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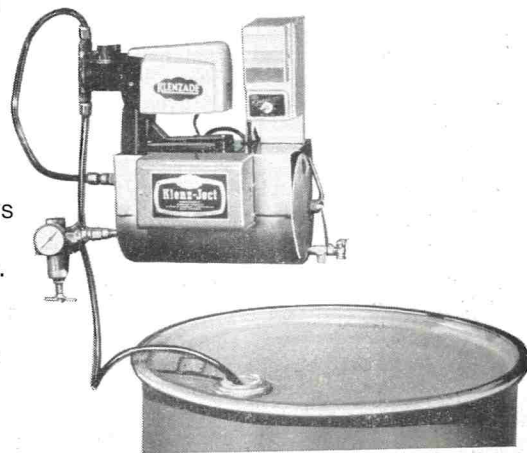laboratory supply dealers

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COMPARATIVE EFFECTIVENESS OF VARIOUS ACIDS IN REMOVING CALCIUM FROM MILK FILMS ON STAINLESS STEEL[1, 2]

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Abstract

Four acids, hydrochloric, nitric, phosphoric and lactic, in two concentrations, 0.1 and 1.0 N, were tested for comparative effectiveness in removal of calcium from stainless steel plates soiled with milk using six different soiling procedures. There were no significant differences among acids or concentrations of acid. Since nitric acid at a concentration of 0.1 N is relatively non-corrosive to stainless steel and has the same potential to remove calcium from a stainless steel surface as has hydrochloric acid, it is recommended as the acid to be used in the method developed by Heinz, et al (2).

Different methods to determine the amount of milk residue remaining on a stainless steel surface after cleaning have been developed to evaluate cleaning (3, 4, 5). A method of removing and subsequently quantifying the calcium from a surface soiled with milk was introduced by Heinz et al. (2). The method was based on the principle that calcium is ionized from caseinate when the pH is reduced to the iso-electric point of casein. Calcium was solubilized in 1 N HCl, and the quantity of calcium in the solution was analyzed by atomic absorption spectroscopy. However, the strong acid caused corrosion of stainless steel surfaces. Therefore, the objectives of this research were to find another acid which would be at least as effective as HCl and to determine whether a concentration of 0.1 N was sufficient.

Material and Methods

Chemicals

Two concentrations, 0.1 N and 1.0 N, of four acids, hydrochloric, nitric, phosphoric, and lactic, were prepared. Raw whole milk was obtained each day from a milk storage tank in the University Dairy Plant. About 60% of the milk came from the University Holstein herd and the remainder from the University Guernsey herd. An alkaline detergent (Klenzade, Liquid K) was used to clean the stainless steel plates before each experiment.

Equipment

The stainless steel plates used were type 304 with a No. 4 finish and measured 22×22 cm. Plates were subjected to a cleaning procedure in an experimental spray stand (Fig. 1) which simulated cleaning a tank wall.

Methods

The statistical experimental procedure was factorial in design with four replications. Each replication of the experiment consisted of using the four different acids at the two concentrations to remove the milk soil applied to the stainless steel surface by six different treatments (Table 1). Thus, in one replication of the experiment, 48 plates were used,

Table 1. Description of Treatments

Treatment 1: Control plates—no milk was placed on these plates. Dried at 38 C.

Treatment 2: 1.0 ml milk—spread evenly over plate with pipette. Dried at 38 C.

Treatment 3: 0.5 ml milk—spread evenly over plate with pipette. Dried at 38 C.

Treatment 4: 0.1 ml milk—spread evenly over plate with pipette. Dried at 38 C.

Treatment 5: Plates were completely submerged in milk for 10 sec and then allowed to drain in a vertical position for 1 min. Dried at 38 C.

Treatment 6: Plates were completely submerged in milk for 10 sec, allowed to drain in a vertical position for 1 min, dried at 38 C then rinsed in the experimental spray unit (Fig. 1).

TABLE 2. QUANTITIES (PPM) OF CALCIUM RECOVERED FROM STAINLESS STEEL PLATES IN FOUR ACIDS IN TWO CONCENTRATIONS
UNDER DIFFERENT CONDITIONS OF SOILING

Treatments	Acids and concentrations[1]							
	1 N HCl	0.1 N HCl	1 N HNO_3	0.1 N HNO_3	1 N Lactic	0.1 N Lactic	1 N H_3PO_4	0.1 N H_3PO_4
1[2]	3.7	6.7	4.8	5.1	5.4	4.1	5.9	5.5
2	48.9	49.8	52.3	57.3	59.4	56.3	54.9	54.8
3	28.6	28.0	26.1	27.2	33.1	29.9	29.7	30.2
4	6.9	5.2	5.8	6.0	5.2	6.2	8.5	5.6
5	51.2	42.1	39.5	49.8	52.4	54.9	39.6	47.1
6	1.5	0.1	1.6	1.7	1.7	2.3	2.2	2.5
X[3]	27.7	28.0	27.3	28.7	30.5	28.7	29.4	28.2

[1]Values are means of four replications.
[2]Plates were washed, rinsed, then sampled. The value in each column was substracted from the means for each treatment in the respective column to remove effects of residual calcium on plates.
[3]Means of treatments 2, 3, and 4.

TABLE 3. QUANTITIES (PPM) OF CALCIUM RECOVERED FROM FOUR ACIDS IN TWO CONCENTRATIONS WHEN MILK WAS ADDED
DIRECTLY TO THE ACIDS

Quantity of milk (ml)	Acids and concentrations								
	1 N HCl	0.1 N HCl	1 N HNO_3	0.1 N HNO_3	1 N Lactic	0.1 N Lactic	1 N H_3PO_4	0.1 N H_3PO_4	S. D.[1]
1.0	52.7	54.2	48.7	49.4	49.7	48.5	50.8	48.5	6.9
0.5	23.0	23.6	24.5	25.1	28.1	24.4	26.9	25.4	2.6
0.1	6.2	7.3	6.2	7.0	7.7	7.0	6.8	7.1	1.5

[1]S. D.—Standard Deviation

and in replicating the experiment four times, 192 plates were used. Only 16 of the 48 plates in one replication could be soiled and sampled in one day. Therefore, the soiling procedure used and the type and concentration of acid used to remove the soil were randomly selected for each day. Each replication of the experiment was completed before the next was begun.

The experimental procedure for each day consisted of cleaning the 16 plates by brushing them with alkaline detergent, rinsing them with tap water, and then allowing the plates to drain dry. Next, these plates were subjected to one of the six different treatments and then placed in a drying oven for 1 hr at 38 C. After drying, those plates which received treatments 1 - 5 were sampled by the method described by Anderson et al. (1). Those plates which received treatment 6 were placed in the spray stand to be rinsed with tap water at 35 C for 30 sec. The plates were then sampled.

Control samples for treatments 2 - 4 were obtained by directly pipetting the given quantity of milk into the sample bag. The four replications of the eight combinations of acid for these three treatments produced 96 samples. Control samples were not included for the other treatments because an accurate sample could not be obtained.

An additional experiment was conducted with 0.1 N nitric acid. The experiment was of factorial design with four replications. Each mean value represents four observations. Different milk was used in each replication although it was from the same source, the University Dairy Plant.

Sample analysis

Calcium analyses were preformed on a Perkin-Elmer 290 atomic absorption spectrophotometer equipped with a calcium lamp and a strip chart recorder. Acetylene was the fuel gas and filtered compressed air was the supporting gas.

RESULTS AND DISCUSSION

Data in Table 2 are corrected mean concentrations of calcium (ppm) recovered by the different acids and concentrations of acids from the stainless steel plates, after they were exposed to different soiling conditions, or from solutions to which milk was added directly. Because plates which had been cleaned but not soiled (control plates) contained an average of 5.1 ppm of calcium, it was necessary to subtract this mean from the quantity recovered from the plates soiled with various quantities of milk. These values are shown in Table 2. There were no significant differences (P <0.05) in the corrected means for 1.0, 0.5, and 0.1 ml of milk removed from plates by the various acids at either of their concentrations. There were no significant differences among replications.

A comparison of methods of application of 1.0, 0.5, and 0.1 ml of milk, "dried on" (Table 2) vs "directly injected" (Table 3) indicated that more milk was removed than was placed on the plates (P <0.05). When plates were soiled with 1.0 ml of milk, an average of 54.2 ppm of calcium was removed by all concentrations of acid as compared to 50.3 ppm in samples containing milk injected directly into the bag. When 0.5 ml of milk was dried on the plate, the amount of calcium removed was 29.1 ppm as compared to 25.1 ppm in samples into which milk was directly injected. No significant differences (P <0.05) were noted between "dried on" (7.1 ppm) and "directly injected" (6.9 ppm) samples when sample size was 0.1 ml. When 1.0 ml and 0.5 ml of milk were directly injected into the acids, a flaky pre-

TABLE 4. QUANTITIES (PPM) OF CALCIUM RECOVERED[1] IN 0.1 N NITRIC ACID FROM STAINLESS STEEL PLATES SOILED WITH DIFFERENT QUANTITIES OF MILK IN FOUR REPLICATIONS

Treatments	Replications			
	1	2	3	4
1[2]	5.1	2.5	2.6	4.4
2	57.3	61.2	53.3	52.5
3	27.2	37.1	36.1	31.6
4	6.0	6.3	3.9	5.5
5	49.8	65.0	52.9	44.4
6	1.7	−.4	1.1	−.8
X[3]	28.7	32.5	31.6	29.6

[1]Average of four plates for each replication
[2]Average quantity of calcium on clean plates
[3]Means of 1.0, 0.5, and 0.1 ml
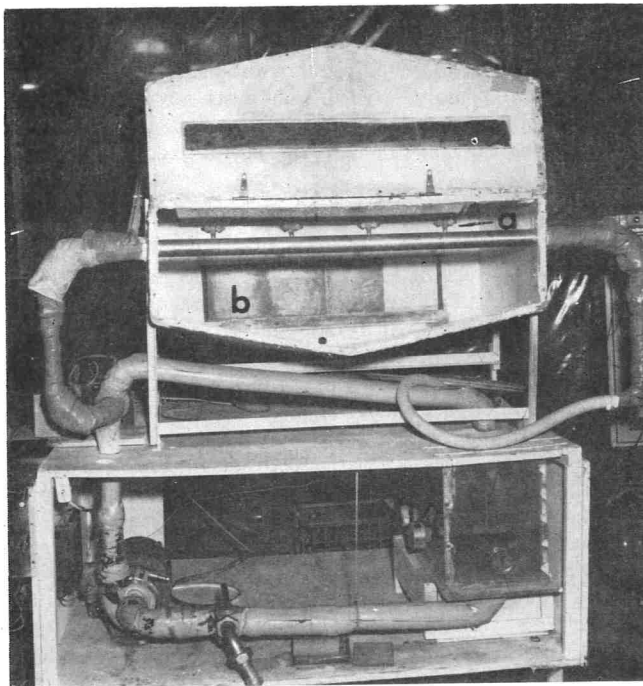
Figure 1. Experimental spray unit: (a) spray nozzle, (b) stainless steel plate.

cipitate formed suggesting that calcium could have been trapped inside the precipitate. This would account for the lower values in these samples. Also, proportionately more calcium was recovered when 0.1 ml of milk was applied compared to 1.0 ml (62 vs. 54 ppm, proportionately). This suggests the method is subject to concentration error which is probably due to entrapment of calcium in coagulated protein.

However, this does not decrease the usefulness of the test for detecting calcium residue.

There was no significant interaction (P <0.05) between concentration of milk placed on the plates and type or concentration of acid used in the removing of the milk.

The corrected means for plates dipped in milk ranged from 39.5 to 54.9 ppm of residual calcium. The corrected means for the residual calcium on plates dipped, dried, and rinsed range from 0.1 ppm to 2.5 ppm; this indicated that the 30-sec rinsing period removed most of the calcium from the plates.

Because this experiment indicated 0.1 N nitric acid effectively removed milk from the stainless steel plates in all concentrations and because this acid is preferred over the others tested, an additional experiment was conducted using only 0.1 N nitric acid. There were no significant differences (P <0.05) in mean quantities of calcium removed between replications when 1.0, 0.5, and 0.1 ml of milk were dried on the plate (Table 4).

The data indicated that the amount of calcium recovered from milk films on stainless steel plates with 0.1 N nitric acid was not significantly less than that recovered with other acids. Also, nitric acid at a concentration of 0.1 N is relatively non-corrosive to stainless steel (6). It is low in cost and easy to obtain. Therefore, nitric acid is recommended for removing calcium residue from stainless surfaces for analysis.

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MINIATURIZED TECHNIQUES FOR IMViC TESTS[1]

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Abstract

A miniaturized method for performing IMViC tests is proposed. Twenty-four gram-negative bacterial species and strains were tested by use of miniaturized and conventional methods; the results were comparable. The miniaturized method effects savings of time, space, materials, and effort.

The advantages and applications of microbiological tests using small volumes of media have been discussed in detail by Hartman *(6)*. The combination of small volumes of media in Microtiter plates and multiple inoculation techniques has been used by Fung and Miller *(4)* for rapid carbohydrate fermentation tests. Davies et al. *(2)* have also proposed a Microtiter method for carbohydrate fermentation.

IMViC (indole, methyl red, Voges-Proskauer, citrate) tests have been miniaturized *(1, 6)*, but a combined multiple inoculation Microtiter test system has not been proposed. This paper describes the use of both miniaturized techniques and multiple inoculation procedures in performing IMViC tests.

Materials and Methods

Bacterial cultures

Twenty-four gram-negative bacterial species and strains (Table 1), representing a variety of metabolic patterns, were tested. The inocula were grown in trypticase soy broth (Difco) for 24 hr at 37 C.

Vessels and multipoint inoculator

Sterile, individually wrapped, flat or round bottomed Microtiter plates and plastic covers and Microtiter loops (0.05 ml) were obtained from Cooke Engineering Co., Alexandria, Va. The multipoint inoculator was constructed by using pins fixed on the bottom of the wells of a Microtiter plate, as previously reported *(4)*, or nailed on a wood block patterned to fit into the wells of a Microtiter plate. Sterilization of the multipoint inoculator was by alcohol flaming.

Mass inoculation procedure

A master plate was prepared by placing four drops of a bacterial culture into each of the 96 wells of a Microtiter plate. Twenty-four cultures were tested in quadruplicate on each plate. These cultures were transferred from the master plate to other Microtiter plate containing liquid test media with the sterilized multipoint inoculator. Each pin head of the inoculator carries ca. 0.0006 ml of liquid.

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[1]Portion of the paper was presented at the 71st Annual Meeting of the American Society for Microbiology in Minneapolis, Minn., 2-7, May 1971.

Indole test

Sterile tryptone broth (Difco) was introduced into the wells (0.2 ml per well) of a series of Microtiter plates; the plates were then inoculated, covered, and incubated at 37 C. At intervals of 8, 12, and 24 hr, Microtiter plates were removed from the incubator for testing. To detect indole production, 2 drops of Kovac reagent were transferred by a Pasteur pipette to each well of the Microtiter plate. A red layer formed on top of the broth in the well indicated a positive reaction. Parallel conventional tests for indole production, as well as other IMViC tests, were performed according to the Difco Manual *(3)*, in each species and strain in four replicates.

Methyl red-Voges-Proskauer tests

Sterile MR-VP medium (Difco), in 0.2 ml aliquots, was placed in the wells of a series of Microtiter plates. Inoculation and incubation were performed as in the indole test. At 24-hr intervals (up to 5 days for the MR test) Microtiter culture plates were removed from the incubator to perform the MR test on one set of plates and the VP test on another set. The multipoint inoculation device was used as the "instrument" for simultaneously transferring methyl red to all 96 micro-cultures. A large petri dish (150 × 15 mm) was filled with methyl red; 4 transfers with the multipoint inoculation device carried about 0.003 ml of methyl red to the wells containing the cultures. This amount of methyl red was optimum for observation for color change. Pink or red color development in the wells indicated a positive reaction. For the VP test, Microtiter loops (0.05 ml) were used to transfer α-napthol and KOH to the cultures to detect the presence of acetyl-methyl-carbinol (pink or red color reactions); 1 loop of α-napthol followed by 1 loop of KOH gave the best results. Twelve loops were operated simultaneously to increase efficiency.

Citrate test

Koser citrate (Difco) was used for citrate utilization test. Addition of medium to the Microtiter plates, mass inoculation, and incubation were the same as for the indole test. After 8, 12, and 24 hr incubation, growth of cultures in the Microtiter wells was observed.

Results and Discussion

Results of the IMViC tests on 24 bacterial species and strains, comparing the miniaturized methods with the conventional methods, are recorded in Table 1. Results obtained with the miniaturized tests correspond directly to results obtained with the conventional tests indicating the reliability of the miniaturized tests *(1, 6)*. The incubation periods necessary for obtaining definite results were shorter for the miniaturized tests than for the conventional

Table 1. Comparison of Miniaturized and Conventional Methods for IMViC Tests[1]

Test organism	Indole		M-R		V-P		Citrate	
	Mini	Conv	Mini	Conv	Mini	Conv	Mini	Conv
	8-12 hr	24 hr	48 hr.	120 hr	24 hr	48 hr	8-12 hr	24 hr
Achromobacter parvulus	−	−	−	−	+	+	−	−
Agrobacterium radiobacter	−	−	−	−	−	−	−	−
Alcaligenes faecalis	−	−	−	−	−	−	+	+
Corynebacterium xerosis	−	−	−	−	−	−	−	−
Enterobacter aerogenes 11	−	−	−	−	+	+	+	+
Enterobacter aerogenes 11a	−	−	−	−	+	+	+	+
Enterobacter cloacae	−	−	−	−	+	+	+	+
Escherichia coli A-1	+	+	+	+	−	−	−	−
Escherichia coli B	+	+	+	+	−	−	−	−
Escherichia coli C-30	−	−	+	+	−	−	−	−
Proteus vulgaris 9434	−	−	+	+	−	−	−	−
Proteus vulgaris X19	−	−	+	+	−	−	−	−
Pseudomonas aeruginosa	−	−	−	−	−	−	+	+
Salmonella paratyphi	−	−	+	+	−	−	+	+
Salmonella pullorum	−	−	+	+	−	−	−	−
Salmonella thompson	+	+	+	+	−	−	+	+
Salmonella typhimurium	−	−	+	+	−	−	+	+
Salmonella typhosa	−	−	+	+	−	−	−	−
Serratia marcescens	−	−	−	−	−	−	+	+
Shigella alkalescens	+	+	−	−	−	−	−	−
Shigella flexneri V	−	−	+	+	−	−	−	−
Shigella flexneri W	+	+	−	−	−	−	−	−
Shigella flexneri 9748	+	+	−	−	−	−	−	−
Shigella sonnei	−	−	+	+	−	−	−	−

[1]The indole, M-R, V-P, and citrate tests were made as described in the text. Mini = miniaturized method; Conv = conventional method. The times given are the minimum incubation periods necessary for all positive cultures to respond to the miniaturized test or the recommended incubation period for the conventional test. + = positive test; − = negative test.

tests. Positive indole and citrate miniaturized tests could be observed as early as 8 hr of incubation for most cultures although 12 hr incubation time provided more definite results. "Overnight" incubation time would be a convenient schedule for these two tests. The miniaturized MR and VP tests were reduced to 48 hr and 24 hr, respectively, compared to 120 hr and 48 hr stipulated in the conventional procedures (3).

The amount of reagent to be added to the culture after growth proved to be rather critical in a study of this nature. Because of the small volume of culture, excess reagent will cause erroneous readings. The authors tested many different "instruments" such as pin-heads, pin-tails, Pasteur pipettes, tooth picks, and loops to add reagents to these cultures. The amounts added by these "instruments" was also critically tested. The data presented in Table 1 were obtained using the optimum procedures and quantities of reagents that provided results identical to those obtained using the conventional methods.

The advantages of the miniaturized methods include savings of time in operation, time in data collection, materials, and effort. The reliability of the miniaturized methods for the IMViC tests was demonstrated by direct comparison of 24 bacterial species and strains grown and tested by both the miniaturized and conventional methods. The multiple inoculation device used in these procedures may also be utilized for conducting simplified tests such as oxidase production, phenylpyruvic acid reaction, oxidation of gluconates (5), and various other routine microbiological tests using liquid media (6).

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A Research Note

THE VALUE OF ADDING IRON AND NONFAT DRY MILK SOLIDS TO A MILK DIET IN THE RECOVERY OF ANEMIC RATS

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Abstract

Weanling rats were fed only homogenized vitamin D milk for 3 weeks at which time the body weights were about 105 g and the hematocrit values were 20.4 for males and 22.8 for females. They were then fed milk plus 10 ppm elemental iron in the form of ferric pyrophosphate, milk plus 10% added nonfat dry milk solids, or milk plus 10 ppm iron plus 10% nonfat dry milk solids. After 3 weeks on the milk-plus-iron diets, the hematocrit values for males and females were about 30 and 40, respectively. Weekly body weights and hematocrit values showed the two iron-supplemented diets to be nutritionally similar (P>.05) when rats were fed *ad libitum*. Rats fed milk plus added nonfat dry milk solids were significantly lower (P<.05) in body weight after 10 weeks of age and significantly lower (P<.05) in hematocrit value after 8 weeks as compared to rats fed either of the iron-supplemented diets.

Gross (2) found that infants fed a diet containing 24 g protein per liter plus a trace of iron had lower hemoglobin concentrations and serum iron levels than did infants that received a diet of 15 g protein per liter plus a trace of iron. A recent report (1) showed that rats fed milk containing added ferric pyrophosphate or ferric phosphate were heavier and had higher hematocrit values than did rats fed milk only. The rats, especially the males, fed the milk-plus-iron were not as heavy as rats fed a standard pelleted rat ration. This raised the question as to whether rats fed an all milk ration might be unable to consume enough protein and calories to supply their needs. The objective of the present investigation was to determine if anemic rats would respond differently, as measured by body weight and hemotocrit value, to a milk-plus-iron diet than they would to a diet containing milk plus iron plus milk solids not fat (MSNF).

Procedure

Seventy weanling rats were fed a milk-only diet for 3 weeks, during which time the average hematocrit value (a measure of the percent of red blood cells in blood) decreased from 41.7 to 21.5. The rats were then divided into three ration groups, equalizing as nearly as possible, the sexes and weights of the three groups. The first ration was homogenized vitamin D milk plus ferric pyrophosphate added at the rate of 10 ppm elemental iron. The second ration was homogenized vitamin D milk plus 10% added nonfat dry milk solids (NFDMS), and the third ration was homogenized vitamin D milk plus 10% added NFDMS and 10 ppm added iron. All rats were given free access to water and kept in teflon-coated metal cages. Body weights and hematocrit
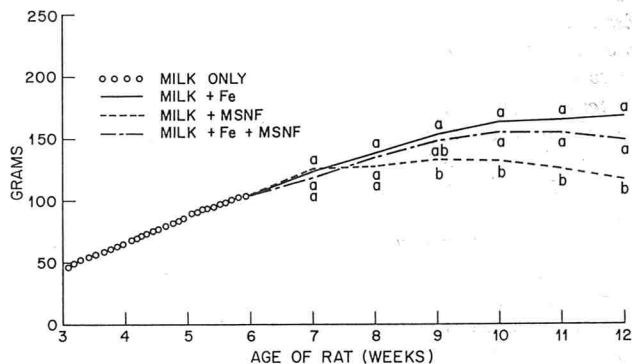
Figure 1. Influence of feeding iron and/or milk solids not fat with fluid milk on the body weight of anemic rats. Points on the same vertical line noted by the same letter are not statistically different (P>.05).
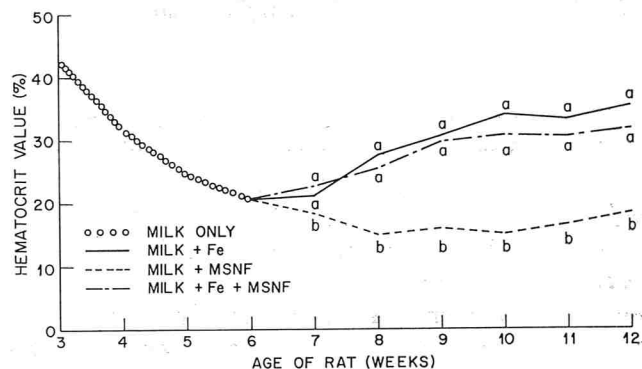
Figure 2. Influence of feeding iron and/or milk solids not fat with fluid milk on the hematocrit value of anemic male rats. Points on the same vertical line noted by the same letter are not statistically different (P>.05).
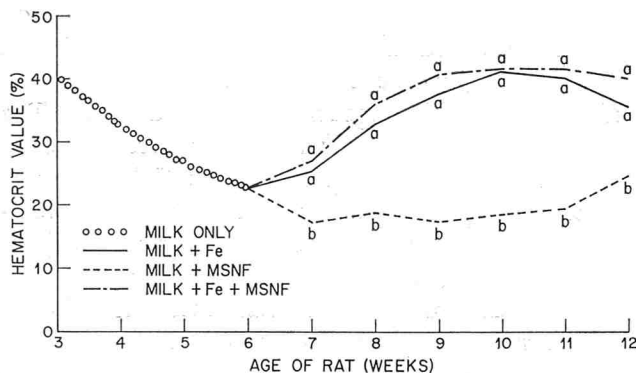
Figure 3. Influence of feeding iron and/or milk solids not fat with fluid milk on the hematocrit value of anemic female rats. Points on the same vertical line noted by the same letter are not statistically different (P>.05).

values were determined weekly until the rats were 12 weeks of age. Assuming the iron content of normal milk to be nil, ration one had approximately 0.30 mg iron per gram of protein and ration three, approximately 0.16 mg iron per gram of protein.

