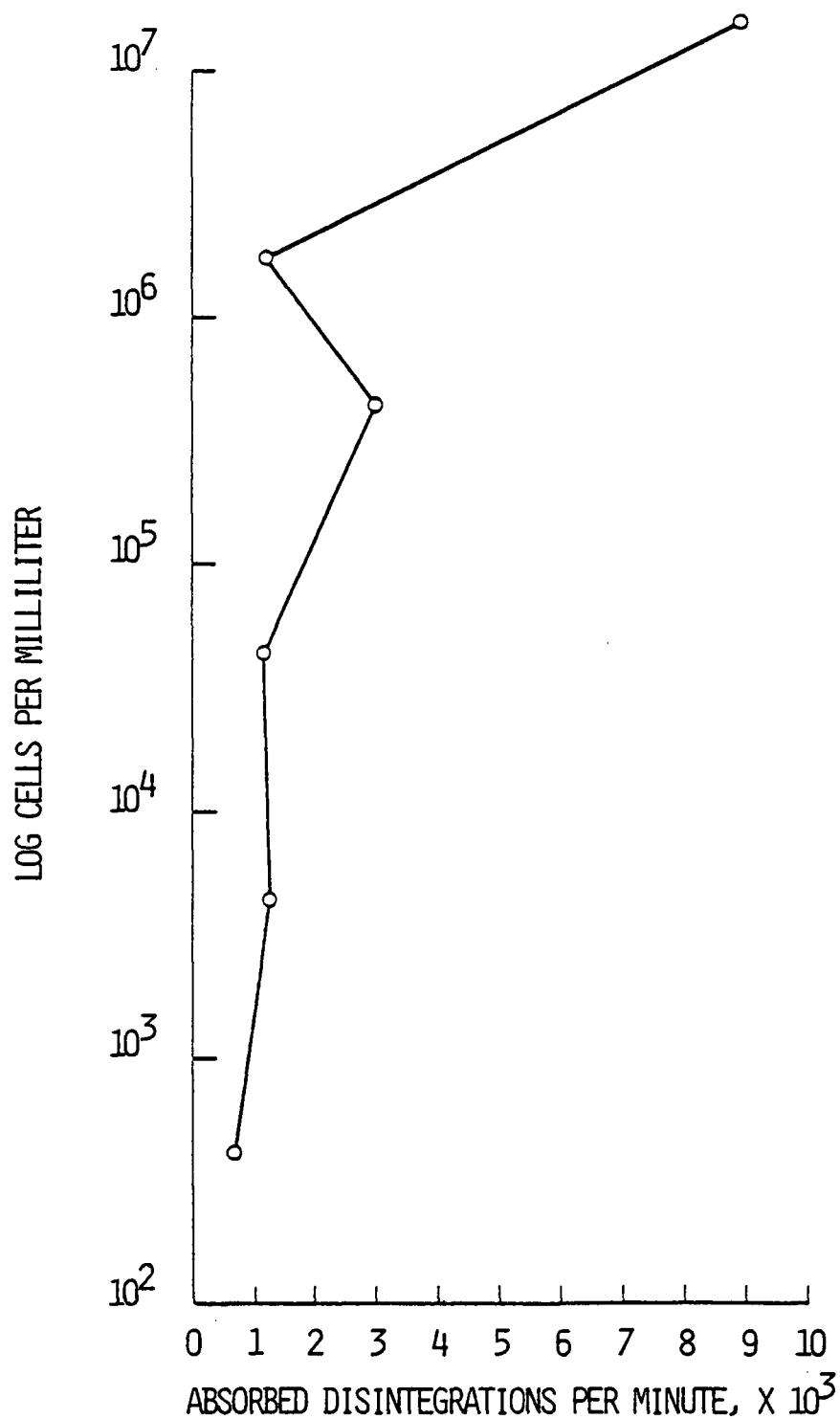Figure 15. Absorption of carbon-14 labeled 2-deoxyglucose versus
log cells/ml (range 10^1 to 10^7) for pure cultures of
E. coli.

approached. The results show highly variable distribution around the plotted line (ie., the curve approximating the relationship between 2-deoxyglucose absorption and cell numbers in this range).

To better observe the relationship between absorption and cell numbers at respective serial dilutions (Figure 15 and Table 16) cell numbers were increased to the range of 10^7 to 10^{11} cells/ml. Table 17 reflects the results of this application. In the first dilution (10^0), problems with blockage of the pores in millipore filters, were encountered. These problems were alleviated with further testing. Significant absorption, as reflected by high bacterial counts, was observed when the cell numbers increased to 10^{11} cells/ml. Figure 16 shows the relationship between absorption and cell numbers as 10^{11} cells/ml are approached. The curve appears to be similar to that in the initial E. coli experiment, or negative logarithmic relationship. In this study with from 10^8 to 10^9 cells/ml there was a significant increase in absorption, then a leveling off effect which approached 10^{10} cells/ml. At approximately 10^{11} cells/ml there was a decrease in absorption as a result of extended filtration time.

To overcome the filtration problem, the initial dilution was changed to approximately 1.33 times that of the second dilution (or 10^1). This modification improved filtration rates and decreased clogging of millipore filter pores. The increased radioactive counts reflected by these improvements further demonstrated the relationship between 2-deoxyglucose absorption and cell numbers above the 10^1 dilution (ie., the extension of the curve above this level).

Table 17. Carbon-14 lableled 2-deoxyglucose test results showing the number of DPM absorbed, original DPM added, percent DPM absorbed, and cells/ml for E. coli. Cell number range is 10^7 to 10^{11}.

Dilution	DPM absorbed	DPM added[a]	Percent absorbed[b]	Log cells per ml
$10^{0[c]}$	4.82	6.11	4.856	11.39
10^1	5.06	6.11	8.940	10.45
10^2	4.94	6.11	6.705	9.45
10^3	4.36	6.11	1.772	8.45
10^4	3.38	6.11	0.186	7.45

[a]Average DPM values calculated for one mean; log count

[b]Percentages corrected using corrected mean DPM values

[c]Dilution not completely filtered

Figure 16. Absorption of carbon-14 labeled 2-deoxyglucose versus log
cells/ml (range 10^7 to 10^{11}) for pure cultures of E. coli.

Table 18 indicates the results of the experiment with P. fluorescens. The curve for P. fluorescens (Figure 17) is logarithmic; while the absorption curve for E. coli (Figure 16) appears negatively logarithmic. P. fluorescens seems to absorb 2-deoxyglucose at a lower rate than E. coli.

Figures 18 and Table 19 include absorption data for P. putrefaciens. This psychrotrophic organism appears less susceptible to 2-deoxyglucose absorption.

Pseudomonas putrefaciens was incubated at 30 C and P. fluorescens was incubated at 22 to 25 C to improve retention of radioactivity. These temperatures approximated the optimal temperatures of growth for both organisms (31). For both pseudomonads, the large increase in absorbed DPM was between 10^9 cells/ml and 10^{10} cells/ml or the second and third dilution as opposed to the third and fourth dilutions in the E. coli experiments. This appears to determine the configuration of the curves for each respective microorganism and the overall relation- ships for absorption.

The decreased absorption in the P. putrefaciens study illustrates the potential difference between microorganisms within the same genus.

Essentially all Gram-negative organisms that utilize glucose will absorb by means of the phosphotransferase mechanism. Retention and absorption will probably be controlled by strict affinity of the organism to glucose utilization, ie. affinity for its analog 2- deoxyglucose.

Table 18. Carbon-14 labeled 2-deoxyglucose test results showing the
number of DPM absorbed, original DPM added, percent DPM
absorbed, and cells/ml for Pseudomonas fluorescens.
Cell number range is 10^7 to 10^{10}.

Dilution	DPM absorbed[a]	DPM added[b]	Percent absorbed[c]	Log cells per ml
1.33×10^1[d]	4.50	5.87	4.320	10.63
1.00×10^1	4.47	5.87	4.045	10.51
1.00×10^2	3.67	5.87	0.631	9.51
1.00×10^3	3.09	5.87	0.169	8.51
1.00×10^4	2.99	5.87	0.133	7.51