RESULTS

An analysis of variance showed that the two sexes were similar in body weight with one exception. At the age of 9 weeks the average weight of the males was 152.6 g and that of the females was 137.7 g; weights that were significantly different $(P < .05)$ as measured by Duncan's Multiple Range test (3). The average weight of both sexes at the various ages, presented in Fig. 1, shows that the two groups consuming iron were similar in weight throughout the experiment. The group consuming milk plus added MSNF weighed less after 10 weeks of age than either of the other ration groups.

The sexes were shown to have different $(P < .05)$ hematocrit values at most of the ten testing points. Thus the hematocrit data are presented separately by sex (Fig. 2 and 3). The small differences in hematocrit values among males on rations of milk plus iron or milk plus iron plus MSNF were not significant $(P > .05)$ (Fig. 2). Male rats on a ration of milk plus MSNF with no added iron had hematocrit values significantly lower $(P < .05)$ than either of the other ration groups at all test points after the rats were 8 weeks of age.

Female anemic rats appeared to respond to iron intake more readily than the males, as measured by hematocrit values (Fig. 3). Even after only one week on either of the iron-added diets, females had significantly greater $(P < .05)$ hematocrit values than did those fed a ration of milk plus MSNF.

The data indicate that, at the levels investigated, the added protein from MSNF did not cause a decrease in availability of iron to the rat, as measured by growth or hematocrit value. The rats on the milk-plus-iron diet apparently were able to consume enough calories for growth since there was no significant difference $(P > .05)$ between the body weights of those receiving milk-plus-iron and those receiving milk plus iron plus MSNF.

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NATIONAL CONFERENCE ON INTERSTATE MILK SHIPMENTS 1973 CONFERENCE

The 1973 Conference is scheduled to be held at the Savery Hotel, Des Moines, Iowa, May 20-24. The hotel management has guaranteed a flat room rate of $11.00 for a single and $15.00 for a twin ($7.50 per person). This rate is a firm commitment and you can be guided by it in requesting out-of-state travel. The registration fee for the 1973 Conference has been set by the Executive Board at $15.00.

John Speer, Program Chairman, and his group are already hard at work arranging the program for next spring. John Brockway, Iowa Dairy Products Association, has been appointed Chairman of the Local Arrangements Committee with Hale Hansen, Iowa State Department of Health, as Co-Chairman.

TIME REQUIRED TO RINSE AND CLEAN RAW MILK FROM STAINLESS STEEL PLATES WITH SOLUTIONS AT 35 C[1,2]

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Stainless steel plates were soiled with milk and skim milk, and the films were dried before being rinsed or rinsed and cleaned in a spray-type apparatus. Rinsing was practically completed after 30 sec and cleaning after 1 min of spraying. There was no significant difference in amounts of soil remaining on plates which had been sprayed for 17 min with detergent solutions tempered to either 35 or 62.8 C.

Of special interest to the milk processor is the possibility of using relatively low temperatures when cleaning equipment, particularly raw milk storage tanks. Cold walls of storage tanks are subjected to internal stress by changes in temperature involved in traditional cleaning. Also, the cost of heating and cooling is considerable.

Patel and Jordan (3) demonstrated the possibility of rinsing milk residue from stainless steel pipes with cold water, 2-14 C; however, they noted that more water was required. Calbert (1) observed that satisfactory cleaning was obtained when the cleaning solution temperature, initially at 54-60 C, was allowed to cool to not lower than 32 C during the cleaning cycle. Polowniak, et al. (4) showed that cleaning a milk contact surface at low temperature with detergents in cold water is feasible.

This paper reports on the investigation of the effects of duration of the rinsing and cleaning phases on soil removal when solutions at 35 C were used to clean stainless steel plates that had been exposed to cold raw milk. The work is a continuation of that reported by Heinz et al. (2) who showed that measurement of the amount of calcium remaining on a milk contact surface is a sensitive and convenient method of determining the amount of milk film on a surface.

MATERIALS AND EQUIPMENT

The experimental spray unit (Fig. 1) consisted of a spray chamber with baffle, a frame (hereafter called the cleaning frame) for positioning four stainless steel plates, a temperature controller, and 8 spray nozzles mounted in a 2-inch stainless steel tube. The piping and spray chamber were insulated to provide a constant temperature for the cleaning solution. Plates were made of 16-gauge stainless steel, type 304 with a No. 4 finish, and measured 22 cm on each side.

Calcium analyses were done with a Perkin-Elmer 290 atomic absorption spectrophotometer equipped with a calcium lamp and strip chart recorder. Acetylene was used as the fuel gas with filtered compressed air as the supporting gas (2).

PROCEDURE

Stainless steel plates were hand washed with an alkaline detergent solution and rinsed in softened tap water. The plates were then placed on end and allowed to dry. The tap water contained 50 ppm calcium, and the average residual calcium on the cleaned plates was 22.0 ± 0.5 mcg/100 cm² (P<0.05).

Two sets of experiments were conducted, one using raw skim milk (0.01% fat) as the soiling agent and the other using raw milk (3.8 to 4.2% fat). Clean plates were submerged in skim milk or milk for 10 sec then drained in a vertical posi-

tion for 1 min. Soiled plates were laid in the drying frame and dried 1 hr at 38 C then placed in the cleaning frame and into the spray chamber.

Tap water was placed in the reservoir of the spray clean-
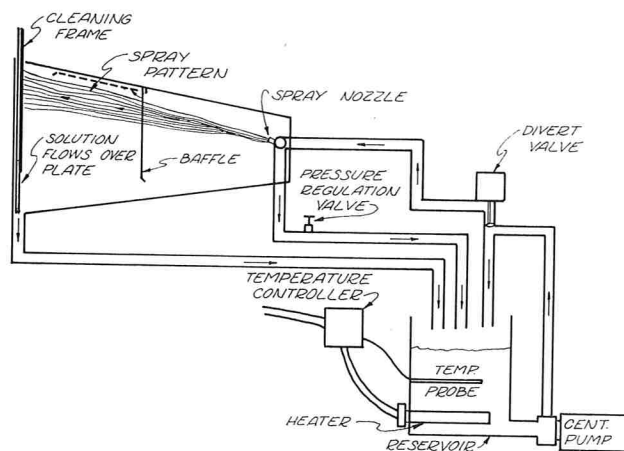
Figure 1. Schematic diagram of experimental spray unit.
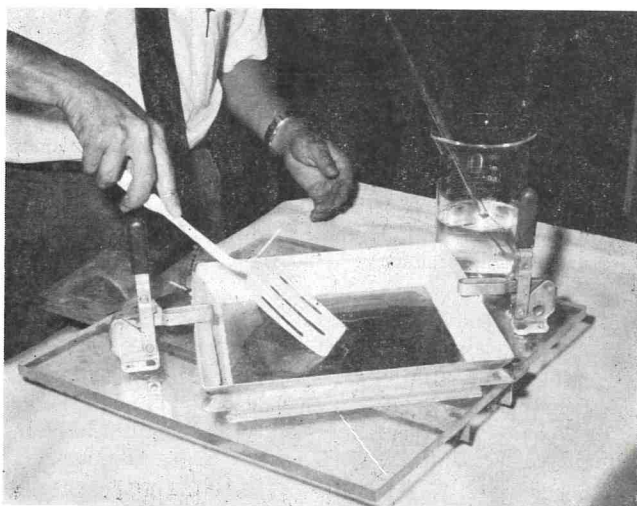
Figure 2. Sampling frame with a stainless steel plate onto which acid was spread with a spatula to solubilize calcium.
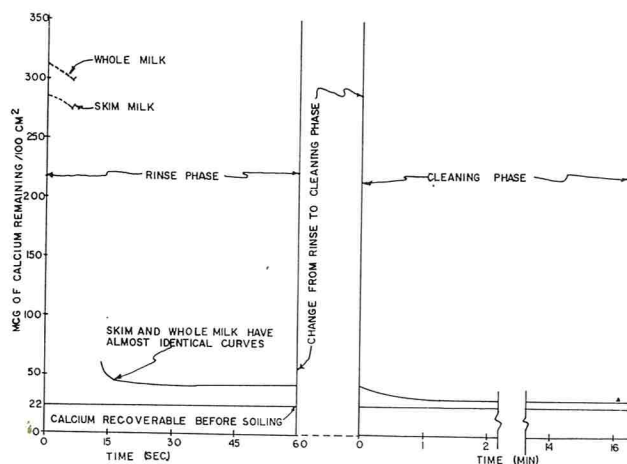
Figure 3. Calcium residual (mcg/100 cm²) on plates as affected by time of rinsing and cleaning at 35 C. (Triangle at lower right indicates mean quantity of calcium from a series of plates cleaned at a temperature of 62.8 C.)

ing unit and heated to 36.4 C as it was circulated through the pump and back to the reservoir (Fig. 1). Preliminary research indicated that it was necessary to provide this higher initial temperature to obtain a temperature of 35 C at the plate surface because of heat loss in the system.

With the rinse solution at 36.4 C, the divert valve was opened and the solution was sprayed onto the baffle inside the chamber. To start a test the baffle was raised, permitting the nozzles (two nozzles to each of the plates) to spray the rinse solution to impact above the plates at a rate of flow of 0.7 gal per minute per nozzle. The nozzle pressure was maintained at 35 psi. The solution flowed over the plates and was returned to the reservoir for recirculation through the system. At the end of the rinse phase the baffle was lowered, and the air-actuated valve was moved to the divert position. The cleaning frame was removed from the spray chamber.

The residue was removed from the plates as follows: (a) a plate was removed from the cleaning frame and placed into the sampling frame (Fig. 2); (b) a plastic bag was coded and attached to the sampling frame; (c) 5 ml of distilled water were pipetted onto the plate and evenly distributed using a spatula, the sampling frame was tilted vertically permitting the solution to run into the bag; (d) 10 ml of 1 N HCl were pipetted onto the plate and distributed with the spatula, the frame was raised, allowing the acid to flow into the bag; the procedure was repeated so that each plate was exposed to 5 ml of water and 20 ml acid, making a sample volume of 25 ml; samples were stored in a refrigerator.

In a second experiment plates were rinsed for 1 min, as described above, then spray-cleaned with an alkaline chlorinated detergent at 35 C. The cleaning proceeded as follows: (a) detergent (Circle 8, Frm Chem, Inc., St. Louis, Missouri) was added to the heated rinse water at the rate of 8.5 oz to 10 gal of water (0.625% cleaning solution), (b) water was circulated approximately 30 sec to dissolve the detergent, (c) the divert valve was energized permitting cleaning solution to flow over the plates for the appropriate length of time, and (d) plates were removed from the spray chamber and milk residue was removed.

In a third experiment, plates were rinsed with water at 35 C then cleaned with detergent at a temperature of 62.8 C. All other conditions were as in the previous experiment.

To determine if the amount of detergent solution remaining on a plate influenced the amount of residual calcium, an additional experiment was conducted. Twenty-four plates were soiled with milk, dried, rinsed, and cleaned with detergent at 35 C. After cleaning, 12 plates, randomly selected, were flushed with distilled water six times. All plates were sampled as previously described.

RESULTS AND DISCUSSION

Figure 3 shows results of exposing plates to rinsing and cleaning solutions at 35 C. Each point represents 12 samples. The independent variable, time, is plotted against the average quantity (mcg/100 cm² of plate surface) of calcium remaining. The average amounts of calcium recovered from plates soiled with milk and skim milk but not rinsed or cleaned were 304.4 ± 18.2 mcg/100 cm² and 287.1 ± 5.4 mcg/100 cm², respectively. Before soiling, the average amount of calcium recovered from plates was 22.0 ± 0.5 mcg/100 cm².

After the plates soiled with milk were rinsed for

15, 30, and 60 sec, the quantities of calcium remaining were 43.8 ± 5.2, 38.5 ± 2.2, and 43.7 ± 2.4 mcg/100 cm², respectively. Thus, approximately 92% of the soil was removed in the first 15 sec of rinsing. Only about 6% of the milk remained on the plate after the rinse cycle. Amounts of soil removed by rinsing for 15 and 60 sec were not significantly different (P <0.05).

For plates soiled with skim milk, 47.9 ± 2.9, 41.0 ± 2.6, and 40.3 ± 6.0 mcg of calcium/100 cm² remained after 15, 30, and 60 sec of rinsing, respectively. Therefore, at the end of 15 sec of rinsing the amount of residual calcium was nearly the same, regardless of the fat content of the milk (skim vs. whole).

At the start of cleaning (after rinsing for 1 min) the calcium residual was essentially equal for plates soiled with either skim or whole milk. At the end of 1 min of cleaning the value was 27.3 ± 1.7 mcg. After 17 min of cleaning, 26.2 ± 1.8 mcg of calcium remained. All of the above experiments were conducted at 35 C. After rinsing at 35 C for 1 min, then cleaning for 17 min at the evaluated temperature of 62.8 C, the mean quantity of calcium recovered was 26.8 ± 1.2 mcg/100 cm² (Fig. 3). This quantity did not differ significantly from that recovered after cleaning at the lower temperature (P <0.05). The data indicated that after 1 min of cleaning with detergent, about 2% of the milk remained on the plate. Less than 0.5% of the residue was removed during the next 16 min of cleaning, regardless of temperature.

To test the possibility that cleaning solution left on the plates would contribute to the calcium residue, 12 spray-cleaned plates were rinsed with distilled water and 12 were left unrinsed. Mean quantities recovered from the rinsed and unrinsed plates were

16.1 ± 3.1 and 15.6 ±3.8 mcg/100 cm², respectively. These means were not significantly different. Therefore, rinsing after cleaning with detergent was not necessary to remove traces of calcium contained in the cleaning solution that adhered to the surface after the cleaning cycle. These tests were done several weeks later than the series reported earlier. We are not able to explain why residues were smaller, but based on unreported observations, we feel the differences relate to the composition of the milk.

The conclusions drawn from this work are: (a) the rinse phase was essentially complete after 30 sec; (b) most of the milk remaining after rinsing was removed during the first minute of the cleaning phase; whether the surfaces were sufficiently clean after this time is uncertain, however, the rate of removal was very slow after 1 min; and (c) plates were cleaned equally well with detergent solutions at temperatures of 62.8 C and 35 C after 17 min of spraying with the detergent solution.

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THE NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH[1]

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Abstract

On January 26, 1971, President Nixon announced the establishment of a National Center for Toxicological Research (NCTR) at Pine Bluff, Arkansas. The Food and Drug Administration will administer this facility in a manner that is responsive to the needs of the FDA, EPA, and other government agencies, when their programs require research and development suitable to the Center. The Center is to be a national resource to be shared and utilized by appropriate government agencies, academic institutions, and industry. It will not duplicate the research capabilities of existing toxicological centers, rather it will build upon this existing capability and technology and augment existing research programs by undertaking projects which are not possible at other research centers.

The author discusses problems that have created the need for this Center and approaches that will be used to try to determine the risk that man runs in his every day exposure to the many hazardous chemicals in his environment.

On January 26, 1971, the following news release was made by the Executive Office of the President:

"Dr. Edward E. David, Jr., Director of the Office of Science and Technology announced today that a new, major project aimed at investigating the health effects of a variety of chemicals will be established in the surplus biological facilities of the Pine Bluff Arsenal, Pine Bluff, Arkansas. The new project is to be known as the National Center for Toxicological Research.

"The Center will examine the biological effects of a number of chemical substances which are found in man's surroundings, such as pesticides, food additives, and therapeutic drugs. The research activities will include both appropriate fundamental investigations aimed at understanding the mechanisms of action of these chemical substances, their metabolism in the animal organism, and their rates of absorption and excretion. In addition, this Center will afford an opportunity to undertake directed studies aimed at an understanding of dose-response relationships, especially for realistically long exposures to low doses of chemicals. This latter category of research requires the use of large numbers of experimental animals in order to assure valid statistical results.

"The National Center for Toxicological Research will be administered by the Food and Drug Administration and is expected to be a useful research resource to that organization in its task of regulating drugs, food additives, and other consumer products. In addition, however, the Center is to be considered as a national resource—to be shared and utilized by other government agencies such as the Environmental Protection Agency. Further, arrangements are being considered through which cooperation with industry and appropriate parts of the academic community can be realized.

"The Center was developed as a result of a growing recognition of two kinds of needs: (a) better approaches to the extrapolation of tests in experimental animals to man, and (b) more extensive facilities for the safety evaluation of many chemicals in man's surroundings. After an extensive review of possible locations, it became apparent that the biological facilities at Pine Bluff which were formerly used by the Department of Defense for the production of biological warfare agents were well suited for this project. The Department of the Army will be engaged in destroying biological warfare agents at this location for approximately one year, though some facilities will become surplus during that time. The Food and Drug Administration will occupy the surplus facilities as the Army vacates them."

In order that you may better understand why the Food and Drug Administration needs a National Center for Toxicological Research, let me outline briefly the responsibilities of FDA in safeguarding the health and welfare of United States citizens.

In 1970, personal consumption expenditures of U.S. consumers reached an estimated $610 billion. On the average, out of every dollar spent by consumers, 38 cents goes for products in some way regulated by FDA. Total retail sales of FDA regulated products are estimated at $240 billion in 1970 and are projected to increase to $300 billion by 1975. As such, FDA is charged with the major consumer re-

sponsibility of the Federal government.

FDA is charged with the responsibility of ensuring that foods are safe and wholesome, drugs are safe and effective, household products are safe or carry adequate warning labels, and all of the above are honestly and informatively labeled and packaged. Safety has been and continues to be the primary concern of FDA. However, drug efficacy and food nutrition are playing an increasingly important role.

To accomplish this mission FDA undertakes a long laundry list of functions, e.g., (a) clears new drugs for safety and effectiveness before they are marketed; (b) establishes safety tolerances for food additives, residues, and color additives; (c) establishes standards of identity, quality, and container fill for food products; (d) inspects factories, warehouses, distributors; (e) samples and examines interstate and imported shipments; (f) conducts research, etc., . . . through a total of 16 similar functions.

RISK VERSUS BENEFIT

As you are well aware, the Food and Drug Administration is one of the most widely criticized of Federal agencies. At any point in time, you can always find some individual, or some group, that is highly critical of what FDA has just done, or hasn't done, as the case may be. Seldom do we hear from people who like what we have done. Quite often FDA's many critics are aware of only one aspect of the situation about which they are complaining, and it is the other generally unrecognized aspects that I want to discuss. In short, I want to consider risks versus benefits, often termed the risk/benefit ratio. One of the primary purposes in establishing the National Center for Toxicological Research is to provide data to use in evaluating the realistic risk that we face today.

Whether we like it or not, we live in a chemical world. There is much preoccupation today with concern about synthetic versus natural chemicals, with the latter heavily favored. However, poisonous plants and animals have been recognized for centuries and they aren't man-made. Neither are the elements, such as the heavy metals, and they are also generally toxic when they are encountered in certain compounds with other, usually non-toxic, elements. Even when our environment is preserved in a natural state, it can be dangerous but we have generally learned to live with it. While it is true that we are constantly adding new chemicals to that environment, and that we have made mistakes, man has never had it so good.

We hear much today about pollution, and the growing recognition that much of the problem is "people pollution." There are certain features of this that must be dealt with if people are to survive. In spite of war, pestilence, automobiles, cigarettes, and pollution, people are now living longer than they did only half a century ago. Most of this increased life expectancy comes from reduced neonatal mortality but even the oldster can generally expect to live a little longer than his father. This "living longer" is now threatened by the so-called "population explosion." Even if current efforts at population control are more effective than expected, the world's population will double before the end of the century.

We should stop and consider what that entails. In the first place, there are fixed quantities of land, water, and natural resources such as the petrochemicals. In the second place, our best agricultural land is disappearing under new subdivisions, shopping centers, streets, and highways at the rate of thousands of acres a day. Thirdly, in much of the world, there is a severe shortage of protein for food and, unless something spectacular is accomplished, the United States will soon begin to share that shortage. It is small comfort that the world-wide food shortage situation today would be much worse were it not for the miracles of modern transportation which many of us, and our children especially, are roundly condemning as the source of much of our pollution. Here we must again consider the risk/benefit ratio.

The National Center for Toxicological Research is being designed to study the effects, or risks, which result from the long-term or life-time exposure that man experiences with some of the chemicals in his environment. Some of these chemicals, in fact most of them, have produced very appreciable benefits for man but it is their lingering residues about which we are concerned. Other toxic chemicals may occur naturally and may be augmented by industrial pollution. The latter is often an undesirable byproduct of manufacturing that has been done for man's benefit.

Of the many classes of these contaminating chemicals, the pesticides have received the greatest amount of public and scientific attention. Without question, pesticides have been used in excessive amounts, but the concern for this excessive use and the efforts to prevent overkill are not as recent as one might think. There has been serious concern with pesticidal uses of arsenic and of arsenic residues for nearly a century resulting in limitations on arsenic tolerances in this country and in Europe. The much more widely used pesticides that were developed during and after World War II have introduced new problems. Some of these new long-lasting pesticides, such as the chlorinated-hydrocarbons, are no more

long-lasting than the arsenicals, but they have been used in much greater quantities and for many more purposes, thus creating greater problems. In complete fairness one must also say that these new pesticides produced greater benefits as well.

FACING REALITY

There is little to be gained at this point, after the fact, by speculating as to whether risks have exceeded benefits from their usage, for we are faced with the reality of chemical residues and the more pressing problem of determining whether or not their widespread but low-levels of occurrence are individually or collectively hazardous to man. Similarly, we have the problem of the many chemicals which have "escaped" into the environment from industrial uses or from the normal use of some naturally occurring substance, such as coal, in which they occur at trace levels. Mercury is one such chemical. It occurs widely in trace amounts in soil, rocks, and in plants and animals, as well as in the coal and petroleum formed from the latter. Mercury has been widely used in industry and in such places as research laboratories and hospitals. Naturally occurring mercury contamination is volatilized when coal and oil are burned. Until recently little thought was given to its fate when it was flushed down the drain, was volatilized into the air, or escaped with the spent brine when mercury electrodes are used in chlorine and caustic soda manufacture. Its density and its low water-solubility led most people to believe that it would combine with the bottom mud and not get into our water supplies. However, in combining with the silt and mud on the stream bed, it has been found to do something that was unexpected. In this mud there are often microorganisms which live and reproduce in a medium which is rich in organic material but in which there is little or no oxygen. These anaerobic organisms slowly convert inorganic or elemental mercury into organic mercury, chiefly methyl mercury. This is picked up by the aerobic or oxygen-using animals that live in the water. Bigger animals eat smaller animals in a fairly rapid sequence. This phenomenon is known as the food-chain and man is the animal at the top. Because of the affinity of methyl mercury for neural tissue, where it is stored and where it can, in sufficient quantities, produce generally irreversible harmful effects, methyl mercury tends to accumulate in larger and larger amounts as it progresses up the food chain. This is called "biomagnification" and it is the reason that the swordfish, which is both large and predaceous, tends to have fairly high concentrations of methyl mercury in its neural and other tissues. Tuna fish, fortunately, are not quite as high in the food chain and relatively few of them exceed what is believed to be a safe level of methyl mercury. If we ate sharks or seals, we would find them to have a high mercury content also.

In addition to the neurological damage which may occur to the individual consuming too much methyl mercury, there is a demonstrated ability of methyl mercury to accumulate in fetal or embryo tissues at levels several times those found in the mother. Although there is no evidence that methyl mercury poisoning is the cause of our problems of mental retardation, the action of methyl mercury could produce that unfortunate condition.

Other heavy metals, such as lead and cadimum, have been widely used for centuries, and must be watched. There is evidence that lead leached from food and drinking utensils may have contributed to the fall of the Roman Empire. The risk of lead poisoning from pottery glaze is still with us and FDA has seized or has recalled large quantities of pottery in recent months.

THE NEWEST PROBLEMS

However, of much greater concern are the plastics and the chemicals associated with them. One class of these chemicals is the polychlorinated biphenyls, or PCB's, which have been found to be the most widely dispersed contaminant of any man-made substance next only to DDT. Since the PCB's, like DDT, are chlorinated hydrocarbons, we know that until a year or two ago many determinations of DDT by chemical analyses may have been, in whole or in part, due to the presence of PCB's. We can now separate them in performing chemical analyses of food, water, and other substances and we find traces of PCB's in a wide range of substances. We don't know nearly as much about the damage they do as we do of their usefulness. Even so, relatively few people have ever heard of them, so you see how little we know. This class of chemicals has a wide spectrum of usage. One type is used as the "oil" or fluid in electrical transformers, in heat exchangers, and in closed hydraulic systems others are used as plasticizers for various plastics and as fire retardant materials in plywood and chipboard. A surprising usage has been as the encapsulating material for the tiny droplets of ink that make pressure tracings on carbonless copy paper. One common characteristic of most of these uses, including the plastics, is that they are subject to volatilization by heat resulting from fire or friction and may precipitate or be "rained down" into bodies of water, onto food plants, or inhaled as part of the air we breathe. In addition

the "closed systems," in which they are still used, have something in common—they leak!

There are many other chemical substances of similar types about which we know little but of which we must, of necessity learn a great deal quickly. However, we must, in the public interest, always weigh risks against benefits when allowing their continued use.

ASSESSMENT OF RISKS

At the National Center for Toxicological Research we must develop methods of assessing these risks. I should point out that it is impossible to prove absolute safety. In fact safety is a relative term and it is highly doubtful that unlimited quantities of any substance are ever safe for any life form. Water is a necessity, yet too much of it, either outside or inside the body, can be fatal to man and to most other mammals. We can only assume safety in the absence of contrary evidence. In a way, and in spite of its absurdity, presumed "safety" is often equivalent to ignorance.