[a]Log count

[b]Average DPM values calculated for one mean

[c]Percentages corrected using corrected mean DPM value

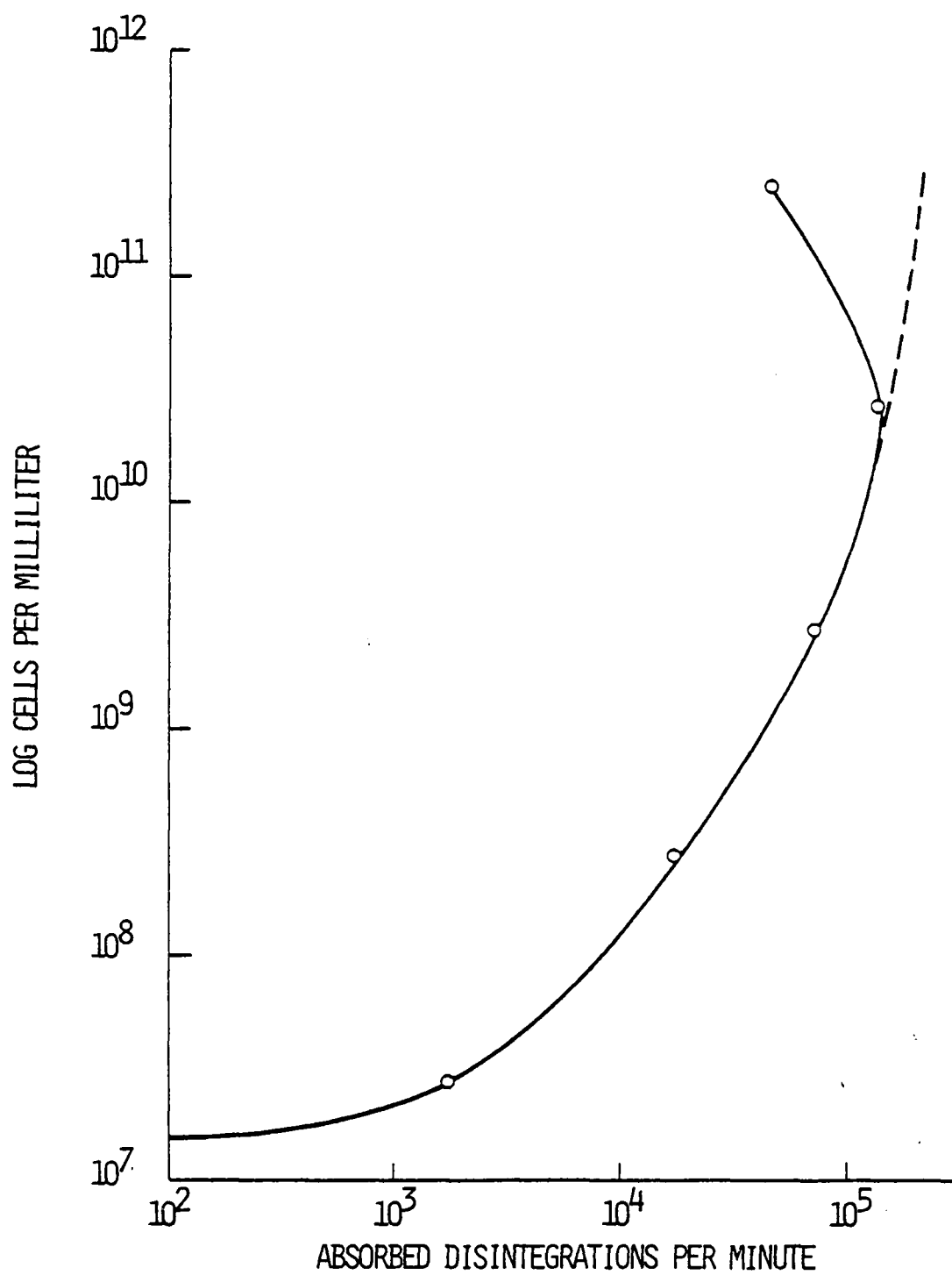[d]Filtration difficulty circumvented by higher dilution

Figure 17. Absorption of carbon-14 labeled 2-deoxyglucose versus log cells/ml (range 10^7 to 10^{10}) for pure cultures of P. fluorescens.

Table 19. Carbon-14 2-deoxyglucose affixative test using
Pseudomonas putrefaciens as test organism with cell
number range of 10^7 to 10^{10}.