Until now we have tested the so-called safety of chemicals by two types of tests; acute toxicity tests and chronic toxicity tests. In the acute test an experimental animal, usually a small rodent such as the mouse, is tested in groups of 10 to 50 animals and to each such group there is administered a different level of the test substance. At the zero, or check, level no test substance is included in the medium that is administered and this group serves as the controls for the test. To help determine whether or not the medium in which the chemical is administered may itself be toxic a group of untreated animals is used. Usually three to five different levels of substances are administered, each to a separate group, for a specified period of time but not exceeding 90 days. If the substance administered proves to be highly toxic it may kill all the animals receiving it at all levels. If the substance is relatively non-toxic it may kill all of the test animals only at the very high levels of administration and only part of them at lower levels. The level of administration at which it kills no more animals than die in the control group is called the "no effect" level.

The chronic toxicity test in use today is conducted similarly except that it is conducted over a longer period of time, often the life time of the test animal, and involves lower dosages. Obviously the highest dosage must be no higher than the no effect level determined by the acute toxicity test.

Generally, except for substances that cause cancer, terata, or measurable genetic damage, we add a margin of safety by permitting only a small fraction of the "no effect" level of the substance to be present in food or drink.

If a substance, which is intended as a food additive, is found to cause cancer in man or in animals, irrespective of the dosage level and without clear limitations as to the method of administration, it cannot be added to a food. As you know, this is the explicit provision of the DeLaney Clause of the Food, Drug and Cosmetic Act. This clause does not, however, apply to pesticides which may contaminate the same food for which the additive was intended.

You may have noted that I carefully used the term "administered" when referring to the way in which the experimental animals received the test substance. It has been the practice in recent years to administer chemicals in ways often quite unlike the way in which man is exposed to the chemical, and in quantities far beyond those that man encounters. For example, recent tests have seen the test substance dissolved in dimethyl sulfoxide and injected into the peritoneal cavity of the animal. Not only is the solvent a bizarre chemical almost never encountered by man, but also man gets very little of any material by intraperitoneal injection. Feeding, inhalation, or skin application would approximate man's exposure more closely. This practice of unusual administration was justified on the basis that any recognized carcinogen, teratogen, or mutagen, regardless of the level of it that man was exposed to, must be excluded from man's environment because testing in animals gave no assurance of man's high threshold of response to the substance. Of course, and by the same reasoning, neither its positive nor negative effect on animals necessarily means that man responds in the same way. As wags have often said: "Many men are rats, but no rats are men!"

This exclusion of a substance from the environment was thought possible prior to refinements in analytical techniques which have occurred in recent years. The older methods measured in micrograms per gram, or 1-millionth of a gram. Technology now permits us to identify and measure many substances down to picograms and nanograms, or to parts per billion and per trillion, respectively. Instead of being able to detect only a glassful of vermouth in the tank-car of gin, which is said to be the definition of a very dry martini and which approximates 1 part of vermouth per 1 million parts of gin, you can see that we're getting our abilities down to detecting really infinitesimal traces.

With these techniques, it is now evident that many substances which we thought were not present in our environment are already present, and "banning" them will not keep them out. The question we must now

face is "what risk do they constitute at these low levels?"

This is the name of the game at Pine Bluff! We must develop tests that will permit us to detect adverse effects in animals that will occur as infrequently as in only one out of a thousand or more animals. Many types of cancer, for example, occur much less frequently than one per thousand in man. We must determine whether or not the compounds that are known to cause cancer at high doses, many of which occur in our foods, actually cause cancer in test animals at low dosages and in a large enough sample of animals to have statistical validity. We must also look for other and simpler test procedures, such as the use of cell cultures, in which acute sensitivity or immune responses may parallel whole animal tests. This latter must be done simultaneously with the animal tests if we are to develop tests which are not financially impossible in the testing of existing and new substances.

The Environmental Protection Agency shares the National Center for Toxicological Research with the Food and Drug Administration, although the latter agency will administer it. There is a wide area of overlapping interest between the two agencies and a continuing cooperative effort in carrying out their respective missions is essential to the success of each agency.

PLANNING FOR THE NATIONAL CENTER

One of the conditions of the transfer from the Army to the Department of Health, Education, and Welfare is that FDA will not have full use of the facility until the stock-pile of biological agents has been completely destroyed and no contamination remains. While this will delay acquisition of very important knowledge it also has some good features; it will give us time to plan our activities very carefully.

A very important part of this planning will be carried out with the assistance of the University of Arkansas, whose Medical Center in Little Rock is only 35 miles away. We have contracted with the University to: *(a)* establish a Scientific Advisory Board to the National Center for Toxicological Re-

search, together with appropriate subsidiary panels of experts, with all members chosen in accordance with the need for a truly National Center; *(b)* to host a National (or perhaps International) Conference in the next few months where all of the problems that I have attempted to discuss today will be considered and recommendations made concerning them; and *(c)* to establish a way of interfacing the NCTR with the rest of the scientific community, not only in universities and other non-industrial research centers but also with the industrial scientific community itself where the lessons learned must be applied. With the help of these scientists we hope to create a truly National Center for Toxicological Research which will have International stature.

There are problems to be considered and of these, one is already identified as being of over-riding importance, i.e., how to accomplish the necessary pathology work on such large numbers of animals. Pathology is relatively poorly automated today and what we are asking is not only that we must speed up these preparations and examinations but also that we improve their accuracy, or their ability to detect small evidences of adverse effects, at the same time. I am happy to say that the pathology groups are accepting the challenge with great enthusiasm and are fully aware that pathology is at present a serious bottleneck.

Finally, let me introduce only one more of the problems in establishing the NCTR, i.e., how to operate it. Of the several possibilities open, such as direct operation by federal employees, by prime contractor such as Oak Ridge (Union Carbide) or Los Alamos (University of California), or by a mixture of both civil service and contractor employees, we are giving serious consideration to the latter. It is hoped that the contractor will be a consortium of universities with proven interest and expertise in the problems involved, but such arrangements are not developed overnight. Thus, while we are identifying the problems to be encountered and planning their solutions there will be an opportunity to determine the possibilities of a consortium tailor-made to the need. If the institutions represented by readers are interested in membership, we will welcome an indication of that interest.

HEART DISEASE—IS DIET A FACTOR?[1]

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ABSTRACT

Data derived from extensive population surveys and animal experiments have provided indirect evidence associating elevated blood cholesterol levels with the increased incidences of atherosclerotic heart disease. As a result of this apparent relationship, recommendations have been made regarding the kind and amount of fatty acids and cholesterol which should be consumed for optimal health. Before massive changes are made in the eating habits of our population, the actual causal relationship between dietary fat and cholesterol to subsequent blood cholesterol levels and atherosclerotic heart disease should be determined. Although several risk factors have been characterized which will possibly aid in identification of those individuals most prone to development of atherosclerotic heart disease, the potential benefit from reducing a single risk factor in the prevention of the disease has not yet been conclusively demonstrated. The relative importance of dairy products as contributors of dietary saturated fatty acids and cholesterol is discussed in relationship to their association to heart disease.

Recently there has been an increased interest among the medical, scientific, and lay communities regarding the relationship between our dietary habits and this nation's number one killer — coronary heart disease. This renewal of interest concerning a problem which has existed since the beginning of mankind is primarily a result of the latest reports from the Framingham Study on heart disease and the recent recommendations which have been made to the public by the federally-financed Inter-Society Commission for Heart Disease Resources.

HEART DISORDERS

There are many different types of disorders of the heart such as congenital heart disease, angina pectoris, hypertensive heart disease, and atherosclerotic heart disease. The basic characteristic of atherosclerotic heart disease is the formation of obstructions or atheroma in the major blood vessels of the heart. In the simplest of terms, atheroma formation is believed to begin by the buildup of fat and other lipid-soluble materials in the inner linings of the walls of the blood vessels. Early stages of this disease are noted by the appearance of yellow fatty streaks in isolated areas. As the disease progresses, the fatty streaks become hardened and more obstructive.

Eventually, the blood vessel will become completely obstructed, causing the flow of blood which is carrying oxygen and other nutrients to a particular area of the heart to stop. The affected tissues then die, resulting in a sudden and unpredictable heart attack. A fact often overlooked is that almost everyone in the world over the age of 12 has atherosclerosis to some degree (13). The National Health Examination Survey of 1960-62 estimated that 3.1 million Americans between the ages of 18-79 had definite coronary heart disease and 2.4 million had suspected coronary disease which represented approximately 5% of the population at that time (4).

ATHEROSCLEROTIC HEART DISEASE

Atherosclerotic heart disease is unquestionably the most important health problem facing this country today. Last year, of the 850,000-plus people who died of causes other than accidents, more than 165,000 of the deaths were a direct result of a coronary heart attack occurring in individuals under 65 years old. Today, it has been estimated that one man in every five will suffer some degree of disability before the age of 60 as a result of a coronary attack, and one in every 15 will meet an early death. Because of the magnitude of this health problem, not only should every attempt be made to learn its real causes through science and research but individuals should reappraise what is already known and take steps to implement programs which may provide some degree of protection from this disease.

Causes

Research studies to date have been unable to clearly define the causes for the buildup of atheroma which eventually leads to atherosclerotic heart disease. The countless numbers of surveys and laboratory studies with man and lower animals suggest there are a multiplicity of factors which may increase a person's "risk" of developing a coronary condition (29). The most comprehensive study conducted in this country to date which attempted to characterize the factors associated with heart disease was the well-known population survey commonly referred to as the Framingham Study (16) This investigation of 5,127 men and women living in Framingham, Massachusetts, was initiated in 1949. No attempt was made to modify the normal habits of this free-

living population, for the study attempted to characterize every-day conditions existing in individuals who were eventually afflicted with coronary attacks. Based upon the results, it was anticipated that some clues regarding the cause of this disease would be obtained. During the ensuing 20 years, extensive clinical evaluation of the subjects and statistical correlation of the information gathered clearly identified several parameters that could be associated with the increased incidence of heart disease. Based upon a composite of the results from this and similar studies, the likelihood of having a coronary heart attack appears to be associated with: *age* — older people are more susceptible; *sex* — men have a higher incidence of heart attacks than women, until the late 40's; *genetics* — those who come from a family with a history of coronary problems are more susceptible; *stress* — the status conscious young man living under constant deadlines and tension may be more prone to coronary attack; *smoking* — heavy cigarette smokers appear to be more susceptible; *lack of exercise and obesity commonly associated with one's physical condition*, may lead to early death; and the most discussed risk factor, *dietary habits*, especially the consumption of cholesterol and saturated fatty acids which are largely derived from animal products (7).

Cholesterol

More than a century ago cholesterol and other fatty materials were identified as major constituents of the atheroma blockages in the blood vessels. In 1913, the Russian scientist Anitschkow demonstrated that feeding cholesterol to rabbits could increase blood cholesterol concentrations and produce atherosclerotic lesions of the heart and major blood vessels. Numerous human population surveys, or epidemiological studies, conducted in various parts of the world have demonstrated that many populations having high incidences of coronary heart attacks also have high blood cholesterol levels. Animal and human studies conducted under carefully controlled laboratory conditions have shown that the type and amount of dietary fat consumed will have a significant effect on blood cholesterol concentration. Diets containing a large amount of polyunsaturated fatty acids tend to decrease blood cholesterol concentrations whereas diets high in saturated fatty acids tend to increase blood cholesterol. Thus the association between dietary fats, cholesterol, and conorary heart disease has come to be regarded as an irrefutable fact (1, 18).

GUIDELINES FOR PREVENTION AND CARE
OF HEART DISEASE

In 1965, a contract was negotiated between the

Federal Division of Regional Medical Programs and the American Heart Association in an attempt to coordinate the efforts of several national professional organizations in developing guidelines for the care and prevention of cardiovascular disease. The American Heart Association created the Inter-Society Commission for Heart Disease Resources to accomplish this mission. The Commission is composed of 29 leading medical, nursing, and allied health organizations, and other experts selected for their special knowledge of heart disease (2).

Last December Commission members assigned to the epidemiology and atherosclerosis study groups issued a declaration for a national commitment to primary prevention as the principal means of controlling coronary heart disease. These Commission members expressed belief that hypertension, cigarette smoking, and high blood cholesterol levels were the three major "risk factors" associated with development of premature atherosclerotic heart disease.

In an attempt to reduce the incidence of coronary heart disease, the members of the two Inter-Society Commission study groups made the following recommendations regarding dietary changes:

(a) An individual's calorie intake should be adjusted to a level which would achieve and maintain optimal weight. For correction of obesity is known to be frequently associated with significant control of other related coronary heart disease risk factors such as diabetes.

(b) An individual should restrict dietary cholesterol intake to less than 300 mg per day. Currently, it is estimated that the average daily consumption of cholesterol is 600 mg per day. (An egg contains approximately 275 mg cholesterol and an 8-oz glass of whole milk 27 mg cholesterol.)

(c) An individual should substantially reduce dietary intake of saturated fatty acids in an attempt to lower blood cholesterol levels. It was recommended that the present 40% of total calories as fat which is now consumed be reduced to less than 35%, with no more than 10% coming from saturated fatty acids.

To accomplish these goals, it was further recommended:

(a) That the food industry make available products which would meet the specifications of lower saturated fatty acids and cholesterol necessary to lower blood cholesterol levels.

(b) That the dairy industry develop low-fat, low-cholesterol milk and milk products, and switch to cows that produce large amounts of high-protein, low-fat milk.

(c) That modified cheeses, containing lower saturated fatty acid and cholesterol content, be developed to aid in the reduction of dietary intake of saturated fatty acids and cholesterol.

(d) That industrial and governmental regulations which currently use butterfat content of milk as a pricing standard be modified to use protein content as the standard.

(e) That labeling and advertising be regulated in a man-

ner allowing the consumer to know the actual fat composition of his diet.

The Inter-Society Report also recommended that the Government initiate massive public and professional education campaigns directed toward instructing consumers to decrease their intake of saturated fatty acids and cholesterol by reducing the amount of meat, dairy products, baked goods, eggs, and cooking fats consumed. It was also suggested that school lunch, food stamp, other supplementary food programs, and government-administered feeding programs in the armed forces and veteran hospitals be revised to encourage a low saturated fatty acid, low cholesterol diet.

The perspective of this report further stated that American agriculture and the food industry are already fully able to implement the changes necessary for this preventive program. With a decisive national commitment, there was reason to believe that significant progress toward reducing atherosclerotic heart disease could be made as early as 1975, according to the report.

If these Commission recommendations were to become national policy, the impact on the food industry is obvious. Eggs, with their high concentration of cholesterol, and dairy foods, such as whole milk, cheeses, and ice cream, which have been singled out as villains in the etiology of heart disease, would have even less appeal in the marketplace than they do today. Highly marbled beef would undoubtedly come into disfavor.

Before the dietary habits of the nation are overturned, a closer inspection of the evidence which has been used to associate diet and heart disease should be made.

DIET AND HEART DISEASE

Dr. D. S. Fredrickson, Director of Intramural Research, National Heart and Lung Institute, recently reviewed the Inter-Society Commission recommendations. In a lecture presented to the Royal College of Physicians of London, Dr. Fredrickson stated, (12), "In the light of what is actually known, the injunctions on consumption of cholesterol and fats seem too radical as they stand. What evidence do we have that an egg yolk a day spells jeopardy for all Americans? Do we have enough information about marginal hyperglyceridaemia or incipient diabetes to advise everyone to eat a diet which will tend to provide more than half of the calories as carbohydrates? What of sucklings and older infants? The Commission's report leads to an inference that a third of their calories from fat should also be polyunsaturated. Are we convinced of the safety of a diet containing 10% of polyunsaturates to the extent that we want to insist on this in baby's formula? Finally, are we certain enough of the efficacy of such sweeping changes that we, as physicians, can convincingly follow them ourselves?"

Cholesterol

The concept of dietary saturated fatty acids and cholesterol as a primary cause of atherosclerotic heart disease is not universally accepted in the medical and scientific communities (5, 24). Unfortunately, the research which has been conducted to date regarding the relationship between our dietary habits and the risk of having a coronary attack has given rise to a tremendous volume of conflicting data which has thrust the scientific and medical communities into deep controversy.

The original findings of Anitschkow, who demonstrated the atherogenic effect of feeding rabbits diets high in cholesterol, would never have occurred, had the studies been conducted on an animal other than the rabbit. The rabbit is by nature a herbivorous animal, living almost exclusively on green plants and vegetables low in cholesterol. Thus, it is hardly surprising that this species of animal would respond abnormally when fed an atypical diet containing a large amount of cholesterol. Other laboratory animals, such as the guinea pig, which have been used to confirm the dietary cholesterol-heart disease hypothesis, are also extremely intolerant to dietary cholesterol; relatively small amounts of dietary cholesterol will result in significant rises in blood cholesterol concentrations. By contrast, the dog, rat, and other carnivorous animals that normally consume diets containing cholesterol are extremely tolerant to dietary sources and rarely respond with elevated blood cholesterol levels. It is often necessary to interfere with normal thyroid activity before any changes in blood cholesterol can be seen in these latter species. The human diet normally contains both plant and animal foods. Thus, man should respond to dietary cholesterol somewhere between the two extremes (10).

It should be recognized that cholesterol is not a foreign or toxic substance to the living organism. The chicken egg, which carries the entire nutrient supply for a developing embryo, contains large quantities of cholesterol. All body cells have been shown able to manufacture cholesterol. A point often overlooked is that cholesterol actually plays a vital role in the normal metabolic processes of man. It is found in and may be required in large quantities for normal development and function of brain tissue and is part of the membranes surrounding every cell in the human body. Cholesterol is the building block for the

manufacture of our sex hormones and it is an absolute requirement for the production of bile acids which are necessary for normal fat digestion. The human body is able to manufacture cholesterol in much greater amounts than dietary sources will provide and regardless of diet, cholesterol synthesis by the liver and other organs is controlled by the requirements of the body (28).

Epidemiological or population survey studies (17, 26, 27) have shown that considerable differences exist between serum cholesterol levels and deaths from coronary heart disease in different populations. Specifically, it has been found that people living in less technically developed populations tend to have lower blood cholesterol levels than those populations living in highly developed countries. The fact that the incidence of heart disease parallels the level of blood cholesterol has been accepted as evidence of a causal association between the two. It would, however, be preposterous to believe that diet was the only factor differing among the population groups studied and is thus responsible for differences in the incidence of heart disease.

Details of the following studies provide insight into the evidence cited in support of the causal association between cholesterol, saturated fatty acids and coronary heart disease. In a study conducted in New York City (8) as part of a total program to prevent heart disease, 814 men were assigned to an experimental low-cholesterol, high-polyunsaturated fatty acid diet and were given close and frequent medical and dietary supervision. The obese individuals in this group were placed on a low calorie diet until their desired body weight was attained. By contrast, the individuals serving as controls in this study remained on their customary normal diet and were examined only once a year, with little or no other supervision. Thus, it is evident that the experiment was inadequately controlled from the beginning. During the four-year follow-up period, there was significantly greater incidence of coronary heart disease in the control group which was not supervised than in the test group which had received diet and other therapy. The researchers themselves did not attribute the reduced incidence of heart attacks solely to the fat-modified diet since there were at least four other important variables involved in the program which had been initiated to prevent heart attacks. Nevertheless, proponents of fat-modified diets quote this study in support of their case. The results obtained in this study could have been attributed, just as soundly, to the correction of obesity and/or to the psychological effects of frequent medical and dietetic review as to the low cholesterol and polyunsaturated fatty acid content of the diet.

The study of Leren, often quoted in support of the relationship between diet and heart disease (23), involved 412 heart attack survivors under 64 years of age. The subjects were randomly allotted to one of two groups with different dietary treatments. One, an experimental group, was fed a low saturated fatty acid diet plus 70 ml of polyunsaturated soybean oil per day coupled with close dietary supervision. A control group remained on the normal diet with little dietary supervision. More individuals in the experimental group were initially underweight and fewer were overweight. Although there was no deliberate attempt to regulate weight in either group, there was a rapid weight loss of almost 6 lb. in the experimental group (thought to result from the diarrheic effect of the added soybean oil), which did not occur in the control. Therefore, although weight reduction was a persistent factor, it was not considered in this study. Lack of adequate control is once again evident in this experiment. During the five-year follow-up period, a higher relapse rate of heart attacks was reported in the control group than in the experimental group fed the low-fat, low-cholesterol diet. The author's conclusion that the cholesterol-lowering diet reduced the incidence of heart attacks is subject to serious question, for this difference in coronary attacks may just as likely have been attributed to differences in total caloric intake and weight reduction.

In another study (11), dietary saturated fatty acids were substituted with unsaturated fatty acids "to the maximal degree compatible with palatability." The diet fed to the subjects in the experimental group was also substantially lower in cholesterol content (365 mg cholesterol per day) than the diet fed the control group (653 mg cholesterol per day). Double-blind conditions were adhered to, so that the researchers were unaware of which people were in either group. Although both groups showed a gradual decline in serum cholesterol over the eight years of the study, the experimental group receiving the high polyunsaturated fatty acid diet exhibited a 13% reduction in blood cholesterol. Seventy deaths in the control group were attributed to acute atherosclerotic events, compared with only 48 such deaths in the experimental group. Deaths attributed to non-atherosclerotic and uncertain causes were more numerous in the experimental group than among control subjects so that total mortality was nearly identical. Thus, lifespan was not increased by diet therapy. Closer inspection of this study reveals that total overall adherence to the diets was only about 50%. In addition, there were twice as many withdrawals from the experimental group as from the control group during the duration of the trial, and withdrawal status was disregarded in data analysis of the report.

Although recruitment was carried on continuously during the eight-year experiment, most recruitment occurred during the first two years. As a result, some people were in the experiment for longer periods than others. In addition, the weight of many participants changed considerably during the trial period. There was also a significant difference in smoking habits between the groups; the control group had more smokers in the one-to-two or more packs per day category. The questionable aspects of this experiment are admitted by the researchers, but are scarcely mentioned by those who use the results as further "proof" of the beneficial effects of polyunsaturated fatty acids.

In the often-quoted National Diet-Heart Feasibility Study (3), subjects were voluntarily placed on a rigid low-cholesterol, high-polyunsaturated fatty acid diet in an attempt to reduce their blood cholesterol levels and thus prevent coronary heart disease. The results of the study were inconclusive for several reasons. Ten percent of the volunteers withdrew from the program during the first year. Only 50% of the subjects were classified as fair-to-good adherers to the programs initiated, with at least 25% classified as poor adherers. Of the men who finished the first year, 41% did not volunteer for a second year. Another 9% dropped out the second year. More noteworthy, however, was that the blood cholesterol levels of the population which adhered to the fat-modified diet were only 11 to 14% lower and less than 8% in those who lost no weight.

Thus, it can be seen that the large numbers of uncontrolled risk factors and the inherent complexities involved in intervening in free-living populations have seriously affected the interpretation of the results of many of these studies. Cornfield and Mitchell (9) have pointed out several other shortcomings of the often-cited clinical studies supporting the association between diet and heart disease. Inadequate sample sizes, inadequate randomization of experimental subjects and exclusion from final analyses of subjects who have withdrawn from the studies have seriously affected interpretations. Statistical procedures used to evaluate results have tended to cause misinterpretation of the results. Consequently, many studies report difference due to dietary manipulation when only differences by "chance" actually exist.

In the studies which purportedly support the relationship between diet and blood cholesterol, it is obvious that only modest achievement has been attained with volunteer participants, who are supposed to be keenly interested in the program. How, then, can a clinician be expected to prescribe therapeutic diets for his patients with any confidence of adequate lipid reduction? The studies just cited are

in agreement that dietary treatment will decrease blood cholesterol levels approximately 10%. However, the Food and Drug Administration would surely not approve the marketing of a drug which showed so modest a therapeutic value (25).

It has recently been estimated that if elevated blood cholesterol levels could be completely removed as a risk factor, the total effect would be to reduce the rate of incidence of heart disease from 14 to 11 per 100 men in 10 years (29). This would occur providing that 100% cooperation was obtained, provided that the serum levels *could* indeed be lowered and maintained through dietary manipulation, and provided that lowered serum cholesterol levels would, in fact, decrease the incidence of heart disease—*all points which are currently open to considerable speculation.* Thus, the concept of heart disease prevention through dietary change—through removal of one risk factor which, at best, would have only a minimal effect—not only remains theoretically unproven, but is also, from a practical standpoint, an unrealistic solution for a complex society that loves an abundance of food. The true decrease in coronary deaths which could realistically be expected as a result of dietary changes is important, especially to those individuals who would have to sacrifice their customary lifestyles in order to adhere to dietary changes.

The public is frequently advised to reduce total fat intake by avoiding butter, whole milk, cheese made from whole milk, and cream, as a means of reducing the risk of developing heart disease. Thus it would be of interest to examine more closely the direct relationship between the consumption of dairy foods and atherosclerotic heart disease.

Dairy foods

A causal relationship between dietary cholesterol and fat and the incidence of heart disease was not borne out by Kahn (14). Nutrient composition of foods available for civilian consumption, or nutrient "disappearance" figures, were compiled from 1909 to 1965. Cholesterol and dietary fat disappearance values were then calculated from these data. The study demonstrated that in the past 50 to 60 years in this country, serum cholesterol changes associated with changes in dietary fat and cholesterol have not been very great. Dr. Kahn concluded that, "This, in turn, indicates that the increased risk of coronary heart disease reported to have occurred over this period is not related to dietary fat changes to a very important degree. Changes in fat consumption may well be a means of lowering present-day risk of coronary disease, but other environmental factors are more probably associated with having raised the risk from that of 50 years ago to the present level."

Recent studies demonstrate that per capita con-

sumption of butter and saturated fatty acids has continually declined since 1950 (19). However, during the same period total fat consumption has risen slightly, primarily because of the increased consumption of vegetable oils. This trend in substitution of polyunsaturated vegetable oils for saturated fatty acids has been accompanied by significant increases in numbers of deaths from atherosclerotic heart disease. Why hasn't this association been realized? These figures show that the increased incidence of coronary heart disease occurred during a period when per capita consumption of dairy foods and other animal fats declined. How can these products, therefore, be related to heart disease? The rise in incidence of coronary heart disease is not consistent with trends in dietary fat and cholesterol consumption.

In addition, a comparison (based upon 1966 statistics) of longevity of life, and death from cardiovascular diseases, between countries with the highest consumption of whole milk equivalent (1. Finland-1513 lb.; 2. Ireland-1356 lb.; 3. New Zealand-1312 lb.; 4. Denmark-973 lb.; 5. Norway-969 lb.) and the United States (at 609 lb., 16th in consumption), reveals that, except for Finland, the countries with the highest consumption of whole milk equivalent had fewer male deaths and a lower death rate from cardiovascular diseases from birth to 64 years of age than the U.S. Except for Finland, average life span projections for the male are higher in countries consuming considerably more whole milk equivalents than the United States. Australia, Switzerland, and France also consume more whole milk equivalents than the United States (836 lb., 921 lb., and 923 lb. versus 582 lb., respectively), yet experience a lower death rate of males aged 45 to 54 years from coronary heart disease (325, 126, and 2 versus 352/100,000 population, respectively).

Another point to consider when attempting to put dairy product fats and cholesterol into proper perspective is just how much fat and cholesterol do dairy foods contribute to the American diet in relation to other foods? In 1965, the average American daily consumed 160 mg of cholesterol from meat; 270 mg from eggs; and only 70 mg from dairy products and 18 mg from butter (14). Also, two 8-oz glasses of milk, recommended daily for adults, contain only 17 gm fat and 54 mg cholesterol. Approximately 40% of our daily caloric intake comes from fat. Considering all fat sources, only 13.0% of the total fat calories and only 4.5% of the total daily calories are derived from dairy product fats (excluding butter). The majority of fat calories are contributed by meat and cooking oils. An important point to consider is that one-third of the fatty acids of milk-fat are monounsaturated fatty acids, which neither raise nor lower

blood cholesterol levels. Milk contains 4% polyunsaturated fatty acids, which tend to lower serum cholesterol levels. Therefore, only about 63% of milk fatty acids are saturated fatty acids. Furthermore, nearly 10% of the fatty acids in milk are small, short-chain fatty acids which are known to be metabolized in a manner which has no effect on blood cholesterol levels. Only one-third or less of milk's fatty acids are of the kind suspected of elevating blood cholesterol, if indeed they affect serum cholesterol levels at all.

Framingham study

Many of the factors associated with increased heart attack "risk" have been based on the results of the Framingham Study on Heart Disease. Part of the Framingham Study was a critical evaluation of the dietary habits of a sample of the Study's subjects to determine normal consumption totals for fat, saturated and polyunsaturated fatty acids, and cholesterol.

Publication of the 24th Report from the Framingham Study (15) in April, 1970, revealed that consumption of these nutrients was relatively uniform in the population group tested. However, significant differences in blood cholesterol levels were noted. It was thus concluded: "With one exception, there was no discernible association between reported dietary intake and serum cholesterol level in the Framingham Diet Study Group." The one exception was a weak negative association between caloric intake and serum cholesterol level in men. The report also concluded that there was "no suggestion of any relation between diet and the subsequent development of coronary heart disease in the study group, despite a distinct elevation of serum cholesterol in men developing coronary heart disease." Thus, although the Framingham Study has achieved significance through its identification of the risk factors associated with the development of coronary heart disease, it has been unable to implicate diet as a risk factor in the development of coronary heart disease.

Clarity on what these findings actually mean is essential. They do mean that in the population tested in this study, normal dietary habits of a free-living individual did not appear to be related to the blood levels of cholesterol found in the subjects tested. In other words, within the range of dietary intakes covered by this study, intake of dietary cholesterol did not seem to affect blood cholesterol levels. These results do not imply, however, that serum cholesterol levels could not be influenced by diet under other circumstances. Yet it is evident from this study that something other than diet was responsible for the wide ranges in observed blood cholesterol values. And, although blood cholesterol values can regularly

be manipulated in controlled or laboratory animal studies, a decrease in blood cholesterol levels in unrestricted, free-living humans would appear to be exceedingly difficult to produce. If the influence of diet on blood cholesterol levels and on concurrent heart disease were of major importance, the results of this and other studies would transcend the weaknesses and deficiencies of the experiments and a clear-cut effect of diet change would be seen. As stated previously, if dietary manipulation *could* actually lower serum cholesterol values and, in turn, decrease the incidence of coronary heart disease, its effect would be very small indeed.

Hazards from diet manipulation

In criticism of recommendations that consumption of polyunsaturated fatty acids be increased: there has been very little experience with the types of diets recommended by the Inter-Society Commission for Heart Disease Resources. The Inter-Society Report recommendations encourage consumption of polyunsaturated fatty acids up to 10% of total calories consumed. It is possible that diets high in polyunsaturated fatty acids may decrease blood cholesterol concentrations as a result of the redistribution of cholesterol into other tissue pools (28). The health significance of alterations in specific cholesterol pools when the total body cholesterol pool remains the same is unknown.

Many fats and oils currently available commercially have been subjected to the process of partial hydrogenation, which results in the production of geometric (trans) and positional (conjugated) isomers of the unsaturated fatty acids. It has been reported that margarines produced in the United States may contain as much as 46.9% trans fatty acids and that some commercial oils contain as much as 62% positional fatty acid isomers. Certain metabolic reactions are known to be affected by the configuration of the fatty acids. When incorporated into cell membranes, permeability characteristics and other biological functions may be seriously altered (20, 21).

HYPERLIPOPROTEINEMIA

Recently, scientists have become increasingly aware that individuals have specific blood lipid characteristics which respond differently to dietary therapy. Normally, cholesterol and other lipid substances are insoluble in the fluids of the body unless complexed with a protein molecule. The complexes of lipid material and protein referred to as liproproteins are readily soluble. Based upon their relative composition of triglycerides, cholesterol, phospholipids, and protein, the various classes of lipoproteins can be separated and identified by electrophoretic and ultracentrifugation techniques.

These disorders, commonly referred to as Type I through Type V hyperlipoproteinemia, or high lipid-protein concentrations, have made it possible to identify the particular type of blood lipid disorder which an individual may have and then prescribe therapy tailored to the individual's specific needs. The increased plasma concentration of certain fractions of lipoproteins, in particular the *beta*-lipoprotein fraction, has been associated with increased risk of atherosclerotic heart disease because of its high cholesterol content.

Types I, III, and V are relatively uncommon and will not be considered further here. The blood lipid disorder termed Type II is very common and is believed to be expressed as a dominant genetically inherited trait. This disorder has been associated with premature death from atherosclerotic heart disease. The plasma from fasting patients with this condition is clear and the cholesterol value may be excessively high, as indicated by a distinctive *beta*-lipoprotein band. The recommended dietary treatment for the condition requires a reduction in cholesterol and saturated fatty acid intake accompanied by an increased uptake of polyunsaturated fatty acids. Changing the ratio of polyunsaturated to saturated fatty acids from the usual 0.2 ratio to 2.0 and reducing dietary cholesterol to less than 200 mg per day have been regarded as acceptable guidelines to follow in correcting this type of hyperlipoproteinemia.

The Type IV, or endogenous hyperlipidemia, is considered the most common blood lipid disorder. These patients will have normal cholesterol levels accompanied by grossly elevated plasma triglyceride concentrations. Patients with this condition commonly suffer from coronary artery disease. Dietary treatment in this instance is of importance since most of the patients are obese. It has been recommended that caloric intake be restricted, for weight reduction alone may effect complete regression of this condition (22).

DAIRY INDUSTRY AND HEART DISEASE

The dairy industry is contributing toward unraveling the mystery of the causes of heart disease through its membership in National Dairy Council (NDC). NDC maintains an active extramural research program designed to learn more about the relationship of our dietary habits and heart disease as well as supporting research directed toward ascertaining the importance of other possible causes. This organization continues to keep up-to-date regarding this issue and avails itself to the industry for interpretation and guidance relating to the diet/heart association.

NDC shares with many eminent scientists the view

that much yet needs to be learned about the cause or causes of coronary heart disease. We also share the conviction that the alleged answer to the problem of coronary heart disease would, at this time, be far better applied on an individual, physician-patient basis, as depicted in the individuality of blood-lipid disorders. An individual at risk must be identified and given individual medical advice as to all the things he can do to reduce his chances of developing coronary heart disease. Unless otherwise directed by a physician, the best advice is to eat a balanced diet from each of the basic food groups in amounts which will maintain desirable body weight and to participate in a program of regular exercise. Thus, the future position of the dairy industry regarding the role of diet and blood cholesterol in relation to coronary heart disease should be:

(a) to continue to give strong support to research directed toward determining the cause or causes of coronary heart disease;

(b) to intensify its effort to tell the scientific community and the general public what is known in this highly controversial area and to keep them appraised of all new developments;

(c) to reject all efforts to bring about massive changes in the dietary habits of the American people, especially with regard to the consumption of dairy foods, on the basis of the inconclusive evidence to date;

(d) to encourage, through a strong program of nutrition education at the national and community level, consumption of a balanced diet chosen from the four food groups, including the recommended two or more glasses of milk a day for adults, and three or more for children, at a caloric level to maintain ideal weight;

(e) and finally, to rely on the inter-dependence of a balanced diet, sensible weight control, regular exercise, and consultation with your physician on special problems as the wisest course to follow on the road to good health.

SUMMARY

In summary, it would thus appear that the relationship of dietary saturated fatty acids and cholesterol is not completely based upon well-established evidence; there are many loop-holes in the existing studies which implicate diet in the etiology of heart disease. Too little is known regarding the possible effects of dietary changes which have been recommended and finally it should be recognized that the effectiveness of such changes is only speculative.

Dr. Edward H. Ahrens, Jr., Chairman of the National Heart Institute Diet-Heart Review Panel, has stated that "available evidence supporting the proposition that the incidence of coronary heart disease might be reduced by dietary measures is suggestive but not convincing," and "that in the absence of conclusive proof on the diet-heart question, any dietary advice to the American public will always lack authenticity and authority, will be conducive to half-measures and will meet opposition which cannot be effectively countered."

Major scientific organizations such as the Food and Nutrition Board of the National Academy of Sciences-National Research Council; The Council on Foods and Nutrition of the American Medical Association, and the Committee on Nutrition of the American Academy of Pediatrics, apparently agree inasmuch as they have not issued specific dietary recommendations to the general public.

Dr. W. B. Kannel, Medical Director of the Framingham Study, stated, "The root cause of the increase in the prevalence of atherosclerotic disease that we see today may very well be laid on the doorstep of progress" (16). One medical man has even gone so far as to state that "the increased incidence of age-related atherosclerotic deaths should shine as a jewel in the crown of medical science. Our knowledge of medicine has eradicated other major causes of death such as tuberculosis and typhoid fever and, in turn, allowed us to live long enough to succumb to such a relatively painless death as atherosclerosis. No other disease in the history of mankind has been so considerate to allow so many to live so long a productive life" (6). This concept may certainly be kept in mind when evaluating the current status of our nation's population; however, regardless of the past successes of medical science, we would be discontent by our very own nature to be satisfied until this disease has been reduced to a minor health problem.

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The fifth edition of "The Modern Way to Efficient Milking" has recently been published by the Milking Machine Manufacturers Council of the Farm and Industrial Equipment Institute. This new 36-page booklet, which contains a uniform set of procedures relating to milking machine operation, has two important revisions. There is a new section on preventive maintenance, and new data on the recommended number of units per slope in pipeline installations.

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INTERACTIONS OF FOOD STARTER CULTURES AND FOOD-BORNE PATHOGENS: *STREPTOCOCCUS DIACETILACTIS* VERSUS FOOD PATHOGENS[1, 2]

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Abstract

The ability of *Streptococcus diacetilactis* to inhibit a variety of food spoilage organisms and pathogens in milk and broth cultures was demonstrated. Test organisms included *Pseudomonas* and *Alcaligenes* species, *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens,* and *Vibrio parahaemolyticus*. In general, approximately 99.0% and 99.9% inhibition was observed in milk and broth, respectively. Possible practical applications of the inhibition were examined. Addition of *S. diacetilactis* extended the shelf life of artificially contaminated cottage cheese and prevented proteolysis in milk at 7.5 C by *Pseudomonas fluorescens*. *Staphylococcus aureus* was inhibited greater than 99% in vanilla cream filling, ham sandwich spread, chicken gravy, soy milk, and ground beef stored at 25 C for 24 hr. Development of the gram-negative flora of ground beef was also inhibited greater than 99% after storage at 7.5 C for 7 days. Possible roles for several factors in the mechanism of inhibition by *S. diacetilactis* are briefly discussed. The effects of pH reached and acids produced by *S. diacetilactis* on the growth of *S. aureus* are described. A greater role for the lactic acid bacteria in fermentations in the food industry is suggested.

The lactic acid bacteria have proven useful to man in fermentations in the food and dairy industries, to add flavor and palatability to existing foods, to make new foods, and as a means of food preservation. These uses have been well studied. Although there are widely held traditions relating to the therapeutic value of fermented milk beverages, it is only recently that the ability of the lactic acid bacteria to suppress many undesirable microorganisms has received much attention from research workers. There are numerous reports on the observed inhibition of *Staphylococcus aureus* (7, 23, 26, 35) *Pseudomonas* (31, 32) and *Salmonella* species (37) by lactic acid bacteria. The studies described in this report were initiated to examine ways in which this inhibitory ability of the lactic acid bacteria may be beneficial to man. One approach studied extensively in the past concerns the ability of some of these organisms to implant in the intestine of animals, including man, and maintain a proper microbial balance, so often destroyed by over use of antibiotics. Another approach concerns the ability of the lactic acid bacteria to inhibit a variety

of food spoilage organisms and pathogens and this will be discussed here. Preliminary reports have been presented (8, 9).

Materials and Methods

Cultures

All cultures used in this study were available from the Microbiology Department, Oregon State University. Table 1 lists the distinguishing characteristics of *Streptococcus diacetilactis,* the inhibitory organism used. It is a member of the lactic *Streptococcus* Group N and is distinguished from *Streptococcus lactis* and *Streptococcus cremoris* by its utilization of citrate and production of diacetyl. Data reported here are for *S. diacetilactis* strain 18-16. Stock cultures of this organism were maintained frozen in 11% nonfat dry milk (Matrix, Galloway West Co., Fond-Du-Lac, Wisconsin) at −20 C. *Vibrio parahaemolyticus* ATCC 17802 was maintained on Trypticase Soy Agar (Baltimore Biological Laboratory) slants with 2.5% added sodium chloride. *Clostridium perfringens* strains were maintained on slants of medium with the following composition: 5.0% trypticase, 1% peptone, 0.5% yeast extract, 0.5% glucose, 0.01% thioglycollate, and 1.5% agar. All other organisms were maintained on lactic agar slants (13). Several strains of *Staphylococcus aureus* including *S. aureus* ATCC 13565, *S. aureus* 14458, *S. aureus* 19095, *S. aureus* 23235, *S. aureus* 265-1, and *S. aureus* 12600 showed similar responses in inhibition studies. The data reported here are for *S. aureus* 265-1.

Inhibition studies

A cross streaking technique (42) in which the test organism and *S. diacetilactis* were cross streaked at right angles to each other was employed. Milk agar plates were prepared by mixing sterile milk (11% solids) and an equal volume of 3% melted agar solution. These were cross-streaked with *S. diacetilactis* and proteolytic organisms to check for inhibition of growth and proteolysis.

Associative growth experiments in milk and broth

Each test organism was grown in association with *S. diacetilactis* in milk and/or lactic broth and viable counts of the two organisms were determined by differential plating techniques. One hundred milliliters of milk or broth in a 250-ml Erlenmeyer flask was inoculated (1%) with an 18-hr culture of both *S. diacetilactis* and the test organism. For *V. parahaemolyticus,* the broth was modified to contain 1% glucose and 3.0% sodium chloride. Control flasks had each organism growing alone. Incubation was static at 21 C or 30 C and viable counts were determined at intervals. Samples were removed and diluted as necessary using Butterfield's phosphate buffer (14). Aliquots (0.1 ml) of the appropriate dilution were placed on prepoured plates and spread with a glass rod. Lactic agar with 0.002% brom-cresol-purple incorporated was used to count *S. diacetilactis. Escherichia coli, Serratia marcesens* and the *Pseudomonas* and *Alcaligenes*

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strains used were counted on plate count agar (Difco) with 1 ppm crystal violet incorporated to prevent growth of *S. diacetilactis*. *Salmonella* species were plated on XLD agar (Difco). *Streptococcus liquefaciens* was counted on lactic agar plates incubated at 45 C for 48 hr. *Staphylococcus aureus* was plated on Vogel Johnson medium (Difco) and on lactic agar with 7% sodium chloride which excluded the growth of *S. diacetilactis*. *Vibrio parahaemolyticus* was plated on Trypticase Soy Agar containing 7% sodium chloride. Also, for this organism, 3% sodium chloride solution was used as diluent. In the case of *C. perfringens*, the associative growth experiments were carried out under 24 units of vacuum in a Jasper P. Marsh, Model 36501, anaerobic chamber (National Appliance Co., Portland, Oregon) at 30 C. Plate counts of *C. perfringens* were determined by the pour plate technique using the maintenance medium detailed above. The plates were placed in jars which were first evacuated, flushed twice with 99.6% nitrogen - 0.3% carbon dioxide, and maintained in this atmosphere at 45 C for 24 hr.

The ability of *S. diacetilactis* to prevent proteolysis of milk by *Pseudomonas fluorescens* at low temperatures was examined by conducting associative growth experiments similar to those described and incubating the flasks at 7.5 C for 4 weeks.

Associative experiments in foods

The technique used for creaming cottage cheese and extending the shelf life has been described by Vedamuthu et al. *(42)*.

Using *S. aureus* as the test organism, associative growth studies with *S. diacetilactis* were carried out with different foods as suspending media. The procedure described for milk and broth studies was modified to suit the food in question.

Dehydrated chicken gravy from two different commercial sources was reconstituted following the manufacturers' instructions. A commercially available soy bean milk was treated in a similar manner. Vanilla cream filling contained sugar, flour, salt, milk, eggs, and vanilla. Aliquots (100 gm) were inoculated with the desired cultures and incubated at 25 C. Lactic broth cultures of *S. aureus* were centrifuged at 6,000 × g for 12 min, resuspended in phosphate buffer, and added to the food at the desired concentration.

Commercial ham spread was weighed into a sterile Waring blendor jar and mixed well after addition of the desired cultures. A 1% inoculum of an 18-hr culture of *S. diacetilactis* was used. The well mixed ham spread was pipetted into test tubes using a wide mouthed pipet (10-ml quantities) and incubated at 25 C.

Ground meat, after receiving the appropriate additions of cultures, was thoroughly mixed by hand using disposable vinyl gloves. The meat (10 g) was then dispensed in sterile petri dishes. Samples were incubated at 25 C to check for inhibition of *S. aureus* and at 7.5 C to check for inhibition of the gram-negative spoilage flora. Representative samples from each storage condition were plated at 1 day intervals. Ten grams of meat were added to 90 ml of sterile phosphate buffer in a Waring blendor jar. Plating was done as described earlier after mixing for 3 min.

Cell-free preparations

Cell-free supernatants of *S. diacetilactis* were obtained by centrifuging lactic broth cultures at 6,000 × g for 12 min and removing the supernatant which was filter-sterilized. The ability of the test organisms to grow in these supernatants was determined using the plating methods described above.

Associative growth experiments at controlled pH

Staphylococcus aureus was grown in association with *S. diacetilactis* in a New Brunswick batch fermenter with pH maintenance at 6.8 by addition of 35% sodium carbonate. A 1% inoculum of an 18-hr culture of each organism was added to 700 ml of lactic broth. The medium was agitated gently at 30 C. Control experiments were carried out using each organism growing alone and the two in association without pH control. Viable plate counts were determined at intervals.

Effect of added acids

Acetic, formic, lactic, and hydrochloric acids were added to lactic broth to adjust the pH to 4.5·and aliquots neutralized to pH 5.0, 5.5, 6.0, and 6.5 with 35% sodium carbonate. Filter-sterilized 100-ml samples were inoculated with *P. fluorescens* and *S. aureus* and treated as described above.

Estimation of acids produced by S. diacetilactis

The contribution of the acids produced by *S. diacetilactis* to the observed inhibition was studied in three modifications of lactic broth. Medium A contained 2% tryptone, 1% glucose, 0.4% sodium chloride, and 0.25% gelatin. Medium B contained these ingredients plus 0.5% yeast extract and 0.5% sodium citrate. Medium C contained 1.0% sodium citrate in addition to the other ingredients in Medium B.

Lactic acid

Total lactic acid was determined by the Harper and Randolph *(17)* modification of the Ling *(28)* method as outlined by Mattsson *(29)*. The amount of lactic acid in cell-free supernatants was determined colorimetrically from the yellow color of the supernatant on the addition of ferric chloride. A 2% $FeCl_3 \cdot 6H_2O$ solution in water was used. The optical density at 425 nm was read within 15 min using a Bausch and Lomb Spectronic 20 spectrophotometer. An uninoculated blank was treated similarly. Since *S. diacetilactis* produces all L(+) lactic acid *(29)*, determination of L(+) lactic acid was carried out to confirm the total lactic acid results. This was done using the method of Mattsson *(29)*; the nicotinamide adenine dinucleotide (NAD-grade III) and lactic dehydrogenase (stock no. 826-6) were obtained from Sigma Chemical Company. The optical density was read at 340 nm on a Beckman DU spectrophotometer against an uninoculated blank treated in the same way as the sample. The amount of L(+) lactic acid was read from a standard curve.

Acetic acid

Culture supernatants were checked for volatile acids by gas liquid chromatography. A 6 ft × ⅛-inch stainless steel column was filled by vibration with a packing mixture prepared as follows; 2.0 g of neopentyl-glycolsuccinate and 0.2 g of concentrated phosphoric acid were dissolved in about 50 ml of chloroform (enough to form a smooth slurry with Poropak Q). Once dissolved, 10 g of 80-100 mesh Poropak Q were added and the slurry well stirred in a small beaker. The mixture was evaporated to dryness and added to the column by vibration. The column was equilibrated for 12 hr. at 180 C before use. All analyses were made using an F and M High Efficiency Gas Chromatograph (Model 402) with a Honeywell Strip Chart Recorder (Electronik 16) and a Hewlett-Packard 3370A Integrator. Isothermal operation at 160 C was used. Injection port and detector temperatures were 198 C and 195 C, respectively. Gas flow rates were approximately as follows: helium 70 ml/min, hydrogen 20 ml/min, and air 300 ml/min. The injection volume was 1 or 2 μl.

Determination of formic acid

The method of Wood and Best *(43)* as employed by Fryer *(15)* to measure formate production by lactobacilli was used to measure this metabolite in supernatants of *S. diacetilactis*.

TABLE 1. COMPARISON OF S. *diacetilactis* AND OTHER LACTIC ACID BACTERIA.

Organism	Taxonomic tests				
	Acid coag. 48 hr at 30 C	Diacetyl in milk	NH₃ from arginine	Dextran from sucrose	Pptn. with group N Anti-serum
S. *lactis*	Yes	No	Yes	No	+
S. *cremoris*	Yes	No	No	No	+
S. *diacetilactis*	Yes	Yes	Yes[b]	No	+
L. *citrovorum*	No	Yes[a]	No	No	−
L. *dextranicum*	No	Yes[a]	No	Yes	−

[a]Only associatively
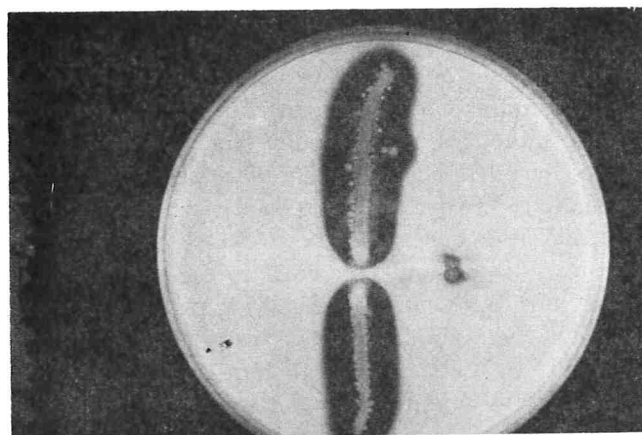[b]A few strains negative

Figure 1. Demonstration of the inhibition of growth and proteolysis of casein by *P. viscosa* (streaked from top to bottom) as a result of cross streaking against S. *diacetilactis* (left to right) on milk agar and incubating at 25 C for 48 hr.

Formate was reduced to formaldehyde which was then reacted with acetyl acetone to produce a colored compound. The optical density at 412 nm was determined in a Bausch and Lomb Spectronic 20 spectrophotometer.

RESULTS AND DISCUSSION

Types of organisms inhibited

An example of the cross-streaking technique used to demonstrate inhibition of *Pseudomonas viscosa*, a common food spoilage organism, by S. *diacetilactis* may be seen in Fig. 1. Inhibition of proteolysis and growth were observed. The ability of S. *diacetilactis* to inhibit a variety of spoilage organisms and pathogens during associative growth in milk and broth cultures is summarized in Table 2. In general, less inhibition was observed in milk as compared to broth cultures at 24 hr. Included are well known spoilage organisms in dairy products, such as *Pseudomonas* and *Alcaligenes* species. These, and other pyschrophilic bacteria, are also important components of the spoilage flora of beef and other meats (2, 25, 38). *Staphylococcus aureus*, *C. perfringens* and *Salmonella* are the most common causes of microbial food-poisoning in the United States (40). *Vibrio parahaemolyticus* is responsible for more than 70% of the food poisoning outbreaks in Japan (36), and is currently receiving much attention from research workers in this country. The presence of this organism in Pacific Northwest Coast waters was documented by Baross and Liston (1).