Dilution	DPM absorbed	DPM added[a]	Percent absorbed[b]	Log cells per ml
1.33×10^1[c]	4.15	5.92	1.717	10.66
1.00×10^1	4.07	5.92	1.410	10.51
1.00×10^2	3.80	5.92	0.760	9.51
1.00×10^3	3.11	5.92	0.158	8.51
1.00×10^4	2.91	5.92	0.098	7.51

[a]Average DPM values calculated for one mean; log count

[b]Percentages corrected using corrected mean DPM value
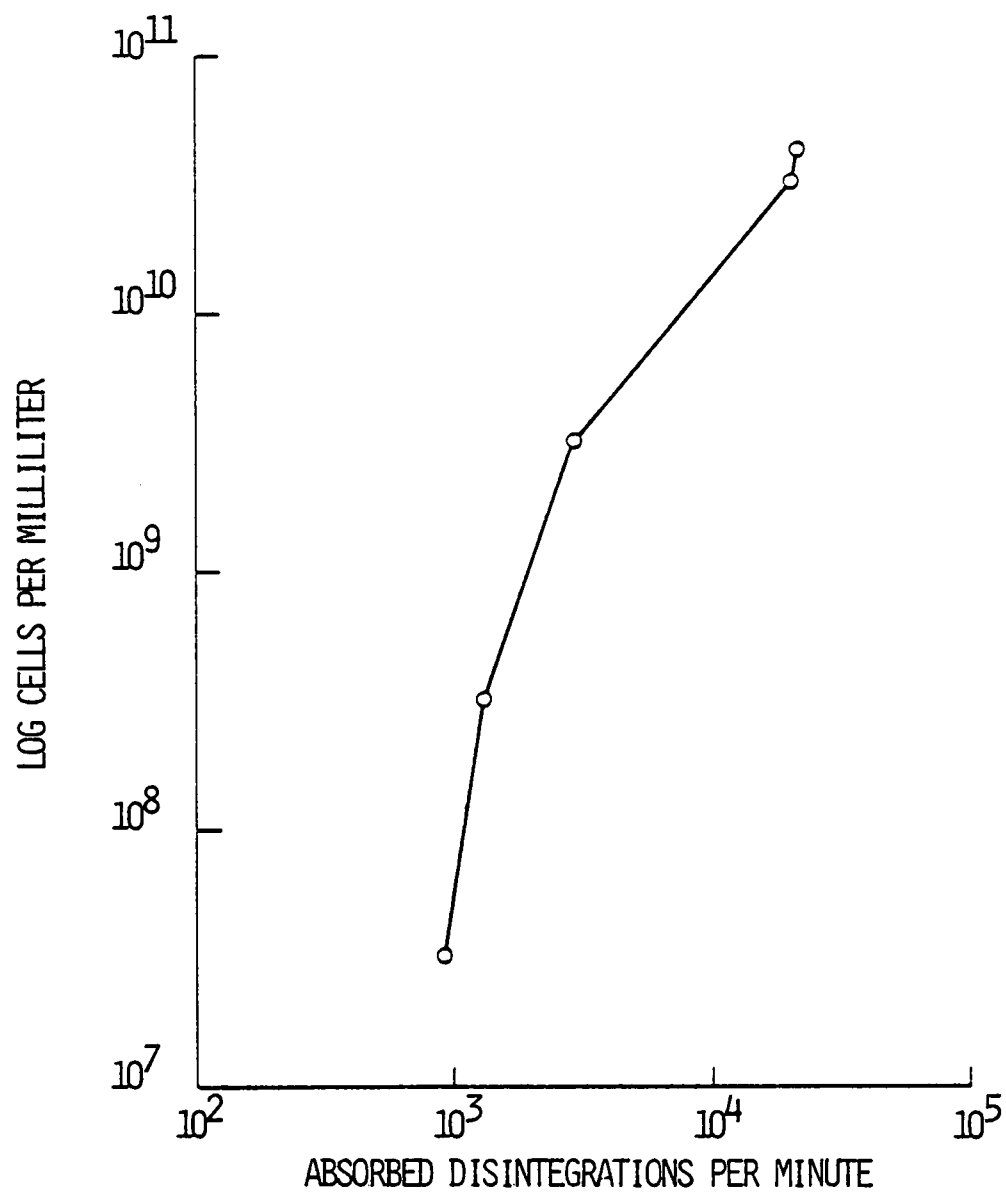
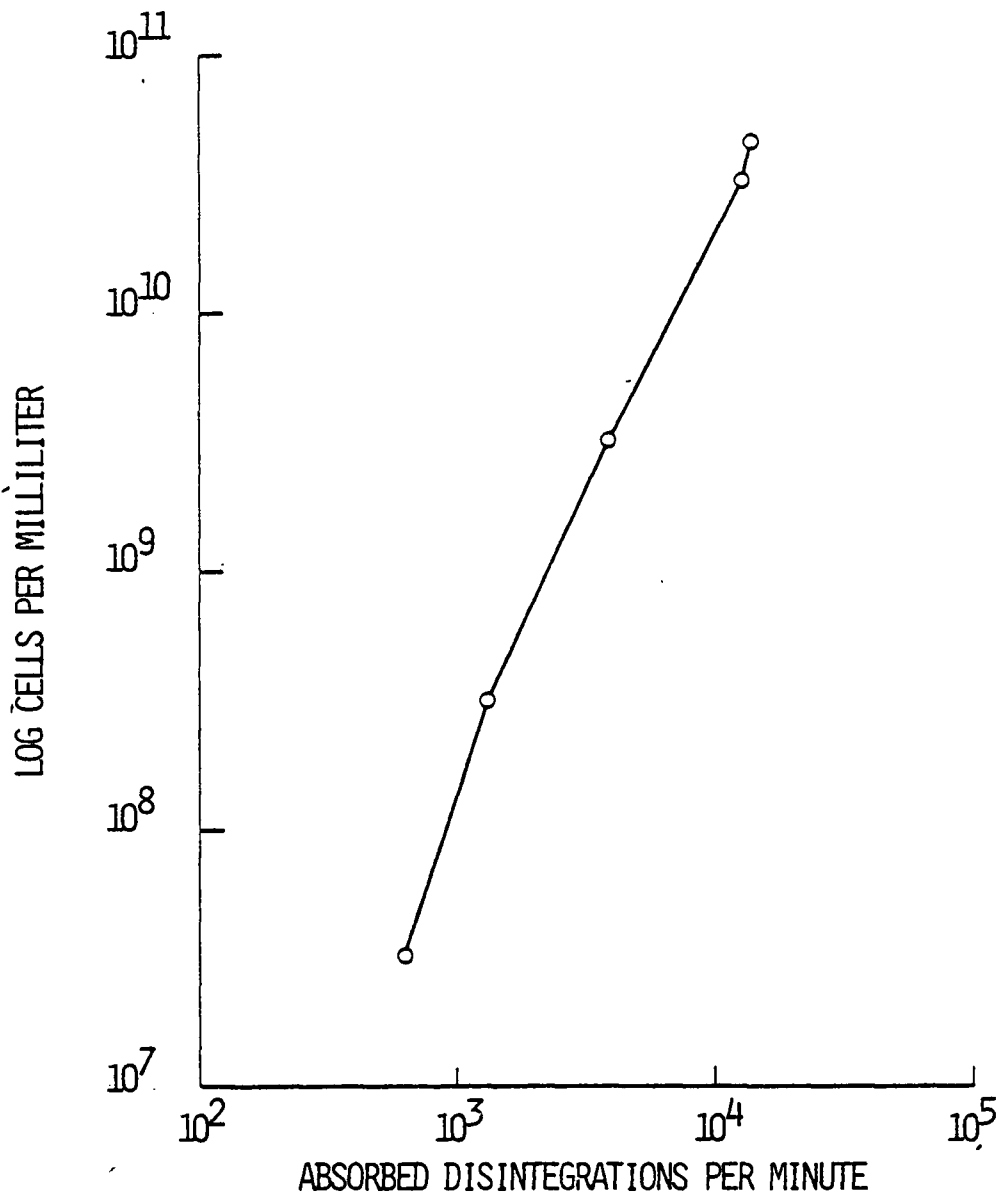[c]Filtration difficulty circumvented by higher dilution