Figures 2 and 3 show typical inhibition patterns for *S. aureus* and *C. perfringens* respectively, when grown in association with S. *diacetilactis*. The test organism grew well in association at first but growth was soon halted and after about 4-6 hr there was a rapid and continued decrease in viable count. The plate counts of *S. aureus* were made on lactic agar with 7% sodium chloride. Since "stress" of *S. aureus* can decrease its tolerance to sodium chloride, as shown in the case of heat-injury by Iandolo and Ordal (24), appropriate dilutions of the associatively growing cultures were plated on lactic agar with the normal 0.4% sodium chloride. These plates were crowded with S. *diacetilactis* colonies but showed no catalase-positive colonies on the addition of hydrogen peroxide. This check indicated that the counts on lactic agar + 7% sodium chloride were accurate reflections of the number of viable *S. aureus* cells in the accociatively growing cultures. The ability of S. *diacetilactis* to inhibit *C. perfringens* under partially anaerobic conditions demonstrates the inhibitory versatility of the lactic acid bacteria. There was no recovery of the

TABLE 2. SUMMARY OF THE ORGANISMS INHIBITED IN THIS STUDY BY S. *diacetilactis*.

Organism[a]	% Inhibition at 24 hr[b]	
	Milk	Broth
Pseudomonas fluorescens	99.9	99.99
P. fragi	99.9	99.99
P. viscosa	99.9	99.99
P. aeruginosa	90.0	99.90
Alcaligenes metalcaligenes	99.9	99.99
A. viscosa	99.9	99.99
Escherichia coli	80.0	99.90
Serratia marcescens	90.0	99.90
Salmonella seftenberg	−	99.99
S. tennesse	70.0	99.90
Staphylococcus aureus	99.9	99.99
Clostridium perfringens	99.0	99.90
Vibrio parahaemolyticus	−	99.90
Streptococcus liquefaciens	95.0	−

[a]*Pseudomonas* and *Alcaligenes* containing cultures were incubated at 21 C while the others were incubated at 30 C.
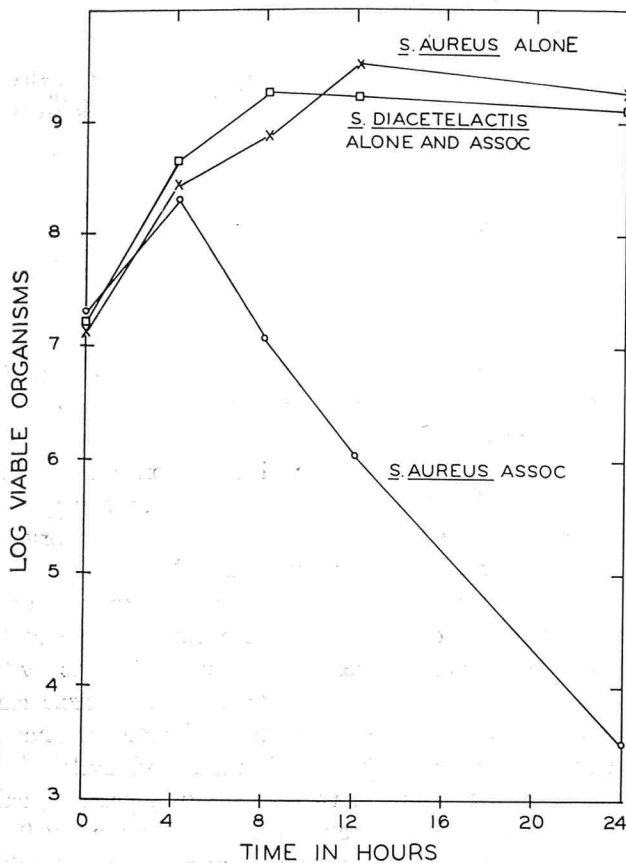[b]Compared to the test organism growing alone.

Figure 2. Inhibition of *Staphylococcus aureus* when grown in association with *Streptococcus diacetilactis* in lactic broth at 30 C. Lactic agar included 7.0% NaCl to differentiate *S. aureus.*

test organisms on extended incubation (96 hr). It is important to observe that a substantial killing effect was achieved in these growth experiments.

Practical applications of the inhibition

A number of experiments have been conducted in our laboratory to determine the practical usefulness of the observed inhibition. The creaming of cottage cheese with cream cultured with S. *diacetilactis* extended the shelf life up to 3 weeks, (Fig. 4), as previously reported from this laboratory by Vedamuthu et al. (42). Addition of S. *diacetilactis* (1%) to 11% nonfat milk inoculated with 1% *P. fluorescens* prevented proteolysis by the spoilage organism, even at 7.5 C as shown in Fig. 5. Plate counts over a 4-week period indicated that growth of *P. fluorescens* was retarded. This demonstration is important because it indicates that inhibition by S. *diacetilactis* can be effective at low temperatures and could have a beneficial role in food preservation.

Associative growth of S. aureus in foods

Associative growth experiments were conducted in a number of food products. *Staphylococus aureus* could be successfully inhibited using ham sandwich

spread as the suspending medium (Fig. 6). A 1% inoculum of an 18-hr milk culture of S. *diacetilactis* was used. This finding is significant considering that ham is a favorable medium for growth and toxin production by S. *aureus* and is often the food incriminated in *Staphylococcus* food-poisoning. The counts of S. *aureus* were considerably reduced when plated on Vogel-Johnson medium. The need for a good medium for plating S. *aureus* from foods and other sources was recently re-emphazised by Keogh (27). Lactic agar + 7% sodium chloride sufficed in the experiments described here using high inocula of pure cultures but would be unsuitable when determining S. *aureus* counts in foods under conventional conditions. In our current studies we are using a successful medium developed in our laboratories by L. R. Chugg, (4).

Table 3 lists some of the food items in which inhibition of S. *aureus* has been demonstrated. Vanilla cream filling, ham sandwich spread, and chicken gravy are representative of food items sometimes associated with food poisoning caused by S. *aureus.* Soymilk is used in several fermentation products, especially in Oriental countries and may find increasing uses as the search for new protein sources continues. Meat products, however, are foods in which
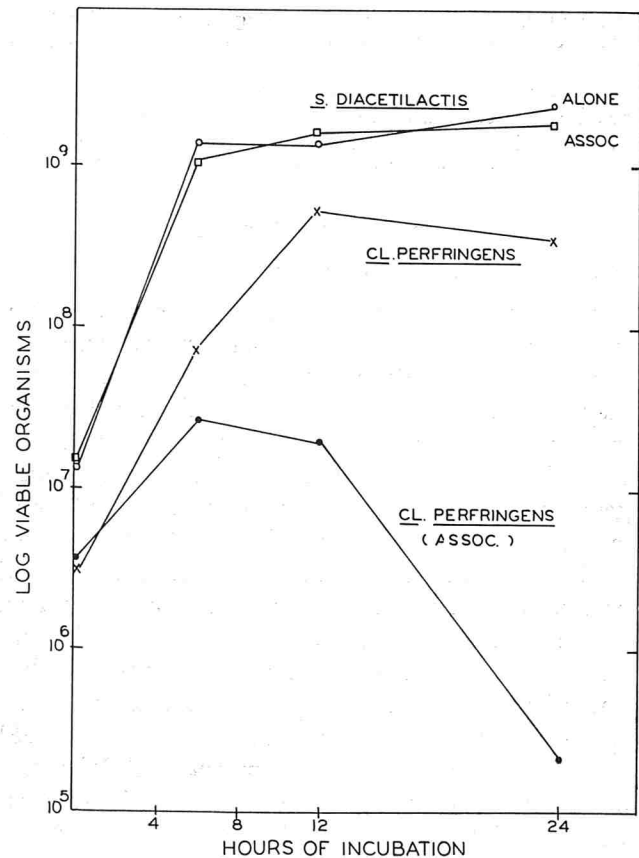

Figure 3. Inhibition of *Clostridium perfringens* when grown in association with *Streptococcus diacetilactis* in lactic broth at 30 C.

TABLE 3.   INHIBITION OF S. *aureus* (IN VARIOUS FOOD PRODUCTS) AND THE GRAM NEGATIVE
FLORA OF GROUND BEEF BY S. *diacetilactis*[a]

Food Product	Organism	Initial inoculum	% Inhibition[b]	Temp. of incubation
Vanilla cream filling	S. *aureus*	$4 \times 10^5/g$	99.9 at 24 hr	25 C
Ham sandwich spread	S. *aureus*	$3 \times 10^6/g$	99.5 at 24 hr	25 C
Chicken gravy I	S. *aureus*	$5 \times 10^6/g$	99.1 at 24 hr	25 C
Chicken gravy II	S. *aureus*	$1 \times 10^5/g$	99.2 at 24 hr	30 C
Soya milk	S. *aureus*	$2 \times 10^5/ml$	99.7 at 24 hr	25 C
Ground beef	S. *aureus*	$4 \times 10^5/g$	99.3 at 24 hr	25 C
Ground beef	gram-negative flora	none added	99.5 at 7 days	7.5 C

[a]A 1% inoculum of an 18 hr culture of S. *diacetilactis* was added to all foods except ground beef for which a 2% inoculum was used.

the lactic acid bacteria may find most application, in the development of new products and improvement of existing ones. Ground meat, in particular, usually contains a high gram-negative spoilage flora. We could successfully control the development of these organisms at low temperatures (7.5 C) using a 2% inoculum of a milk culture of S. *diacetilactis*. Our results confirm the findings of Reddy et al. *(33)*, who used conventional and frozen starter cultures to control the flora of ground beef at 7 C. The availability of several lactic acid bacteria in concentrate form makes their addition to meat and other products feasible, without the problems associated with propagating and carrying these cultures in the conventional manner. The advances in techniques of starter production and storage should be of tremendous benefit to those in the fermented meat industry, in particular. *Staphylococcus aureus* can be inhibited at 25 C in ground meat, indicating an added advantage of starter cultures in making fermented meat products.

Mechanism of Inhibition

While the ability of S. *diacetilactis* and other lactic acid bacteria to inhibit a variety of microorganisms is well established, the exact mechanism involved is less well understood and is the subject of much conjecture. Some of the possible factors involved are discussed below.

Antibiotic production. The ability of certain lactic acid bacteria to produce antibiotic substances has been well established, e.g. nisin production by S. *lactis*. Collins reported strain dominance due to antibiotic production by S. *diacetilactis (6)*. The broad spectrum of inhibition shown in our studies suggests that nisin or a similar compound does not cause all the observed inhibition. Vedamuthu et al. *(42)* were unable to demonstrate inhibition by cell-free supernatants or cell extracts of S. *diacetilactis* using the disc assay technique. Inhibition was observed when live cells of S. *diacetilactis* were used. Recent preliminary reports described the isolation and some properties of acidophilin *(41)* and bulgarican

(34), broad spectrum antibiotic substances produced by *Lactobacillus acidophilus* and *Lactobacillus bul-*
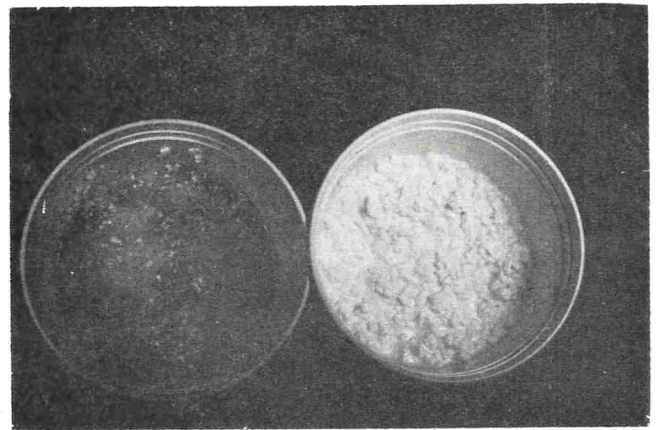
Figure 4.   Appearance of Cottage cheese after storage at 7.3 C for 21 days. Both samples were contaminated with *Pseudomonas putrefaciens*. The sample at left was creamed with 12% fat cream; sample at right was similarly treated except the cream was inoculated with 1% S. *diacetilactis* and incubated 6 hr at 21 C before adding to the cheese.
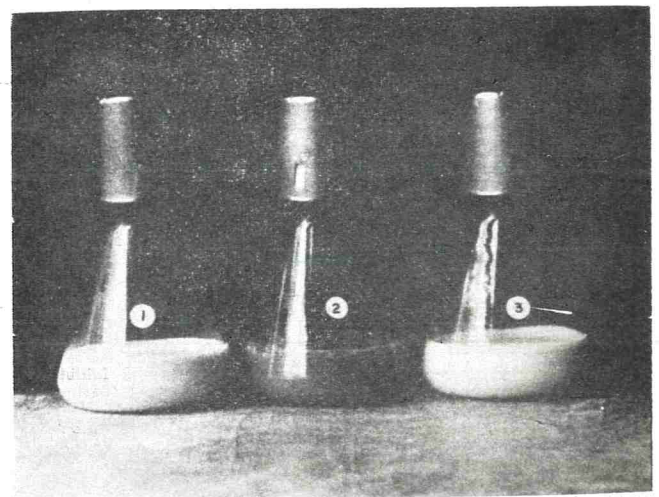
Figure 5.   Prevention of proteolysis of milk due to *Pseudomonas fluorescens*. Sample on the left was sterile milk. Sample in the center was inoculated 1% with *P. fluorescens*. Sample on the right was inoculated (1%) with both *P. fluorescens* and S. *diacetilactis*. The samples were incubated at 7.5 C for 21 days.
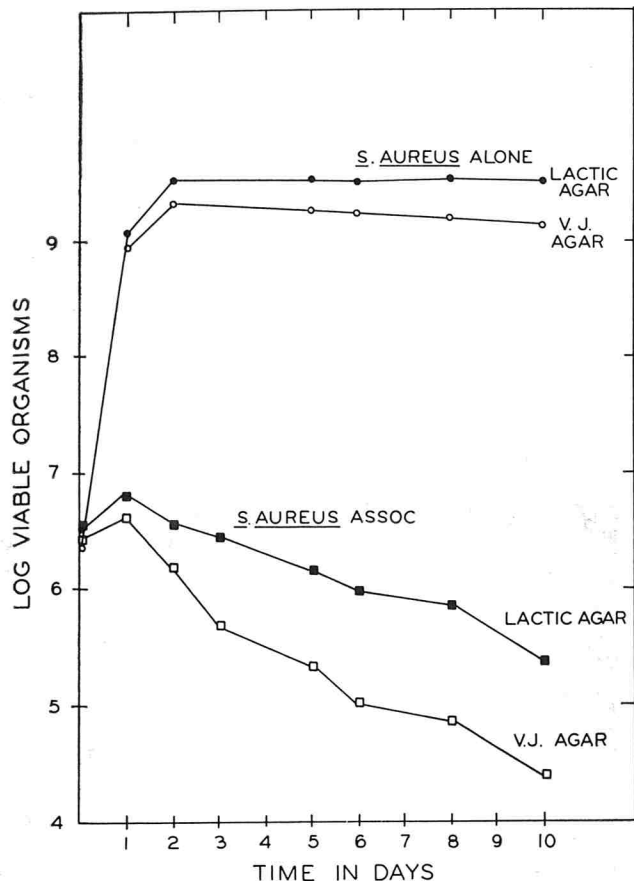
Figure 6. Inhibition of S. *aureus* when grown in association with S. *diacetilactis* in ham sandwich spread at 25 C. A 1% inoculum of S. *diacetilactis* was added. Lactic agar included 7.0% NaCl to differentiate S. *aureus*.

garicus, respectively. The possibility that S. *diacetilactis* produces similar antibiotics is worthy of consideration.

Hydrogen peroxide production. The toxic effects of hydrogen peroxide have been well demonstrated. Dahiya and Speck (7) identified hydrogen peroxide as the inhibitory agent in cultures of *Lactobacillus lactis* and *L. bulgaricus* active against S. *aureus*. More recently Price and Lee (32) showed hydrogen peroxide to be the active substance in the inhibition of pseudomonads by lactobacilli isolated from fish. In our studies, cell-free broth supernatants of S. *diacetilactis*, which will not allow growth of the test organisms, retained their inhibitory properties when heated at 121 C for 15 min and when catalase was added, indicating that hydrogen peroxide is not the inhibitory agent under these conditions.

Nutrient depletion. A difference in metabolic activities may make it possible for an organism to exhaust an essential nutrient from a growth medium and thus retard the growth of others. This effect was observed by Hsu and Lockwood (22). Dubos and Ducluzeau (11) showed that the inhibition of S. *aureus* by a *Micrococcus* species was due to the utilization by the *Micrococcus* of certain amino acids necessary for growth of S. *aureus*. The amino acids involved were glutamic acid, aspartic acid, arginine, and threonine. Likewise, Troller and Frazier (39) attributed the inhibitory properties of *Serratia marcescens* and a *Pseudomonas* species against an enterotoxigenic strain of S. *aureus* to competition for amino acids. Iandolo et al. (23) found depletion of nicotinamide from the medium to be the main cause of inhibition of S. *aureus* MF 31 by S. *diacetilactis*. More extensive inhibition was observed in supernatants at pH 7.3 than in those at pH 5.2, due to the decreased availability of nicotinamide at the higher pH. With our strains of S. *aureus*, the inhibitory effects of the supernatant are reduced by increasing the pH, (see Fig. 7) suggesting that the type of nutrient depletion observed by Iandolo et al. (23) is not involved.

Decrease in oxidation-reduction potential. An organism may cause inhibition of others by reducing the Eh of the medium to a sufficiently low level. Dubos and Ducluzeau (12) demonstrated that the inhibition of a *Micrococcus* species by S. *aureus* was due to lowering of the Eh to values below 100 mv. Hentges showed that the inhibitory properties of coliforms (20) and Klebsiella (18) against *Shigella* were due to a combination of reduced oxidation reduction potential and volatile acid production. The reducing properties of the lactic acid bacteria are well recognized. Dellaglio et al. (10) reported Eh

TABLE 4. RELATIONSHIP BETWEEN pH REACHED, AMOUNT OF ACIDS PRODUCED AND THE INHIBITION OF S. *aureus* BY S. *diacetilactis*.

Medium	% Inhibition (24 hr)[a]	pH Reached (24 hr)	Acids produced 24 Hr		
			Lactate (mg/ml)	Acetate (mg/ml)	Formate (mg/ml)
A[b]	99.99	4.25	3.2	0.75	—
A + 0.5% yeast extract + 0.5% sodium citrate	99.99	4.50	7.8	1.85	—
A + 0.5% yeast extract + 1% sodium citrate	99.20	5.05[c]	10.0	2.90	0.07

[a]Compared to S. *aureus* growing alone.
[b]Medium A contained 2% Tryptone, 1% glucose, 0.4% sodium chloride, and 0.25% gelatin.
[c]This medium buffered by citrate and growth products of S. *diacetilactis*.
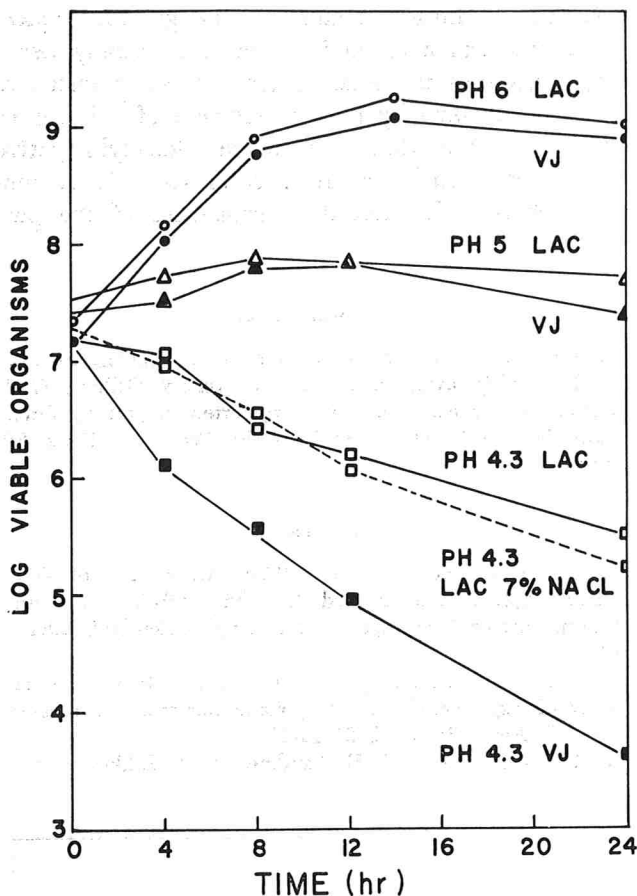
Figure 7. The effect of pH on the growth of S. *aureus* in cell-free supernatants of S. *diacetilactis* grown in a modification of lactic broth (medium A). The pH was adjusted from 4.3 with 35% sodium carbonate. Viable counts of S. *aureus* on lactic agar and Vogel Johnson agar are shown.

values as low as −250 mv for *Lactobacillus casei* grown in milk. We are presently studying the contribution of reduced oxidation reduction potential to the inhibitory properties of S. *diacetilactis* and other lactic acid bacteria.

Acid production and pH alteration

Due to the extensive fermentative capacity of the lactic acid bacteria, the metabolic products, especially the acids produced, must be considered as potential inhibitors. The inhibitory properties of acids such as acetic, formic, and lactic acids are well recognized and they have been implicated as the causative agents of bacterial inhibition in several studies. Sorrels and Speck (37) attributed the inhibitory properties of *Leuconostoc citrovorum* filtrates against *Salmonella gallinarum* to the acetic and lactic acids present, the former being more inhibitory. Similar findings were reported by Pinheiro et al. (31) on the inhibitory properties of S. *diacetilactis* against *Pseudomonas fragi*. Several reports by Hentges (18, 20, 21) demonstrated the role of acetic and formic acids in the inhibition of *Shigella* by coliforms

and *Klebsiella* in the intestinal tract. The inhibitory effects of several acids on *Salmonella typhimurium* and on S. *aureus* were studied by Goepfert and Hicks (16) and Minor and Marth (30), respectively.

In our studies, cultures of S. *aureus* and P. *fluorescens* failed to grow in cell-free supernatants of S. *diacetilactis* but did grow when the pH was adjusted towards neutrality (see Fig. 7). No inhibition was observed when S. *aureus* was grown in association with S. *diacetilactis* with pH control at 6.8 by automatic addition of 35% sodium carbonate (Fig. 8). A significant difference was observed between the S. *aureus* counts on lactic agar + 7% sodium chloride and those on Vogel-Johnson medium, suggesting that some cells were being injured under these conditions and failed to grow out on the more inhibitory medium.

These experimental results suggested that the acids produced by S. *diacetilactis* had a role in the inhibitory action, at least with respect to pH control. We therefore tested the effects of acetic, formic, lactic, and hydrochloric acids on S. *aureus*. The effect of each acid was shown to be pH dependent as shown in Fig. 9 for acetic acid. Greatest inhibition was observed at low pH, consistent with the inhibitory effects of the undissociated form of the acids. Similar effects of pH on inhibition by acids have been reported by several workers (16, 19, 37). Formic and lactic acids gave growth inhibition patterns similar to acetic acid, but hydrochloric acid allowed growth at all pH levels tested, indicating that low pH *per se* was not sufficient to inhibit S. *aureus*. This is in agreement with the results of Chung and Goepfert (5) who showed that the minimum pH at which *Salmonella* would initiate growth varied with the acid used to lower the pH.

The interaction between inhibition of S. *aureus*, pH reached, and amount of acid produced was studied when S. *diacetilactis* was grown in three modifications of lactic broth (Table 4). Medium A, in which there was least acid production, showed extensive inhibition, probably because of the low pH reached. The other media were buffered to an extent by the citrate present and probably also by neutral and basic products due to extensive growth of S. *diacetilactis* in these media. When 3.2 mg/ml of lactic acid and 0.75 mg/ml of acetic acid (the amounts produced by S. *diacetilactis* in 24 hr, Table 4) were added to medium A, this acidified medium was less inhibitory to S. *aureus* than supernatants of S. *diacetilactis* as determined by viable plate counts of S. *aureus* at intervals up to 48 hr. This suggested that the acids alone were not responsible for all the inhibition observed with growing cultures of S. *diacetilactis*. It appeared however, that the acids
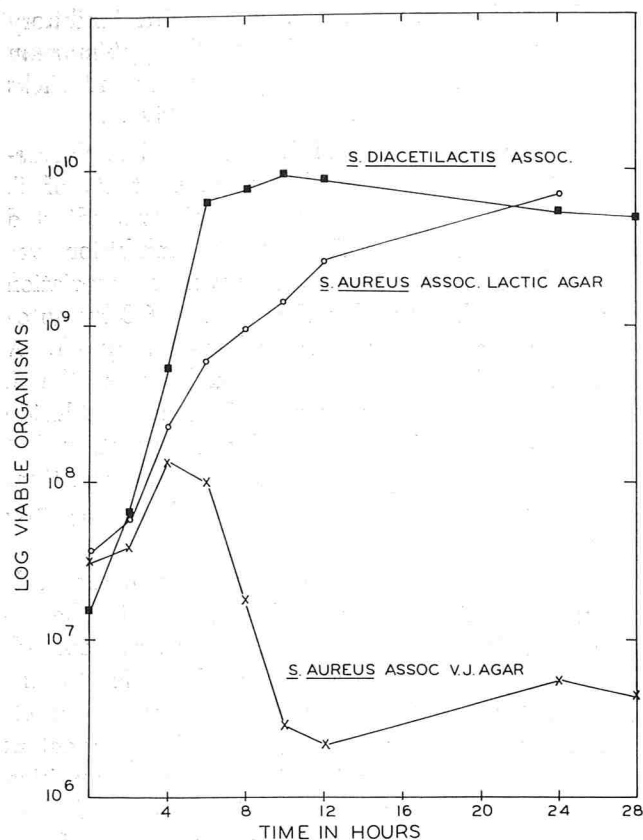
Figure 8. Growth of *S. aureus* in association with *S. diacetilactis* in a batch fermenter with constant pH maintenance at 6.8. Lactic agar included 7.0% NaCl to differentiate *S. aureus*.

produced were important in at least two roles. First, the acids themselves were toxic substances at a low pH and second, they provided the low pH environment that seems essential for the lactic acid bacteria to exert their inhibitory properties to the greatest extent, probably by allowing some metabolite effective at low pH to act.