Figure 18. Absorption of carbon-14 labeled 2-deoxyglucose versus log cells/ml (range 10^7 to 10^{10}) for pure cultures of of P. putrefaciens.

As Leder (115) demonstrated, strict osmotic control was important to the permeability of E. coli and could possibly be applied to many Gram-negative bacteria including the pseudomonads. Standard methods phosphate buffered water (5) was used in dilution blanks for the E. coli and pseudomonad studies. It was reasonable to assume that phosphate buffered water would not interfere with the test, since 0.05 M phosphate was used as a rinsing agent in Mickelson's study (126). Earlier use of 0.1% peptone in dilution blanks and rinse may or may not have imparted improved osmotic stability to the system.

Application to pasteurized fluid milk - For preliminary application of the procedure to pasteurized fluid milk, the test was first conducted on milk held one day at 4 C and then on the same source of milk stored for eight days at 7 C.

Initial absorption (day-one) was indistinct (showed little differentiation between cell dilutions) as indicated by Figure 19 and Table 20. Absorption of 2-deoxyglucose was more consistent when milk samples were stored at 7 C for eight days. Clear differentiation between serial dilutions of bacteria was observed.

In the fresh product experiment (day-one), the maximal bacterial counts achieved only 10^2 cells/ml, while at day-eight (storage at 7 C), the maximal bacterial count reached an upper limit of 10^7 cells/ml, total bacterial count. However, the maximal radioactive counts achieved for 10^2 and 10^7 cells/ml were approximately equivalent. This discrepancy led to a control experiment illustrated in Table 21. Milk

Table 20. Carbon-14 labeled 2-deoxyglucose affixative test results showing the number of DPM absorbed, original DPM added, percent DPM absorbed, and cells/ml for commercial pasteurized milk sample. All counts and DPM are logarithmic.

| Dilution | Day 1 | | | |
	DPM absorbed	DPM added[a]	Percent absorbed[b]	Log cells per ml
10^{0c}	3.96	5.95	1.021	2.28
10^1	3.00	5.95	0.113	1.28
10^2	2.98	5.95	0.107	0.28
10^3	2.87	5.95	0.082	0.00
10^4	2.96	5.95	0.103	0.00

	Day 8			
1.33×10^{1d}	3.91	6.01	0.792	6.95
1.00×10^1	3.55	6.01	0.344	6.83
1.00×10^2	3.41	6.01	0.251	5.83
1.00×10^3	3.04	6.01	0.106	4.83
1.00×10^4	3.01	6.01	0.100	3.83

[a]Average DPM values calculated for one mean; log count

[b]Percentages corrected using corrected mean DPM value

[c]Cell recovery from milk sample was 29.3%

[d]Filtration difficulty circumvented by higher dilution and cell recovery from milk sample was 56.0%
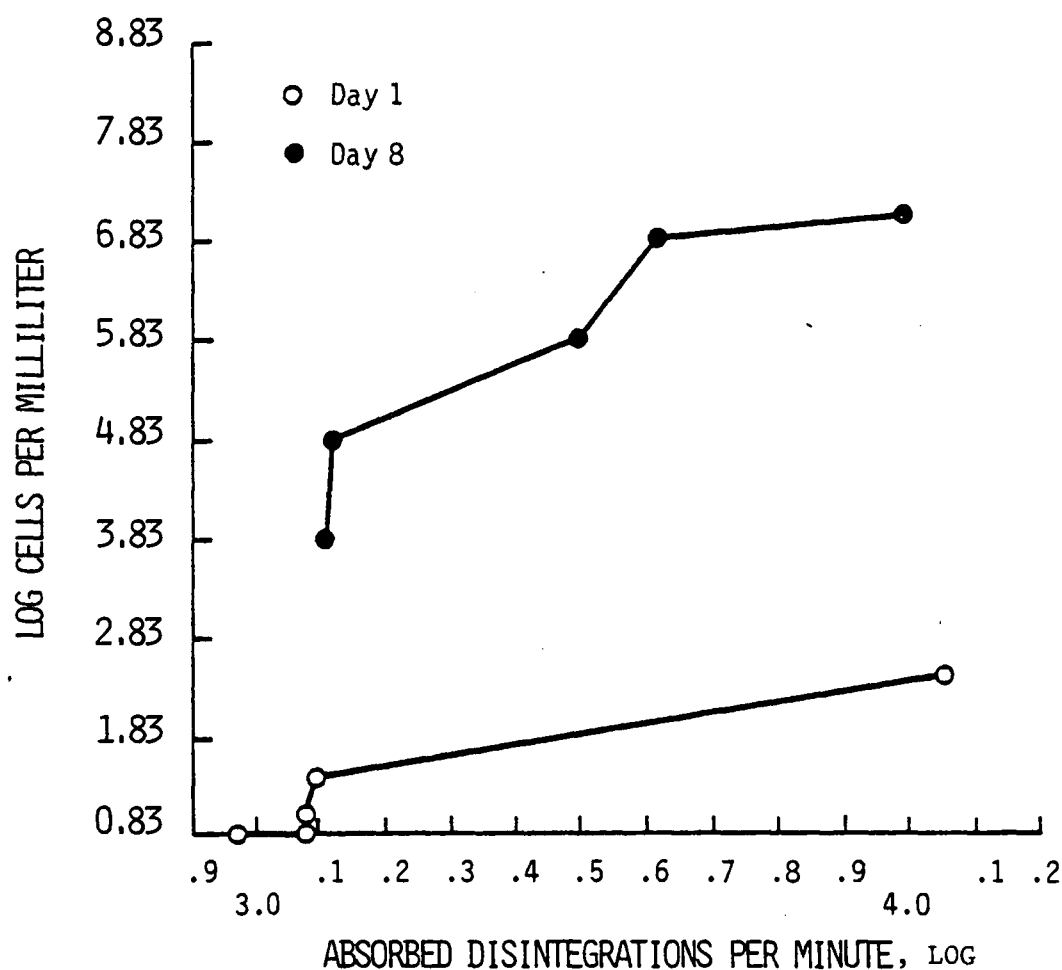
Figure 19. Absorption of carbon-14 labeled 2-deoxyglucose by bacterial cells found in concentrated commercial fluid milk samples, (different samples, but same lot): stored for one and eight days at 4 C and 7 C, respectively.