Whatever the exact mechanisms involved, this tremendous ability of the lactic acid bacteria to inhibit a variety of undesirable microorganisms should not be allowed to go untapped by food microbiologists. We have been too slow to extend our knowledge of these extremely versatile bacteria to new products and too conservative in our efforts to use them in food fermentations and the development of new food sources. Another important aspect relates to lack of refrigeration in underdeveloped countries. Food preservation by controlled population dominance may be very useful here. The availability of frozen concentrates of most of the lactic acid bacteria would facilitate their use as described here. Such concentrates are easy to handle and provide large numbers of physiologically active organisms. While we have used *S. diacetilactis* in most of these studies so far, other lactic acid bacteria including lactobacilli, pediococci and leuconostocs may be used as

different conditions demand. Though the typical flavor and aroma of lactic cultures is usually desirable, objections to certain flavors in some instances may be overcome by proper selection of cultures or by the use of mutants such as the diacetyl-negative strains described by Burrows et al. *(3)*. These mutants retained the inhibitory properties of the parent strains.

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Figure 9. Influence of pH on the effect of acetic acid on *S. aureus*. Lactic broth was acidified to pH 4.5 and aliquots neutralized to the pH's shown. Plate counts were those on lactic agar. The counts on Vogel-Johnson agar of the pH 4.5 sample are also shown.

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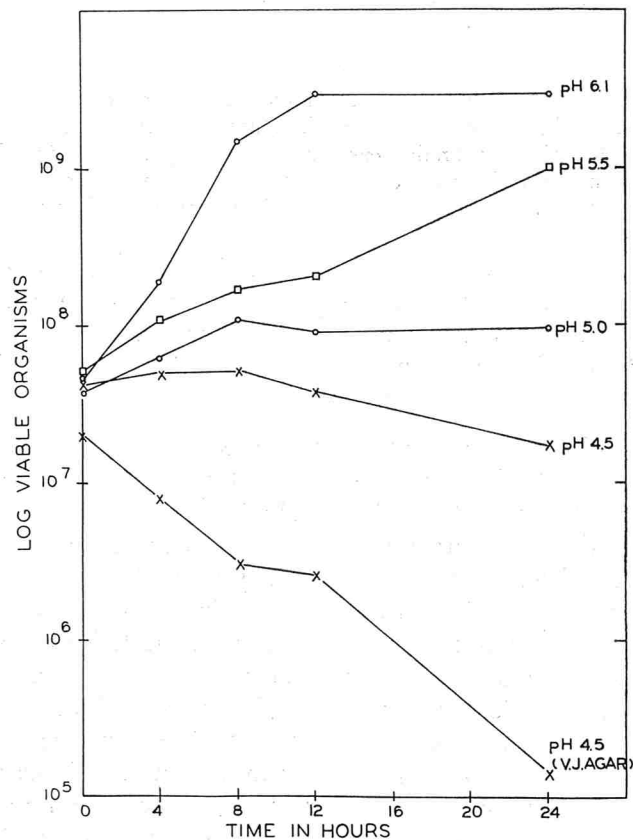4. Chugg, L. R. 1971. Rapid, sensitive methods for enumeration and enterotoxin assay for *Staphylococcus aureus*. Ph.D thesis. Dept. of Microbiology, Oregon State University. 82 p.

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THE PROPIONIC-ACID BACTERIA—A REVIEW

II. METABOLISM[1]

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Abstract

This paper, the second of three sections of a review, deals with propionate and acetate formation, carboxylation, decarboxylation, and transcarboxylation, the roles of biotin and vitamin B_{12}, and the enzymes involved in the propionic-acid fermentation.

Pathways for propionate, acetate, and carbon dioxide formation by *Propionibacterium* have been thoroughly investigated and established. These investigations led to discovery of transcarboxylase, a biotin-containing enzyme, and were the result of studies showing that the formation of propionate does not involve the expected turnover of CO_2. This enzyme appears to be unique to propionibacteria; it could be speculated that it is the first discovered of a group of transcarboxylases. One of the breakthroughs relative to the mechanism of biotin fixation reactions was the discovery that biotin can be carboxylated enzymatically.

Fitz *(64)* in 1878 began what is considered today as the first work of importance on the biochemistry of the propionibacteria. The fermentations studied were those of calcium malate and calcium lactate. Fitz made a careful study of the salts of the propionic and acetic acids, fractionally distilled the volatile acids, formed silver salts of the fractions, and thus definitely proved by the boiling point and silver content of the compounds that he was dealing with propionic and acetic acids. He concluded that, from three moles of malic acid, two moles of propionic acid and one mole of acetic acid were formed; in the process, four moles of carbon dioxide and one mole of water were formed. From his work using calcium lactate, he suggested the following equation for the propionic-acid fermentation:

$$3 \text{ lactate} \rightarrow 2 \text{ propionate} + \text{acetic} + CO_2 + H_2O$$
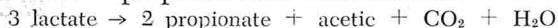
After the experiments of Fitz, study of the propionibacteria was completely overlooked until von Freudenreich and Orla-Jensen investigated other aspects of the propionic-acid fermentation. Orla-Jensen *(150)* first conducted research concerning the formation of eyes in Emmental cheese and, later, von Freudenreich and Orla-Jensen *(239)* were able to isolate pure cultures and to give the first extensive description of the propionic-acid-producing organisms. Most of the attention given these organisms was directed to their effects in Emmental cheese and their taxonomy *(55, 150, 239)*, until Virtanen *(233)*, van Niel *(231)*, and, later, Wood and Werkman *(277)* extensively investigated their fermentative abilities.

Virtanen's *(233)* efforts were directed toward understanding the actual chemical mechanism of the propionic-acid fermentation. He was particularly interested in determining if pyruvic acid and acetaldehyde played important intermediate roles in this fermentation. To investigate the probable occurrence of pyruvic acid as an intermediate product, Virtanen tested utilization of this keto acid by propionibacteria and found that it was easily fermented. The products were qualitatively the same, but quantitatively the reverse, of the lactic-acid fermentation; that is, twice as much acetic acid as propionic acid was formed. This quantitative difference led Virtanen to conclude that glucose is fermented probably by conversion first through lactic acid rather than directly via pyruvic acid. The propionic-acid fermentation of sugar was expressed as occurring in two distinct steps.

Van Niel *(231)* probably has made the most comprehensive contribution to our knowledge of this genus. His dissertation contains a full discussion of the characteristics of the species and a key for their differentiation. Methods of isolation also are discussed fully. His work also includes a comprehensive study of the biochemistry of the propionibacteria. Quantitative studies on the fermentation of lactic and pyruvic acids, glucose, glycerol, and starch enabled van Niel to draw conclusions as to the mechanism of the propionic-acid fermentation. Van Niel found that, in this C_3-acid fermentation, the ratio between acetic acid and CO_2 conforms to the theoretical value of one but that the ratio between propionic and acetic acids does not. He found the latter ratio to lie between 1.6 and 1.8. The balances established by Wood and Werkman *(277)* showed that the ratios of propionate to acetate vary from 2.1 to 14.7, and, the ratios of CO_2 to acetate, from 0.9 to 6.3, and that the succinate fluctuates from 7.9 mM to 26.1 mM per 100 mM of fermented glucose. These ratios were representative of the extreme differences

observed in two species of propionibacteria. They suggested that this wide difference in ratios with the two species resulted from a fundamental difference in the mechanism of fermentation. Later, this hypothesis was supported on the basis of isotope distribution in the products of the same two species by Wood et al. *(271)*. Virtanen *(233)* and van Niel *(231)* found the ratios of CO_2 to acetate to be substantially 1:1. The ratio of propionic to acetic acid usually had been found to be approximately 2:1, although van Niel *(231)* found ratios as high as 5:1. This increased yield of reduced products was explained by a donation of hydrogen by compounds occurring in the yeast-water medium. Such an explanation was not acceptable in the light of results of Wood and Werkman *(277)*, since their data on redox indexes show that the majority of schemes of the propionic-acid fermentation were not satisfactory, as it was impossible to apply experimental values to these schemes. Also, oxidation-reduction balances calculated from van Niel's results are not satisfactory and suggest that the nitrogen source materially affected carbohydrate metabolism.

For a more complete review of the early literature concerning the production of propionic acid by propionibacteria, reference should be made to the work of van Niel *(231)* and that of Wood *(258)*. Wood and his associates have conducted most of the research on fermentation by propionibacteria.

PROPIONATE FORMATION

Various routes of pyruvate and other carbohydrates to propionate formation exist in the metabolism of propionibacteria. The evidence indicates that hexoses are converted to pyruvate mainly by the Embden-Meyerhof pathway. Early studies with dried cells by Virtanen *(233, 234)* and by Pett and Wynne *(162)* established the uptake of organic phosphate and the accumulation and fermentation of hexose phosphates. Investigation by Wood and Werkman *(277)* pertaining to the dissimilation of glucose by the propionic-acid bacteria showed that schemes previously proposed for the mode of hexose dissimilation did not fully agree with their data. Furthermore, isolation by Stone and Werkman *(214, 215)* of phosphoglyceric acid during fermentation of glucose and hexose diphosphate necessitated reconsideration of previously proposed schemes for propionic-acid fermentation. Werkman and his collaborators *(214, 215, 216, 249, 250, 255)* showed that added fluoride plus a hydrogen acceptor prevented formation of normal products and caused 3-phosphoglycerate to accumulate. In the absence of fluoride, hexose diphosphate, 3-phosphoglycerate, and α-glycerol phosphate were fermented slowly to the normal products.

Lactate and pyruvate were readily converted into propionate by propionibacteria; hence in 1936, it was generally assumed that lactate was an intermediate in the reduction of pyruvate to propionate; i.e. pyruvate → lactate → propionate. Werkman and Wood *(250)* reviewed the various mechanisms suggested for formation of propionic acid and made two proposals. The first was that water is removed from lactate to form acrylic acid, which is then reduced to propionic acid. They regarded this as the most probable mechanism, though the reduction of acrylic acid by propionibacteria had not been demonstrated. The second proposal suggested was that propionic acid is formed by decarboxylation of a symmetrical dicarboxylic acid. This possibility was based on their experiments with isotopes and on unpublished evidence that decarboxylation of succinate can occur by a reaction with a pH optimum of 5.2.

Numerous observations concerning the ability of this genus to ferment succinic acid with the formation of propionic acid have been made *(69, 80, 188)*. Wood and Werkman *(281)* determined the products of the anaerobic breakdown of succinic acid and reported the production of propionic acid and CO_2 in approximately equimolar amounts along with small amounts of acetic acid. Apparently, no other products were recovered. Delwiche *(42)* described conditions under which the decarboxylation of succinate could constitute a major pattern of propionate formation by *Propionibacterium pentosaceum*. From the data presented, the succinic decarboxylase system seemed sufficiently active to produce propionic acid from succinate at a rate comparable to the rate of production of propionic acid from pyruvate.

Barker and Lipmann *(14)* showed that dried *P. pentosaceum* transferred phosphate from ATP to form an ester linkage with glucose, arabinose, glycerol, and several other substrates. In contrast to the definite labeling patterns obtained with lactic-acid bacteria, the fermentation of position-labeled glucose by *Propionibacterium arabinosum* revealed that all the carbon atoms of hexose were present to some extent in all the carbon atoms of the products. Many difficulties were encountered in interpreting isotope data, and the observation of Leaver et al. *(121)* that ^{14}C from lactate-3-^{14}C is found in all carbons of propionate and succinate as well as both carbons of acetate and CO_2 demonstrates these difficulties. A great deal of study has been necessary to interpret the results; it was, however, apparent with later evidence to be discussed that the labeling patterns and flouride inhibition studies reflected

a substantial conversion of glucose to pyruvate via the Embden-Meyerhof pathway.

Generally, the Embden-Meyerhof scheme is considered to be the major pathway of hexose dissimilation by members of the genus *Propionibacterium*. Two lines of evidence supporting this premise are: *(a)* isolation of several of the intermediates of glycolysis *(14, 273)* and *(b)* distribution of a major portion of the isotopic labeling of various intermediates commensurate with such a pathway *(271)*.

Evidence has, however, accumulated that negates the operation of a single route for hexose degradation. This is indicated by the relative insensitivity of glucose degradative mechanisms to glycolytic inhibitors such as fluoride *(71, 235, 255, 273)*. Also, isotopic data *(207, 271)* do not conform to any one carbohydrate dissimilative pathway. With glucose-1-^{14}C, for instance, considerable radioactivity appears in the CO_2, as would be expected if an anaerobic hexose monophosphate pathway were functioning *(267, 271)*. It was clearly pointed out at the time that another fermentation mechanism must exist.

Fukui *(71)*, while studying the ability of various species of the genus *Propionibacterium* to adapt to aerobic growth, noted that some were able to utilize sodium gluconate as an energy source. Since utilization of gluconate is generally considered indicative of a pathway or pathways alternate to conventional glycolysis *(74)*, an enzymatic study of gluconate dissimilation by propionibacteria was carried out by Vandemark and Fukui *(230)*. They found that cell-free preparations of gluconate-grown *P. pentosaceum* can oxidize the various phosphorylated intermediates of the Embden-Meyerhof and hexose-monophosphate pathways. Various enzymes essential to these pathways, as well as transketolase and transaldolase, were present in preparations. Volk supported these data *(236, 237)* by determining the occurrence of D-arabinose-5-phosphate as an intermediate in the metabolism of pentoses by propionibacteria. He later purified and described an enzyme which isomerized D-arabinose-5-phosphate to D-ribulose-5-phosphate *(238)*.

A nonreducing sugar formed in the propionic-acid fermentation *(276, 281)* has been identified as trehalose by Stjernholm *(206)*. To study additional pathways of propionate metabolism, attempts were made to trace the ^{14}C pattern in trehalose, which, in turn, could be used to reflect the hexosephosphate pools indicative of the specific metabolic route used by propionibacteria. One major difficulty in investigating the propionic-acid fermentation has been the randomization of the label in the substrate to every position in the propionate and succinate molecules *(271)*. Stjernholm and Wood *(209)* isolated

and degraded trehalose from labeled glucose medium to determine the ^{14}C distribution pattern. The ^{14}C distribution patterns both in the glucose and in the fructose units of the trehalose and in the fructose indicated that there is an Embden-Meyerhof type of cleavage to triose phosphates. The phosphorylated C_3-compounds are then equilibrated by triosephosphate isomerase, and recondensed by aldolase. Approximately 20% of the ^{14}C was randomized in accord with an Embden-Meyerhof cleavage, followed by resynthesis after equilibration of the triosephosphates.

To support the foregoing evidence for presence of additional pathways, Stjernholm and Flanders *(208)* took ribose-1-^{14}C and potassium gluconate labeled in different positions and incubated each with cell-free extracts of *Propionibacterium shermanii*. The resulting propionate, acetate, and succinate were isolated, and the ^{14}C distributions were determined from the data. They proposed that the extensive randomization observed is caused by conversion of the labeled substrates to fructose-6-phosphate via the transketolase-transaldolase sequence followed by the Embden-Meyerhof pathway and a transcarboxylation cycle [see Swick and Wood *(221)*]. Results of the isotope distribution in the metabolic acids of the fermentation of arabinose-1-^{14}C by actively growing cells of *P. pentosaceum* by Rappoport and Barker *(177)* in 1954 is in accord with the results of Stjernholm and Flanders *(208)* and can be explained by the same sequence of reactions.

The possibility of fermentation of substrates by members of this genus under aerobic conditions, such as through the citric-acid cycle has been investigated. Some of the early studies with propionibacteria pointed out observations contrary to a generalization of anaerobiosis in this genus. In this connection, the reader should refer to van Niel *(231)*, who perhaps made the first definitive observations on the oxidative activities of this genus. He discussed in detail his findings on oxidation of lactate and glycerol in the presence of both free oxygen and nitrate. Chaix and Fromageot *(35)* and Chaix-Audemard *(37)* made manometric measurements of oxygen uptake rates, revealing relatively active oxidation on a variety of substrates including glucose, lactate, and pyruvate. Generally, these studies gave comprehensive rate data, but not total oxygen consumption, thus rendering difficult any interpretation concerning oxidative pathways. Delwiche and Carson *(45)* found, as a result of several different approaches, that *P. pentosaceum* contains enzymes of the citric-acid cycle. They found that intact cells, when grown aerobically, can oxidize intermediates of the citric-acid cycle with the ex-

ception of citrate. Cell-free extracts quantitatively degrade citrate to α-ketoglutarate, and acetone-dried cells can synthesize citrate from pyruvate and oxaloacetate, and from acetate and oxaloacetate. Even though citrate is not utilized aerobically, Hietaranta and Antila (79) noted that citric acid is fermented and converted to propionate by propionibacteria in Emmental cheese.
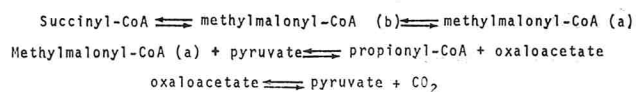
Carboxylation, Decarboxylation, and Transcarboxylation Reactions

Wood and co-workers (216, 250, 261, 263, 273-282) made the first major contribution to clarifying the pathway for conversion of pyruvate to succinate and propionate by their discovery of heterotrophic carbon-dioxide fixation in propionibacteria. They found an increase in carbon content in the products greater than that of the consumed substrate (278). This observation was later confirmed by Phelps et al. (166). Vorob'eva (240) obtained similar results when carbon balances for these reactions were determined with four strains of propionibacteria isolated from Russian cheese. Utilization of CO_2 was demonstrated with a net decrease in atmospheric carbon dioxide and a net increase in succinate in the medium (278, 281). The information was obtained from an experiment in which serial analyses were made of the products of a glycerol fermentation and values were computed on the basis of the amount of fermentation that occurred during each time interval. The amounts of CO_2 utilized and succinate produced were equivalent, which indicated that the CO_2 was fixed by condensation with a C_3 compound derived from glycerol. This hypothesis was further supported by the observation that, when the fermentation occurred in phosphate buffer in the absence of CO_2, little if any succinate was formed (279). It therefore seemed that CO_2 played a direct role in succinate formation. In addition, NaF was found to inhibit the fixation of CO_2 and to cause, simultaneously, an equivalent decrease in the yield of succinate (281). Wood et al. (282, 283, 284) found that, when $^{13}CO_2$ was used as a tracer of CO_2 fixed in products, succinate was formed by the union of pyruvate and CO_2 and that the fixed C was exclusively in the carboxyl groups. When glucose or glycerol was fermented, the succinate and propionate became carboxyl labeled. Most of the evidence indicated that succinate is a precursor of propionate. The early work of Werkman and Wood (250) suggested that CO_2 fixation with pyruvate as the acceptor should form oxaloacetate, which, in turn, would yield succinate. To support this contention, Krebs and Eggleston (111) showed that the rate of succinate for-

mation from oxaloacetate, fumarate, and malate was sufficiently high to allow the assumption that these acids are intermediates in formation of succinate from glycerol. They, however, rejected the view that succinate is synthesized from 2 moles of acetate and suggested that the low yield of acetate was not the result of succinate formation, but probably because of complete oxidation to CO_2. Experiments of Carson and associates (30-34) support these views. Studies by Delwiche (42) and by Johns (86) further confirmed the existence of such a dicarboxylic-acid pathway to succinate.

Succinate decarboxylation has been demonstrated in *Propionibacterium* species and *Micrococcus lactilyticus (Veillonella alcalescens)* (13, 45, 164, 251, 252). Also, labeled succinate could be formed to some extent by labeled propionate and carbon dioxide (13, 34, 120, 121, 164, 265, 272). Studies with cell-free extracts from propionibacteria by Whiteley (251) and by Phares and associates (46, 163, 164) reveal that the process of conversion of pyruvate to propionate is highly complicated and that coenzyme A derivatives of propionate and succinate play an important role. Additional data obtained with cell-free preparations suggest that carbon dioxide and the carboxyl group of methyl malonyl-CoA may give rise to other one-carbon compounds as part of the decarboxylation process.

Conversion of succinate to propionate is shown to occur via a transcarboxylation, and free CO_2 is not a product (221). The propionibacteria, however, do decarboxylate succinate with formation of CO_2 in a biotin-dependent reaction (42, 44, 128). This decarboxylation is much slower than that catalyzed by *M. lactilyticus* (86, 251, 252). Allen et al. (3) postulated that succinate is decarboxylated in propionibacteria in the following manner:

$$\text{Succinyl-CoA} \rightleftharpoons \text{methylmalonyl-CoA (b)} \rightleftharpoons \text{methylmalonyl-CoA (a)}$$
$$\text{Methylmalonyl-CoA (a)} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxaloacetate}$$
$$\text{oxaloacetate} \rightleftharpoons \text{pyruvate} + CO_2$$

They also postulated that there could be a direct decarboxylation of methylmalonyl-CoA. This mechanism of decarboxylation of succinate to form propionate and CO_2 in propionibacteria requires further investigation.

When succinate accumulates as an end product of the fermentation, the cycle is broken, and the oxaloacetate must be generated by fixation of CO_2. The fixation is catalyzed by the enzyme phosphoenolpyruvic carboxytransphosphorylase described by Siu et al. (196, 197). The following equation represents the primary reaction of CO_2 assimilation occurring in this system:

$$\text{Phosphoenolpyruvate} + CO_2 + HPO_4 \rightleftharpoons \text{oxaloacetate} + \text{pyrophosphate}$$
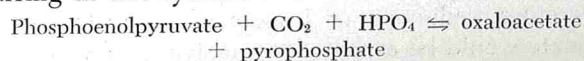
Phosphoenolpyruvate and CO_2 are converted to ox-aloacetate. Inorganic phosphate is required and acts as an acceptor of the phosphate from the phospho-enolpyruvate, yielding pyrophosphate. Addition of pyrophosphatase stimulates conversion of phospho-pyruvate to oxaloacetate. The reaction is reversible (197).

Under certain conditions, succinate is a minor product of the propionic-acid fermentation, and this may account for recovery of < 6 moles of ATP from 1.5 moles of glucose by the propionibacteria (3). Generally, the stoichiometry is 2 moles of pro-pionate to 1 mole of acetate. This stoichiometry does not always hold, since Wood and Werkman (277) found that the ratio of propionate to acetate varied from 2.2 to 14.0 in glucose fermentations. Possible explanations offered by Stjernholm and Wood (212) for the high yields of propionate are that the acetyl-CoA arising from the oxidation of pyruvate is, in turn, utilized via condensation with oxaloacetate to yield citrate, which then is oxidized to succinyl-CoA with simultaneous reduction of more oxaloacetate to succinate. Succinate could be formed from phos-phoenolpyruvate by CO_2 fixation. Succinate could then be converted to propionate. In effect, acetate would be oxidized to CO_2, and thus increase the propionate to acetate ratio.

Swick and Wood (221) studied a new type of bio-chemical reaction in which one compound, a car-boxyl donor, is decarboxylated and a second com-pound, a carboxyl acceptor, is carboxylated. Thus, it is possible to accomplish a direct carboxylation without the intervention of CO_2 or the expenditure of energy to a c t i v a t e the CO_2. Although this re-action does not involve fixation of CO_2, it is con-sidered here because of its similarity to CO_2 fixation and because it may be coupled with the fixation of CO_2:

$$\text{Methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxaloacetate}$$
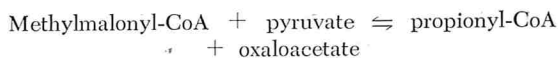
It should be emphasized that neither ATP nor Mg^{++} is required for this transfer and that free CO_2 is not involved.

The transcarboxylation reaction in propionibacter-ia was discovered as a result of studies that showed that the formation of propionate did not involve the expected turnover of CO_2. It had generally been con-sidered that carbohydrates such as glucose, glycerol, and lactate are catabolized to pyruvate, which is then converted to oxaloacetate by fixation of CO_2. It was thought that oxaloacetate was then reduced to succinate, which in turn was esterified with CoA and decarboxylated to yield propionyl-CoA and CO_2. Therefore, reduction of 1 mole of pyruvate to pro-pionate would be expected to involve the fixation and

release of 1 mole of CO_2. Because succinate is sym-metrical, one-half the CO_2 released would be de-rived from the original CO_2 fixation and the other one-half from pyruvate. By $^{14}CO_2$ dilution experi-ments, Wood and Leaver (266) reported that the CO_2 turnover is much lower than would be required for propionate formation via the dicarboxylic-acid path-way. Thus, these data and data from enzyme ex-periments suggest that a C_1 compound other than CO_2, but convertible to CO_2, is involved. Numer-ous other studies likewise indicated that a C_1 com-pound other than CO_2 was formed during the de-carboxylation of succinate (121, 164, 167, 168, 210, 267, 272).