Table 21. Control study applying carbon-14 2-deoxyglucose affixative test to sterile commercial milk sample.[a] All counts and DPM are logarithmic.

Dilution	DPM absorbed	DPM added[c]	Percent absorbed[d]	Log cells per ml
10^{0}[b]	4.22	5.60	4.399	0.00
10^{1}	3.31	5.60	0.452	0.00
10^{2}	2.81	5.60	0.178	0.00
10^{3}	2.78	4.99	0.617	0.00
10^{4}	2.82	4.99	0.683	0.00

[a]Study to evaluate absorption by sterile normal milk of radioactively applied; normal milk having all components intact similar to commercial milk product

[b]Filtration difficulty and incomplete filtration

[c]Average DPM values calculated for one mean for the first three dilutions, then last two dilutions were averaged but radioactive supplies were limited; log count

[d]Percentages corrected using corrected mean DPM value, but percentages increased due to different levels of radio-activity applied
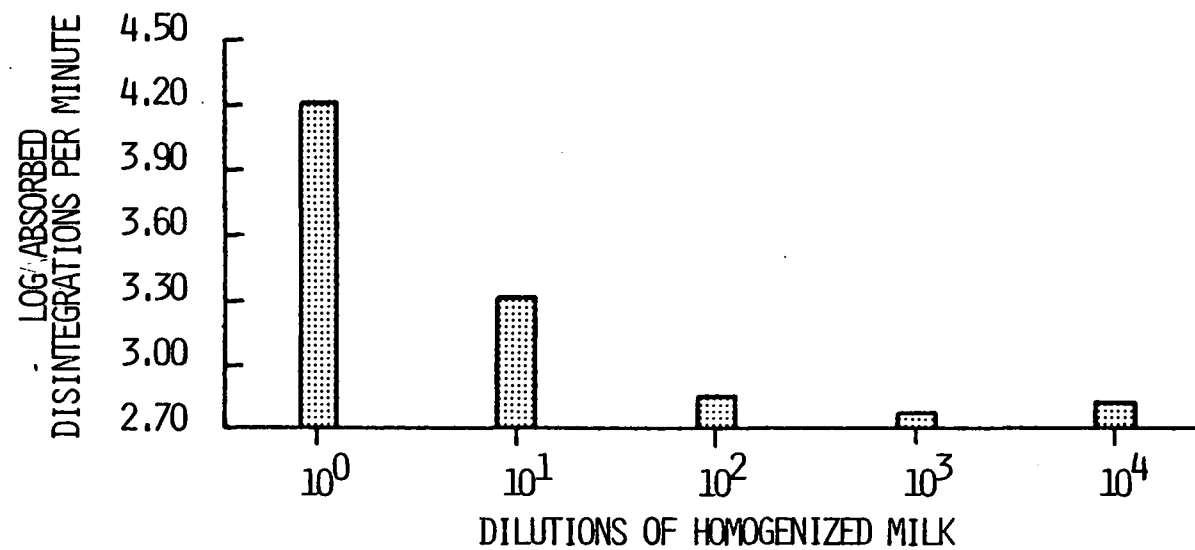
Figure 20. Absorption of carbon-14 labeled 2-deoxyglucose by dilutions of sterile homogenized fluid milk samples.