The explanation for the small turnover of CO_2 observed in this system has been provided by the oc-currence of the transcarboxylase reaction. It now is evident that formation of propionate involves two linked cycles, one a C_1 cycle involving methylmalonyl isomerase and methylmalonyl-oxalacetic transcar-boxylase and the other a CoA cycle involving propi-onyl-CoA transferase. The reactions are outlined in Fig. 1. Oxaloacetate is formed by two reactions: one is by the CO_2 fixation catalyzed by phosphoenol-pyruvic carboxytransphosphorylase described by Siu et al. (196) and the other by the transcarboxylase re-action.

Observations have been made relative to the fix-ation of CO_2 in the reactions requiring ATP. The reactions of this group involve biotin-containing en-zymes and usually acyl-CoA compounds. It has been known for a long time that biotin has some role in CO_2 fixation, but how it functions has been a mystery.

ROLE OF BIOTIN

One of the more interesting biochemical problems under investigation is elucidation of the role of biotin in fixation and transcarboxylation of CO_2. Since 1947 (270), biotin has been known to have some function in CO_2 utilization. This section will be confined primarily to the function of biotin in the utilization of CO_2.

Lichstein (128) found that biotin is essential for decarboxylation of succinate by propionibacteria. He mentioned that it was noteworthy that dethio-biotin was found to be more active on a microgram concentration basis than either biotin or oxybiotin were for growth of P. pentosaceum. Cells grown on medium deficient in biotin possessed little or no succinate decarboxylase activity; the activity could be engendered by addition of biotin.

Fixation reactions in which biotin has been impli-cated are those in which ATP is required as a source of energy and include the enzymes propionyl car-

(1) Glycolysis
$$1.5 \text{ glucose} + 3 \text{ NAD}^+ + 3 \text{ P}_i \rightleftharpoons 3 \text{ P-enolpyruvate} + 3 \text{ NADH} + 3 \text{ H}^+$$

(2) Pyruvokinase
$$\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightleftharpoons 3 \text{ pyruvate} + 3 \text{ ATP}$$

(3) Pyruvic dehydrogenase
$$\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

(4) Phosphotransacetylase
$$\text{acetyl-CoA} + \text{P}_i \rightleftharpoons \text{acetyl-P} + \text{CoASH}$$

(5) Acetyl kinase
$$\text{acetyl-P} + \text{ADP} \rightleftharpoons \text{acetate} + \text{ATP}$$

(6) Transcarboxylase
$$2 \text{ pyruvate} + 2 \text{ methylmalonyl-CoA(a)} \rightleftharpoons 2 \text{ oxaloacetate} + 2 \text{ propionyl-CoA}$$

(7) Phosphoenolpyruvic carboxytransphosphorylase
$$3 \text{ P-enolpyruvate} + 3 \text{ CO}_2 + 3 \text{ P}_i \rightleftharpoons 3 \text{ oxaloacetate} + 3\text{PP}_i$$

(8) Malic dehydrogenase
$$2 \text{ oxaloacetate} + 2 \text{ NADH} + 2 \text{ H}^+ \rightleftharpoons 2 \text{ malate} + 2 \text{ NAD}^+$$

(9) Fumarase
$$2 \text{ malate} \rightleftharpoons 2 \text{ fumarate} + 2 \text{ H}_2\text{O}$$
$$2 \text{ NADH} + 2 \text{ H}^+ + 2 \text{ P}_i + 2 \text{ ADP} + 2 \text{ FP} \rightleftharpoons 2 \text{ NAD}^+ + 2 \text{ ATP} + \text{FPH}_2$$

(10) Fumarate reductase
$$2 \text{ fumarate} + \text{FPH}_2 \rightleftharpoons 2 \text{ succinate} + 2 \text{ FP}$$

(11) CoA transferase
$$2 \text{ succinate} + 2 \text{ propionyl-CoA} \rightleftharpoons 2 \text{ succinyl-CoA} + 2 \text{ propionate}$$

(12) Methylmalonyl isomerase
$$2 \text{ succinyl-CoA} \rightleftharpoons 2 \text{ methylmalonyl-CoA (b)}$$

(13) Methylmalonyl racemase
$$2 \text{ methylmalonyl} = 2 \text{ methylmalonyl-CoA (b)} \rightleftharpoons 2 \text{ methylmalonyl-CoA (a)}$$

SUM:
$$1.5 \text{ Glucose} + 6 \text{ P}_i + 6 \text{ ADP} \rightleftharpoons 6 \text{ ATP} + 2 \text{ H}_2\text{O} + \text{CO}_2 = \text{acetate} + 2 \text{ propionate}$$

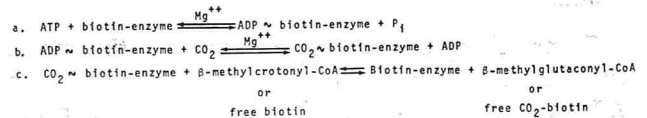Figure 1. Enzymes and reactions involved in propionic-acid fermentation (3).

boxylase, β-methylcrotonyl carboxylase, acetyl carboxylase, and pyruvic carboxylase. All are inhibited by avidin, and the first three enzymes have been shown by direct assay of protein to contain biotin (270). For propionyl carboxylase, the enzyme was of high purity (100). There is no evidence as yet that the other primary CO_2 fixation reactions involve biotin. There was a report (126) that phosphoenolpyruvic carboxykinase contained biotin, but recalculation (127) has shown that the biotin content is low. Cooper et al. (38) resolved the question by the study of phosphoenolpyruvate carboxykinase, carboxytransphosphorylase, and pyruvate carboxylase enzyme systems to determine the active species of "CO_2" utilized. They found that only pyruvate carboxylase is a biotin-requiring enzyme and that biotin was not involved in the other two enzyme systems. Siu et al. (196, 197) supported these results by demonstrating that phosphoenolpyruvic carboxytransphosphorylase is not a biotin enzyme and is not inhibited by avidin.

Although it was recognized in the 1950's that biotin plays a role in the decarboxylation of succinic acid by propionibacteria, the mechanism of action of this vitamin was obscure. The first direct experimental results concerning the mechanism of action of biotin in CO_2-fixation reactions were obtained by Lynen et al. (131) working with *Mycobacterium* when they showed that free biotin in the substrate

may be substituted for β-methylcrotonyl-CoA as a CO_2 acceptor in a reaction catalyzed by the carboxylase. The reaction could be represented as:
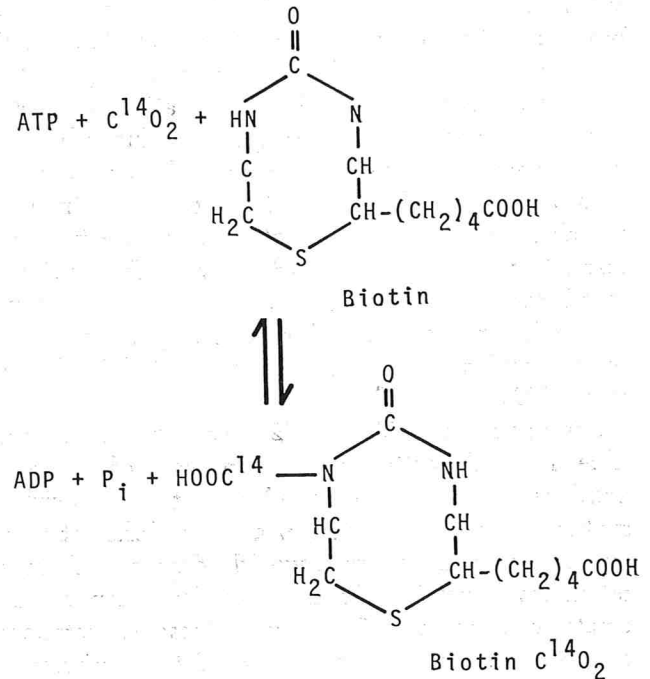
Lynen et al. (131) have suggested the following mechanism for the combination of CO_2 with the biotin complex of the enzyme and for the carboxylation reaction.

a. $\text{ATP} + \text{biotin-enzyme} \xrightleftharpoons{\text{Mg}^{++}} \text{ADP} \sim \text{biotin-enzyme} + \text{P}_i$

b. $\text{ADP} \sim \text{biotin-enzyme} + \text{CO}_2 \xrightleftharpoons{\text{Mg}^{++}} \text{CO}_2 \sim \text{biotin-enzyme} + \text{ADP}$

c. $\text{CO}_2 \sim \text{biotin-enzyme} + \beta\text{-methylcrotonyl-CoA} \rightleftharpoons \text{Biotin-enzyme} + \beta\text{-methylglutaconyl-CoA}$

  or   or

  free biotin   free CO_2-biotin

Shortly after the first studies on β-methylcrotonyl-CoA carboxylase by Lynen and his group in 1959, the mode of action of biotin as the co-enzyme of carboxylation and transcarboxylation processes became well established through investigations on many other biotin enzymes.

Stadtman et al. (202) stated that an additional role of biotin in the propionate exchange system is indicated because the reactivated enzyme is completely inhibited by avidin, but not by avidin pretreated with biotin. A possible role of biotin in the decarboxylation of methylmalonyl-CoA to form propionyl-CoA is suggested by the observation (217) that avidin inhibits a transcarboxylation, catalyzed by extracts of *P. shermanii*, from methylmalonyl-CoA to pyruvate. Similarly, it was determined (202) that the function of biotin as a CO_2-acceptor in conversion of methylmalonyl-CoA to propionyl-CoA is completely analogous to its established role in transformation of β-methylglutaconyl-CoA to β-methylcrotonyl-CoA (131) (i.e., methylmalonyl-CoA

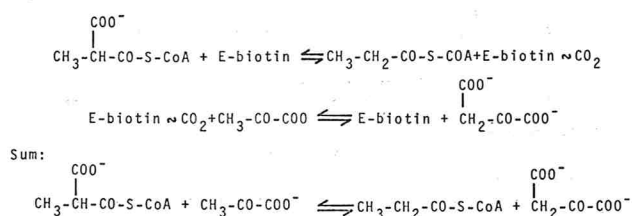+ biotin-enzyme $\leftrightharpoons$ CO_2 ~ biotin-enzyme + propionyl-CoA).

Swick and Wood (221) found that addition of avidin almost completely inhibited transfer of a carboxyl moiety to oxaloacetate from succinate (via methylmalonyl-CoA) in partly purified enzyme preparations. Later (269), this observation was confirmed by using purified enzymes. This inhibition was prevented by the simultaneous addition of biotin with avidin. Addition of biotin to the extract in the absence of avidin did not enhance the transcarboxylation reaction. Inhibition by avidin indicates that biotin also functions as a cofactor in the transcarboxylation reaction and that it may be mediated by a complex similar to that formulated by Lynen et al. (131).

Stjernholm and Wood (212) investigated the possible role of biotin in the synthesis of transcarboxylase. They hypothesized that transcarboxylase synthesis might be limited in a biotin-deficient medium and that the bacteria would adapt to a fermentation involving CO_2 fixation and formation of succinate and acetate. Contrary to these expectations, they found that the largest yield of transcarboxylase was obtained in a medium in which biotin was most carefully excluded through treatment of the hydrolyzed casein with charcoal. It is quite evident that, under these conditions, P. shermanii did not adapt by modifying its metabolism to yield succinate and acetate via CO_2 fixation, but, instead, synthesized biotin.

Transcarboxylase is unique among biotin enzymes since it does not require an added metal ion nor ATP for activity (221, 269). Two facts suggested that transcarboxylase might contain bound metal ions. First, oxalate, a known metal chelator, is a potent inhibitor of transcarboxylase (149). Second, one of the half-reactions catalyzed by transcarboxylase also is catalyzed by pyruvate carboxylase. This common half-reaction (enzyme-biotin-CO_2 + pyruvate $\leftrightharpoons$ enzyme-biotin + oxaloacetate) is inhibited by oxalate with either enzyme (149). Northrop and Wood (148) determined that there are six biotin and six metal moieties [consisting of Co and(or) Zn] per mole of transcarboxylase and that the biotin prosthetic groups definitely have a catalytic function as a carboxyl carrier. The stoichiometry of this enzymatic reaction suggests that metal groups have a catalytic role coordinated with that of biotin, which in turn requires that zinc and cobalt are interchangeable. A proposed role of cobalt (prosthetic group of the enzyme) in catalysis of transcarboxylase is indicated in Fig. 2. The electron-withdrawing properties of the metal may facilitate loss of a proton from the methyl group of pyruvate and aid in ac-

tivation of the substrate as a carboxyl acceptor from the carboxylated biotin.

Wood et al. (268) carboxylated transcarboxylase with methylmalonyl-CoA[14]C and demonstrated that the transferred carboxyl formed an amide with the l'-nitrogen of the urea moiety of biotin. Incubation of the ^{14}C-carboxylated enzyme with pyruvate, NADH, and malate dehydrogenase produced ^{14}C-malate equivalent to the enzyme-bound [14]C. Results of these chemical studies support the following reaction mechanism:

$$CH_3-CH(COO^-)-CO-S-CoA + E\text{-biotin} \leftrightharpoons CH_3-CH_2-CO-S-CoA + E\text{-biotin} \sim CO_2$$

$$E\text{-biotin} \sim CO_2 + CH_3-CO-COO^- \leftrightharpoons E\text{-biotin} + CH_2(COO^-)-CO-COO^-$$

Sum:

$$CH_3-CH(COO^-)-CO-S-CoA + CH_3-CO-COO^- \leftrightharpoons CH_3-CH_2-CO-S-CoA + CH_2(COO^-)-CO-COO^-$$

The mechanism consists of two half-reactions with the formation of a carboxylated biotin bound to the enzyme as an intermediate. Wood et al. (260) performed an experiment in which propionibacteria were grown on a medium containing tritiated biotin. After sufficient growth, radioactive methylmalonyl-oxalo-
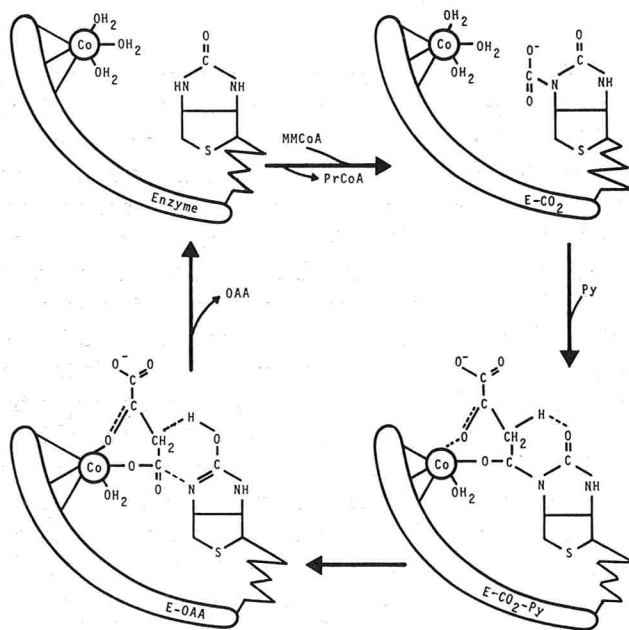
Figure 2. Suggested role of cobalt in the transcarboxylase reaction. The scheme details the keto acid portion of the reaction. The mechanism of participation of the CoA esters is not shown. The biotin is carboxylated by methylmalonyl-CoA, the pyruvate combines with the cobalt which is considered to aid in the departure of a proton from the pyruvate and transfer of the carboxyl from the carboxylated biotin to form oxaloacetate. The reactions are shown in only one direction although all steps are reversible. If zinc and cobalt can occupy common metal sites, then the mechanism also applied to the participation of zinc in the reaction. PrCoA, propionyl-CoA; Py, pyruvate; OAA, oxaloacetate; MMCoA, methylmalonyl-CoA (148).

acetic transcarboxylase was isolated from the micro-organisms. The enzyme was obtained in homogeneous form by application of sedimentation and electrophoretic techniques. It contained about 1.5 μg of biotin per milligram of protein. Purification of the enzyme was evaluated from the increase in enzymic activity as well as from the increase in radioactivity of the protein. At very high purification, the enzyme was unstable. Thus, increased in radioactivity was used as the criterion for the purity of protein.

The biotin is covalently linked to the enzyme as an amide of the e amino group of lysine (268), and the carboxyl is transferred to the 1'-N of the biotin, giving a carboxylated intermediate similar to those formed by enzymes that catalyze CO_2 fixation. Ultracentrifugation of transcarboxylase gives two components with sedimentation coefficients of 18S and 16S at ca. 60,000 rpm; at ca. 40,000 rpm, however, only a single component appears with an $s_{20,w} = 17S$ (72,260). Transcarboxylase dissociates spontaneously at low ionic strength and alkaline pH to inactive subunits with an $s_{20,w} = 6S$. This 6S component is made up of two species, one of which contains the biotin and the other the metals, Co and Zn (72, 149, 260). Gerwin et al. (72) successfully isolated a subunit that arises from the 6S biotin subunit and has a value of 1.35 and a molecular weight of approximately 12,000. It contains essentially all the biotin found in the original enzyme.

Recently (38), the active species of "CO_2" utilized in CO_2 fixation by biotin enzymes in propionibacteria was indicated to be HCO_3 (or H_2CO_3). With such enzymes as phosphoenolpyruvate carboxykinase and carboxytransphosphorylase, which are nonbiotin requiring, the active species is CO_2 rather than HCO_3^- (38). These results substantiate the earlier observations of Kaziro et al. (99) that, with the biotin-containing enzyme, propionyl-CoA carboxylase, HCO_3^- was the active species of CO_2.

Evidently, biotin is not required by propionibacteria for CO_2 fixation in the fermentation of propionic acid as generally found in other CO_2 fixation reactions. As pointed out, the CO_2 fixation reaction involving phosphoenolpyruvic carboxytransphosphorylase is nonbiotin requiring; whereas, a nonfixing CO_2 reaction involving the transcarboxylase is biotin requiring. This seems to be one of the unique characteristics of metabolism by propionibacteria.

This discussion on the role of biotin was primarily concerned with its action on enzymes of propionibacteria. For additional information on the mechanism of biotin action on carboxylases, transcarboxylases, and effect of avidin, the reader is referred to a recent review by Knappe (107).

ROLE OF VITAMIN B12

Propionic-acid formation represents a major metabolic process common to the genus *Propionibacterium*. Because these bacteria contain uniquely high concentrations of vitamin B_{12}, experiments have been performed to determine if this vitamin is involved in propionic-acid metabolism. Stadtman et al. (202) determined the effect that dimethylbenzimidazole-B_{12}-coenzyme (DMBC) had on the incorporation of ^{14}C-propionate into succinate. They found that the conversion of methylmalonyl-CoA to succinyl-CoA is dependent upon the presence of DMBC and that no succinate could be detected during incubation when DMBC was absent. Eggerer et al. (50) suggested a mechanism for isomerization of methylmalonyl-CoA to succinyl-CoA where the role of the cobamide coenzyme is to produce a radical by the oxidation of one electron in γ-carbon of methylmalonyl-CoA. The radical then could isomerize by the following proposed mechanism:
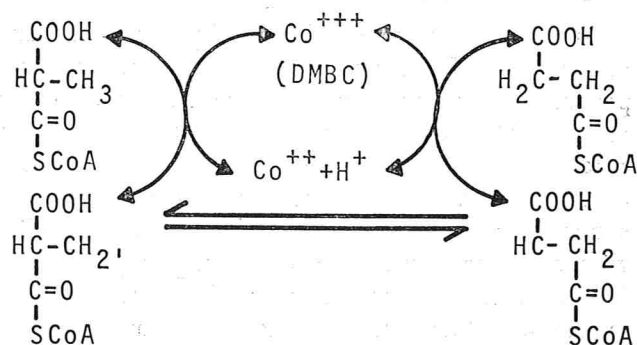
Since the cobalt in cobamide coenzyme has a valence charge of $+3$, it is of further significance in the proposed mechanism that Co^{+3} is known to produce radicals by reaction with a variety of organic compounds (139). Northrop and Wood (148) did not attribute any significance to vitamin B_{12} in the transcarboxylase reaction, but did propose a mechanism whereby cobalt plays an important role in this reaction. The significance of the role of cobalt in this reaction was discussed earlier.

Menon et al. (135) found that cells of anaerobically grown *P. shermanii* contained much greater amounts of the corrin ring of vitamin B_{12} than did aerobically grown cells. The diminished concentration of coenzyme B_{12} in aerobically grown cells was reflected in the fatty-acid composition of the fermentation products. Propionic acid, the predominant volatile fatty acid among the products of the anaerobic fermentation, occurs in much smaller quantities as a by-product of aerobic fermentation. This is consistent with the well-known dependence of the methylmalonyl-CoA and succinyl-CoA isomerase reaction

on cyanocobalamine. Menon et al. *(135)* found that the addition of coenzyme B_{12} to extracts of aerobically grown cells restored the propionate-succinate conversion to about 30 to 50% of that found in extracts of anaerobically grown cells. Results similar to these were found by several other investigators *(75, 124, 199, 285)*.

ENZYMES INVOLVED IN THE PROPIONIC-ACID FERMENTATION

Formation of propionate by propionibacteria has been extensively investigated, and the pathway is known to involve a number of enzymes that have been purified and studied. The enzymes discussed in this section catalyze a wide variety of biochemical reactions found in propionibacteria, but, here, primary emphasis is concentrated on those involved in propionic-acid fermentation. The graphical representation of reactions projected in Fig. 3 provides a simple reference to the systems of primary importance in the fermentation of propionic acid.

Oxaloacetate may be formed by two reactions: one is by the CO_2 fixation catalyzed by phosphoenolpyruvic carboxytransphosphorylase described by Siu et al. *(196)* and the other is by the transcarboxylase reaction. Let us assume for the moment that oxaloacetate is formed by the latter mechanism; i.e., by reaction of a catalytic amount of methylmalonyl-CoA with pyruvate, simultaneously yielding propionyl-CoA. The oxaloacetate then is reduced to succinate via malate and fumarate with accompanying oxidation of pyruvate to CO_2 and acetate. The succinate, in turn, is converted to succinyl-CoA by reaction with the propionyl-CoA, simultaneously yielding propionate, the reaction being catalyzed by CoA transferase *(221)*. The methylmalonyl-CoA is regenerated by conversion of the succinyl-CoA to methylmalonyl-CoA by the enzyme methylmalonyl isomerase. Methylmalonyl isomerase has been demonstrated in propionibacteria by Swick and Wood *(221)*, Stadtman et al. *(202)*, and Phares et al. *(165)*. The unesterified carboxyl group of the C_4-dicarboxylic acids is not released, but is transferred to pyruvate. This mechanism avoids formation of free CO_2 and the subsequent necessity of activating CO_2 for carboxylation of pyruvate. Thus, only catalytic amounts of oxaloacetate need be produced by fixation of CO_2. Likewise, CoA is recycled, and only catalytic amounts of the acyl derivative need be formed by *de novo* synthesis using ATP. Therefore, this sequence of events provides a mechanism for reduction of pyruvate to propionate with a minimum expenditure of energy and without fixation of CO_2.

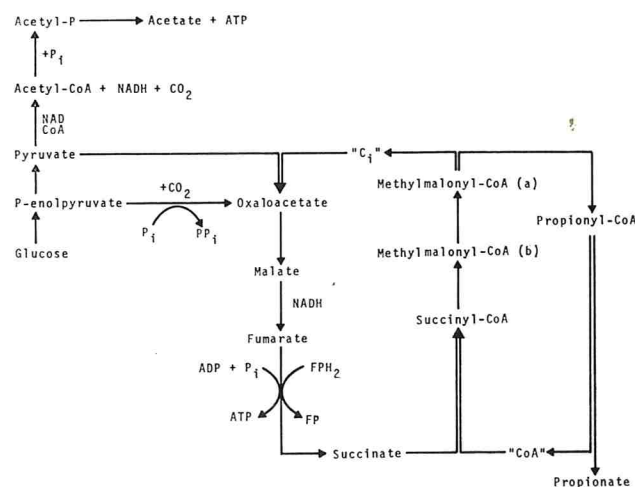An interesting feature of the propionic-acid fermen-

Figure 3. Reactions of the propionic-acid fermentation and the formation of acetate, CO_2, propionate, and ATP. Methylmalonyl-CoA (a) and (b) are the isomers. FP is flavoprotein and FPH_2 is reduced flavoprotein *(3)*.

tation is the ostensibly high yield of ATP derived from glucose. Bauchop and Elsden *(18)* considered, on the basis of cell yields, that approximately 6 moles of ATP are formed per 1.5 moles of glucose fermented. Reactions 2 and 5 would yield 4 moles of ATP per 1.5 moles of glucose. Thus, 2 moles of ATP must, as they suggested, arise from the reactions yielding propionate. One of the unique features of the formation of propionate is that it involves a coupled reduction of fumarate to succinate and oxidation of pyruvate to acetate and CO_2. An electron-transport-coupled phosphorylation may occur during this step as indicated in reaction 8, through reduction of flavoprotein by $NADH + H^+$. This mechanism would yield an additional 2 moles of ATP and, thus, the 6 moles of ATP (See Fig. 1).