sterilized at 121 C for 15 minutes, 16 psi., and the analog applied

to dilutions. The initial dilution (or 10^0) absorbed relatively high

amounts of radioactivity, even though it was not completely filtered.

The same results were observed for the 10^1 dilution. When the sample

became more diluted, the amount of absorption decreased considerably

(Figure 20).

The constituents of the milk seemed to impair millipore filtration,

yet sample preparation seemed to overcome this interference. Radio-

activity was absorbed by sterile diluted milk, unprepared by the pre-

viously mentioned sample concentration procedure. These results would

seem to involve either the milk fat or solids-non-fat portion of milk

as interference substances for this specific analytical procedure.

Testing individual bacterial cell sensitivities of absorption

with adequate controls, will provide data helpful in determining cell

numbers for these microorganisms.

Background radiation was apparently detected when bacterial cells

were filtered onto Millipore pads without the addition of the radio-

active analog. The radioactive counts were insignificant and compara-

ble to normal background counts.

The sensitivity of the test seemed to range from 2 counts per 10^4

cells/ml at the lower end for pure cultures of cells, while sensitivity

appeared to be 8 counts per 10^3 cells at the upper end applied to

mixed milk concentrates and 2 counts per 10^2 cells/ml at the lower end.

CONCLUSIONS

Three rapid methods for the estimation of psychrotrophic bacterial counts in fluid pasteurized milk were studied in this investigation. Methods included the Limulus amoebocyte lysate test (LAL), the B-D-glucose absorption test, and the carbon-14 labeled 2-deoxyglucose affixative test.

The LAL test indicated a cell numbers sensitivity, in the range of 10^1 to 10^3 cells/ml, based on total bacterial psychrotrophic counts. The following disadvantages outweigh apparent advantages demonstrated by the test in its usefulness as a laboratory aid:

1) the reagent is relatively expensive. The gel test with endo-
    toxin standards and negative controls costs $80.00 ($4.00
    per vial). This represents a cost of approximately $12.00-
    $20.00 per test.

2) The results require subjective judgement. The determination
    of coagulation endpoint is made by visual observations.

3) equipment has to be completely sterile and pyrogen free.

4) pyrogen free water is expensive. This also includes any
    other required pyrogen free compounds.

The application of the improved LAL gel test or microslide method seems more practical for routine analysis by industry laboratory personnel. Its simplicity, convenience, reduced cost, and definitive endpoint determination may make it ideal for detection and estimation of psychrotrophic bacteria in fluid milk. Its value as a self-life projection test might be achieved with additional study.

The B-D glucose absorption test showed poor sensitivity for differentiation of serial dilutions of bacteria. Further testing, using higher cell numbers, could provide more meaningful results for absorption.

The carbon-14 labeled 2-deoxyglucose affixative test revealed greater sensitivity for differentiation of serial dilutions of bacteria. Improvements in filtration and dilution technique should provide greater accuracy in estimation of bacterial cell numbers. Application of larger volumes of 2-deoxyglucose with similar specific activities would improve handling proficiency and accuracy. Examining the absorption sensitivities of individual species representative of spoilage microorganisms found in fluid milk would provide an evaluation of the test's usefulness.

To reduce the cost of the procedure, (presently at approximately $7.80/test), a modification such as applying the radioactive analog to bacterial cells, counting and determining the lowest number of cells present, could be developed. This test could serve as a shelf-life projection test detecting the minimal level of contamination and decrease expenditure for the radioactive analog three fold.

Further testing is warranted, yet preliminary results provide a basis of application to estimation of bacterial cell numbers in fluid milk products.

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