Lactic dehydrogenase

Occurrence in *P. pentosaceum* of enzymes able to catalyze an oxidation of lactate in the presence of fumarate has been demonstrated by Barker and Lipmann *(14)*. Evidence presented by these authors suggests the participation in the system of a type of lactic dehydrogenase different from that found in animal tissues. Molinari and Lara *(138)* found that the enzyme (concentrated fifteen-fold) had infinite affinity for methylene blue, 2,6-dichlorophenolindophenol, and 1,2-naphthaquinone-4-sulphonate. The optimum pH for lactate oxidation was found to be 7.7 at 30 C. The preparations were activated by NH_4^+, CN^-, F^-, and Mg^{++} ions and malonate. The purified preparations were thermolabile and inhibited by pyruvate, oxalate, thiol reagents, narcotics, quinacrine, chloroquine, quinine, dicoumarol, Vitamin K_1, pentachlorophenol, thyroxine, and hydrozine.

"Pyruvokinase" enzymes, pyruvate, phosphate dikinase, and pyruvate kinase

The propionibacteria grow well on lactate and pyruvate, and therefore, must be able to convert pyruvate to P-enolpyruvate, which is required for the net formation of 4-carbon compounds, carbohydrates, glycerol, and other cell materials. Investigation by Evans and Wood (58) has revealed that conversion of pyruvate to P-enolpyruvate occurs neither via oxaloacetate, nor by reversal of the pyruvate kinase reaction. They found that crude extracts of propionibacteria contain pyruvate kinase in addition to the dikinase. Allen et al. (3) reported the following reaction as the one primarily catalyzed by pyruvate kinase:

$$\text{Phosphoenolpyruvate} + \text{ADP} \leftrightharpoons \text{pyruvate} + \text{ATP}$$

Evans and Wood (58) stated that the major role of the dikinase in propionibacteria seems to be the reverse of pyruvate kinase:

$$\text{Pyruvate} + \text{ATP} + \text{P}_i \overset{\text{Mg}^{++}}{\leftrightharpoons} \text{phosphoenolpyruvate} + \text{AMP} + \text{PP}_i$$
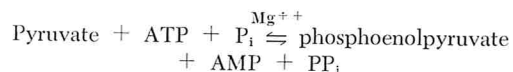
The dikinase is induced ten-fold by growing propionibacteria on lactate rather than on glycerol, presumably because the enzyme is essential for a net formation of phosphoenolpyruvate from lactate. The equilibrium of the dikinase reaction may be close to unity, or may favor pyruvate formation, but P-enolpyruvate formation would be favored by hydrolysis of the pyrophosphate formed in the reaction, as well as by removal of the P-enolpyruvate for biosynthesis of cellular components and synthesis of 4-carbon intermediates.
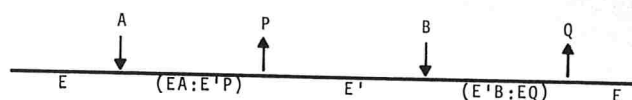
Methylmalonyl-oxaloacetic transcarboxylase

Transcarboxylase is a unique biotin-containing enzyme occurring in propionibacteria that catalyzes the reversible transfer of a carboxyl group from methylmalonyl-CoA to pyruvate, yielding propionyl-CoA and oxaloacetate (221, 260). It differs from the other biotin enzymes that catalyze utilization of CO_2, in that free CO_2 is not involved and neither ATP nor a divalent metal is required. The biotin is covalently linked to the enzyme as an amide of the e amino group of lysine, and the carboxyl is transferred to the 1'-N of the biotin, giving a carboxylated intermediate similar to those formed by enzymes that catalyze CO_2 fixation (268). Transcarboxylase catalyzes the following general reversible reaction:

$$\text{Oxaloacetate} + \text{propionyl-CoA} \leftrightharpoons \text{pyruvate} + \text{methylmalonyl-CoA}$$
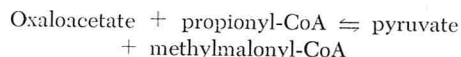
The steady-state kinetics of reaction catalyzed by an enzyme acting alternately as an acceptor and donor of a transferred group has been developed and reviewed (147). The mechanism is designated "ping pong" and may be outlined as follows:

A, B, P, and Q represent substrates and products, which for the transcarboxylase reaction are methylmalonyl-CoA, pyruvate, propionyl-CoA, and oxaloacetate, respectively. E and E' represent two forms of the enzyme: enzyme-biotin and enzyme-biotin $\sim CO_2$. The central feature of a "ping-pong" mechanism from the point of view of kinetic theory is the compulsory release of the first product (P) before addition of the second substrate (B). Consequently, the mechanism is restricted to an exclusive formation of binary enzyme-substrate complexes.

The forward and reverse reactions catalyzed by transcarboxylase have been studied in the presence and absence of products and substrate analogues by Northrop (147). The kinetic patterns of initial velocities at low substrate concentrations support the conclusion that the transcarboxylase reaction proceeds through two half-reactions in a "ping pong" mechanism with an intermediate formation of an enzyme-biotin $\sim CO_2$ complex. Northrop (147), however, determined that the kinetic patterns with inhibitors were inconsistent with the predicted patterns for a "ping pong" mechanism since the inhibition was competitive when the inhibitor and the varied substrate were similar; that is, both are coenzyme-A compounds or both keto acids. Dissimilar combinations of inhibitors and varied substrates resulted in noncompetitive inhibition when the inhibitor was a product of the reaction, and noncompetitive inhibition when the inhibitor was an inactive analogue of the fixed substrate. A pictorial representation of the hypothetical mechanism for the transcarboxylation reaction is shown in Fig. 4. The model does not show all possible kinetic events, as this cannot be done with a single two-dimensional diagram, but shows only the major steps of the conversion of substrates to products. These findings were interpreted as indicating the transcarboxylase has two types of separate and independent binding sites, one for binding each of two groups of substrates and inhibitors; coenzyme-A compounds constitute one group of reactants, and keto acids constitute the other group. Consequently, the mechanism constructed for the reaction may be described as a hybrid between a "ping pong" mechanism and a random, ternary complex mechanism because it involves an intermediate representing a substituted form of the enzyme and allows independent binding of substrates to two distinct sites on the enzyme. Northrop and Wood (149), using an analogue of
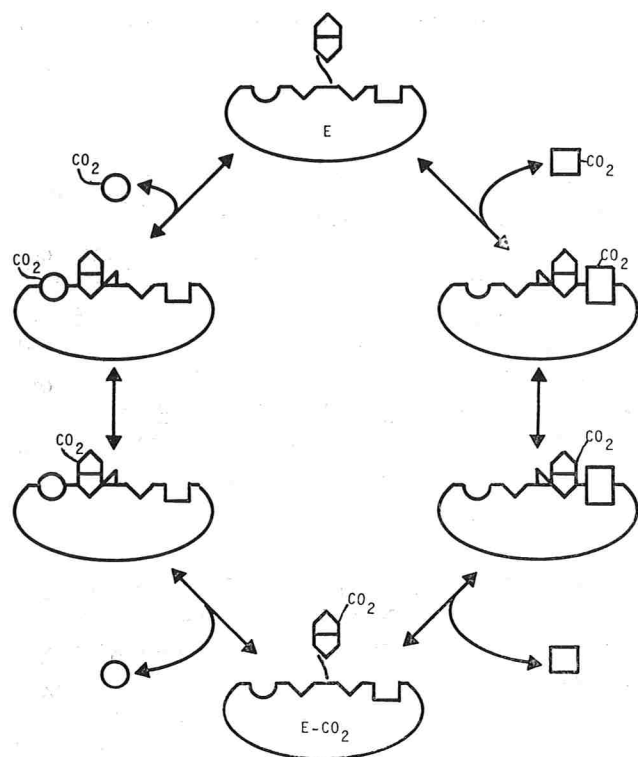
Figure 4. Diagramatic representation of the hypothetical mechanism for the transcarboxylase reaction. *Free circle*, pyruvate; *carboxylated circle*, oxalacetate; *free square*, propionyl-CoA; *carboxylated square*, methylmalonyl-CoA; *hexagonal structure*, biotin; *carboxylated hexagonal structure*, carboxyl-biotin; *E*, one of possibly six reactive enzyme centers of transcarboxylase. The forward reaction is read in a *clockwise direction*. Not shown on the diagram are numerous nonproductive complexes between enzyme and substrates. *(147)*.

pyruvate and 2-ketobutyrate, found that these compounds cause inhibition of pyruvate-oxaloacetate exchange and stimulation of propionyl-CoA:methyl-malonyl-CoA exchange. Oxalate, an analogue of oxaloacetate, caused inhibition of both exchange reactions. They determined that these findings were inconsistent with a standard "ping pong" reaction mechanism, but could be explained by a hybrid "ping-pong" mechanism.

The transcarboxylase seems to have a broad specificity for the CoA esters *(269)*. This is interesting because it permits the carboxyl group of oxaloacetate arising in the Krebs cycle or elsewhere to be utilized in fatty-acid synthesis and vice versa. For example, it is generally accepted that malonyl-CoA is an intermediate in the synthesis of fatty acids; clearly, the propionibacteria could form malonyl-CoA via oxaloacetic transcarboxylation instead of by direct carboxylation of acetyl-CoA with CO_2. At one time, the only type of heterotrophic CO_2 fixation known was that of the propionibacteria. Now, many types of CO_2 fixation are known. The same

development seems possible for transcarboxylation mechanisms. For economy and control of cellular reactions, it would seem advantageous to transfer carboxyl groups just as ester phosphates are transferred. Carboxyl groups like phosphate anhydrides could then be generated and utilized in coupled reactions instead of being generated each time that they are required.

Wood et al. *(260)* found the transcarboxylase to be active with propionyl coenzyme A, acetyl coenzyme A, and butyryl coenzyme A as carboxyl acceptors and with oxaloacetate as the carboxyl donor. They found the K_m values for the acceptors to be 2.7×10^{-4}, 5.0×10^{-4}, and 2.5×10^{-4}, respectively. The K_m for oxaloacetate was 1.0×10^{-4}. With methylmalonyl-CoA and malonyl-CoA as the carboxyl donors and pyruvate as the acceptor, the K_m values are 0.8×10^{-5} and 3.5×10^{-5}, respectively. The K_m for pyruvate is 1.0×10^{-3}. T h e s e K_m values reflect the wide potential that this enzyme has for synthetic reactions that involve carboxylation.

Wood and Stjernholm *(269)* determined the equilibrium constant of the transcarboxylation reaction to be 1.9 at pH 6.5 and 30 C, and the free energy was calculated to be -3.9×10^2 calories. Consequently, there can be an easy flow of carboxyl groups in either direction, the direction of flow depending upon which compounds are continuously being removed or regenerated.

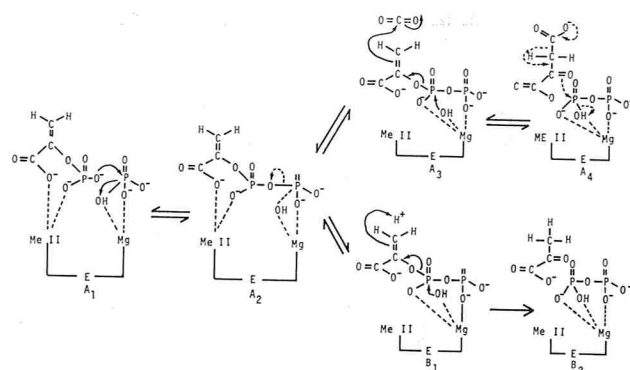Transcarboxylase has been extensively studied and

Figure 5. Possible role of the dissociable type I metal (Mg^{2+}) and the unidentified tightly bound type II metal (Me II) of carboxy-transphosphorylase (E) in the oxaloacetate reaction (A_1 to A_4) and the pyruvate reaction (A_1 to B_2). Conversions A_1 into A_4 are reversible. The dotted arrows indicate electron shifts when the conversion is from right to left and the solid arrows when it is from left to right. Conversion of A_4 into A_3 requires only the type I metal (Mg^{2+}). Thus CO^2 exchanges with the carboxyl group of oxaloacetate when the type II metal is blocked by chelators. On the other hand, the phosphate cleavage requires both the type I and type II metals. Thus $^{32}P_i$ exchange occurs only when both metals are free. The pyruvate reaction A_1 to B_2 is experimentally irreversible and $^{32}PP_i$ is not converted into phosphoenolpyruvate by this sequence *(264)*.

many of its characteristics have previously been discussed in the section entitled "Role of biotin". Hence, repetition of these characteristics is avoided in this section.

Phosphoenolpyruvic carboxytransphosphorylase

T h e phosphoenolpyruvic carboxytransphosphorylase of *P. shermanii (196, 197)* catalyzes the primary reaction in CO_2-fixation sequence, which seems to be the major CO_2-fixing step in this bacterial species. The reaction requires inorganic orthophosphate (P_i) and is reversible:

Phosphoenolpyruvate + CO_2 + P_i ⇋ oxaloacetate + PP_i
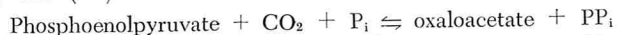
The major characteristics of the enzyme according to Siu and Wood *(197)* are:

1. The optimum pH for the reaction is approximately 7.8.
2. The evident Km values of the components of the system of the forward reaction are: phosphoenolpyruvate, 1.7×10^{-4} M; $NaHCO_3$, 2.9×10^{-4} M; P_i, 1.2×10^{-3} M; Mg^{++}, 2.1×10^{-3} M; and Mn^{++}, 1.4×10^{-4} M. High levels of P_i or PP_i inhibit the formation of oxaloacetate.
3. Phosphoenolpyruvic carboxytransphosphorylase is not a biotin enzyme and is not inhibited by avidin.

Lochmüller et al. *(129)* studied the conditions which influence the yield of the enzyme from *P. shermanii*. They found that the yield is higher with glycerol than with glucose as a substrate and increases with time of fermentation. They also purified and crystallized the enzyme and determined its molecular weight to be approximately 430,000. Treatment with 6 M urea yields a subunit or units that, after dialysis, have a sedimentation constant of $s_{20,w} = 7.0S$. On the basis of extrapolated specific activities, and with the highest value for the reverse reaction, the forward reaction is 3.5 times faster than the reverse reaction; it is 7 times faster based on the directly observed specific activities. The catalysis is inhibited by numerous compounds, including KCl and NaCl at high ionic strength. Sulfate is a non-competitive inhibitor of the forward reaction with a K_i of about 20 nM. Buffers such as Tris-HCl, glycyl-glycine, and imidazole inhibit both forward and reverse reactions. The effect of products and substrates on the rate of the reverse reaction was studied *(129)*. All substrates were inhibitory even in moderate concentrations. A possible explanation of the inhibition is that competition of product with substrate may occur.

Wood et al. *(262)* determined the possible role of inorganic pyrophosphate as a donor of energy-rich phosphate bonds for synthesis of phosphoenolpyruvate by determining the equilibrium constants of three reactions in which CO_2 is fixed into oxaloacetate. They observed that pyruvate carboxylase and carboxytransphosphorylase may be linked and that P-enolpyruvate is synthesized from pyruvate with

utilization of ATP and inorganic pyrophosphate. The free energy of the linked reaction with ATP and PP_i is not as favorable for synthesis of P-enolpyruvate as it is with ATP and inosine triphosphate. The linked reactions with carboxytransphosphorylase may provide a mechanism for the utilization of the energy of inorganic pyrophosphate.

Cooper et al. *(38)* investigated the active species of "CO_2" utilized in reactions catalyzed by pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate carboxytransphosphorylase. By means of radiochemical and spectrophotometric techniques, they obtained evidence that the active species in the carboxykinase and carboxytransphosphorylase reactions is CO_2 and not bicarbonate. Bicarbonate seems to be the active species in the pyruvate carboxylase reaction.

In the carboxylation of phosphoenolpyruvate to form oxaloacetate, the CO_2 may approach the C-3 atom from the side of the plane in which the three substituents of C-2 (phosphate, carboxyl, and vinyl carbon) occur in a clockwise to counterclockwise sequence. These two sides of the plane of the carbon skeleton are designated *re* and *si*, respectively. Using specifically labeled 3-^{3}H-phosphoenolpyruvate, Rose et al. *(184)* determined the stereochemistry of CO_2 (or HCO_3^-) addition for the reactions catalyzed by phosphoenolpyruvate carboxylase, P-enolpyruvate carboxykinase, and P-enolpyruvate carboxytransphosphorylase. In all instances, the addition occurs from the same side of the plane of enzymebound P-enolpyruvate; namely, the *si* side. The conservation of this type of addition reaction through evolutionary changes implies that this stereochemistry may have significance to the reaction mechanism.

A second reaction catalyzed by phosphoenolpyruvate carboxytransphosphorylase has been investigated by Wood and coworkers *(41, 256, 264)* and is described as the pyruvate reaction:

Phosphoenolpyruvate + P_i ⇋ pyruvate + PP_i
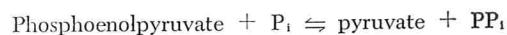
A comparison of the properties of this reaction with those of the CO_2 fixation-oxaloacetate reaction was made *(41)*. The results show that carboxytransphosphorylase catalyzes conversion of phosphoenolpyruvate and phosphate into oxaloacetate and pyrophosphate in the presence of CO_2 (first reaction) but that in the absence of CO_2, pyruvate and pyrophosphate are the products (second reaction). Although the reactions are catalyzed by the same enzyme, it was proposed *(41)* that different forms of the enzyme exist that preferentially catalyze the two reactions. Relationships of the forms were presented, although it was recognized that the evidence was still inconclusive *(41)*. Both forms of the active enzyme cata-

lyzing the two reactions are inhibited strongly by low concentrations of EDTA in the presence of 12 nM Mg^{++} (41, 129). Studies with a variety of chelators (256) indicate that carboxytransphorylase requires two different metal ions designated as type I and type II. Type I metals are freely dissociable and have K$_m$ values of about 1 nM (129). Mg^{++}, Mn^{++}, and Co^{++} meet the type I requirements; the type II metal is firmly bound to the enzyme and has not yet been identified (256). In addition to the requirement for type I and type II metals, a heavy metal seems to stimulate the pyruvate reaction (41). Wood et al. (264) reported a study of the role of the type I and type II metals in the carboxytransphosphorylase reaction as determined by use of ^{32}P$_i$, ^{32}PP$_i$, ^{14}CO$_2$, and pyruvate-1-^{14}C. The detailed mechanism by which these metals function is illustrated in Fig. 5 (264).

Davis et al. (41) determined the stoichiometry of the pyruvate reaction and presented evidence that both reactions are catalyzed by the same enzyme. They found that the CO$_2$-fixation reaction is reversible, but that the pyruvate reaction is experimentally irreversible. Both reactions have about the same pH optimum, and divalent metal is required for either reaction. The manifest K$_m$ values for the pyruvate reaction are Mg^{++}, 6.3 $\times$ 10^{-4} M; Co^{++}, 2.3 $\times$ 10^{-4} M; Mn^{++}, 4 $\times$ 10^{-5} M; phosphoenolpyruvate, 3.6 $\times$ 10^{-5} M; and P$_i$, 6.6 $\times$ 10^{-4} M. These values are quite similar to those previously observed for the CO$_2$-fixation reaction, except for that of phosphoenolpyruvate and Mn^{++}, which are about ten-fold lower for the pyruvate reaction.

The question arises whether the pyruvate reaction has any physiological function in the propionibacteria in vivo. Fermentation by these bacteria normally yields CO$_2$ from carbohydrates and, at least under anaerobic conditions, one would expect that carboxytransphosphorylase would be kept in a reduced state. In the presence of thiols, bicarbonate almost completely inhibits the pyruvate reaction (264). It seems likely that the normal role of the enzyme is to form oxaloacetate from phosphoenolpyruvate. Evans and Wood (58) have found that the propionibacteria also contain a second enzyme (pyruvate, phosphate dikinase), which generates inorganic pyrophosphate. The enzyme is induced by growth on lactate, and it seems likely that in vivo the enzyme catalyzes the conversion of pyruvate into phosphoenolpyruvate rather than the reverse. Davis et al. (41) have linked pyruvate, phosphate dikinase from propionibacteria with carboxytransphosphorylase with formation of oxaloacetate and pyrophosphate:

$$\text{Pyruvate} + \text{ATP} + 2\text{P}_i + \text{CO}_2 \rightleftharpoons \text{oxaloacetate} + \text{AMP} + 2 \text{ PP}_i$$
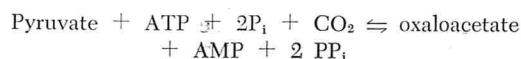
Pyrophosphate is a strong inhibitor of carboxytransphosphorylase (41, 129), and it may serve as part of the control mechanism that regulates the interplay of these enzymes as well as that of pyruvate kinase during the interconversions of pyruvate, oxaloacetate, and phosphoenolpyruvate (259).

Malic dehydrogenase

Malic dehydrogenase from propionibacteria has been purified by Allen et al. (3). This enzyme catalyzes the reduction of oxaloacetate to malate:

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{malate} + \text{NAD}^+$$
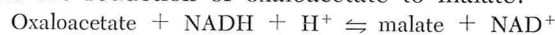
A description of its properties as found in beef-heart are given by Siegel and England (193).

Fumarase

Fumarase is the enzyme that catalyzes the reaction between fumarate and malate. It was first observed in propionibacteria by Krebs and Eggleston (111) who found a "powerful fumarase in P. shermanii." In characterizing some of the properties of this enzyme, Ayres and Lara (12) determined that fumarase is inhibited by thiol reagents and mercurials. They found that measurements of K$_m$ for the reaction of fumarate to malate indicate a nearly constant value from pH 5 to 7.

Fumarate reductase (Succinic dehydrogenase)

Although it has been known for many years that reduction of fumarate to succinate is an essential step in propionic-acid fermentation and that the reaction is catalyzed by succinic dehydrogenase, purification and characterization of the reductase from propionibacteria was not accomplished by Lara (119) until 1959. Although Lara was not able to obtain high recovery of the enzyme, he did show that the properties of the enzyme are intermediate between characteristics of the reductase from yeast and animals on one hand and strict anaerobes on the other. Depending upon what hydrogen acceptor was used, he found that the enzyme had optimum activity between pH 7.4 and 7.8. With the various dyes as acceptors, the K$_m$ of the enzyme ranged from 2.2 $\times$ 10^{-3} to 7 $\times$ 10^{-4} M.

Methylmalonyl isomerase

Methylmalonyl isomerase catalyzes the interconversion of methylmalonyl-CoA (b) and succinyl-CoA (1, 2, 3, 4, 102, 103, 124, 152, 153, 165, 211, 218, 219, 220), a reaction that plays an important role in conversion of pyruvate to propionate in propionibacteria. This enzyme has been the object of considerable interest since it is one of the few enzymes requiring cobamide coenzymes for catalytic activity in highly purified preparations (124, 153, 202, 211).

Stjernholm and Wood (211) obtained methylmalonyl isomerase in "purified form" from propionibacteria, but subsequently found that this preparation contained methylmalonyl racemase (1, 4), an enzyme that catalyzes the interconversion of the two isomeric forms (a and b) of methylmalonyl-CoA. The isomerase also has been obtained in purified form from propionibacteria by Overath et al. (153).
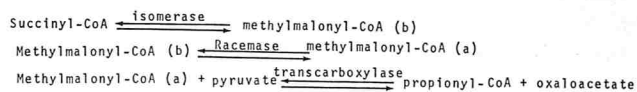
Although there is considerable information pertaining to the mechanism of the isomerase reaction, the role of the cobamide coenzymes is not clear. Swick (218, 219) and Swick and Moss (220) investigated the isomerization reaction with purified enzymes and clearly demonstrated that the rearrangement is the result of the migration of the thioester group. Similar results were reported by Eggerer et al. (50). Swick (219) determined that the isomerization does not involve free acrylic acid as an intermediate. Kellermeyer and W o o d (103) have shown by mass analysis of the succinyl-CoA, formed from specifically ^{13}C-labeled methylmalonyl-CoA, that the conversion occurs by an *intra*molecular shift. Thus, this result supports the free-radical mechanism model of Eggerer et al. (50), representing one type of *intra*molecular shift of the CoA-carboxyl of methylmalonyl-CoA. Also, this model does propose a method by which the B$_{12}$ coenzyme can function in the isomerization.

The primary work on purification and characterization of this enzyme in propionibacteria was accomplished by Kellermeyer et al. (102). They found the following properties: The purified preparation migrated as a single peak in the analytical ultracentrifuge with an $s_{20,w} = 7.0S$ and a molecular weight equal to 56,000. Inhibition studies with parahydroxymercuribenzoate and N-ethylmaleimide indicated that the enzyme is not dependent on sulfhydryl groups for activity. The enzyme does not require K^+ or NH_4^+. Ethylenediaminetetraacetic acid at a concentration of 8×10^{-2} M did not effect the isomerase activity. The pH optimum was approximately 7.4, and the activity diminished only slightly at pH 6.0 and 8.0. The K_m for succinyl-CoA and methylmalonyl-CoA are 3.45×10^{-5} and 8×10^{-5} M, respectively. No spectral shifts were noted during interaction of the enzyme and cobamide coenzyme or substrate.

Methylmalonyl racemase

Methylmalonyl isomerase catalyzes the interconversion of succinyl-CoA and methylmalonyl-CoA. It has been demonstrated that preparations of the isomerase contained a second enzyme, methylmalonyl racemase, which catalyzes the interconversion of the two isomeric forms of methylmalonyl-CoA (4, 134,

152). This reaction is an essential step in the formation of propionate from pyruvate by propionibacteria:

$$\text{Succinyl-CoA} \underset{\text{isomerase}}{\rightleftarrows} \text{methylmalonyl-CoA (b)}$$
$$\text{Methylmalonyl-CoA (b)} \underset{\text{Racemase}}{\rightleftarrows} \text{methylmalonyl-CoA (a)}$$
$$\text{Methylmalonyl-CoA (a) + pyruvate} \underset{\text{transcarboxylase}}{\rightleftarrows} \text{propionyl-CoA + oxaloacetate}$$

Allen et al. (1) found separation of methylmalonyl racemase from other enzymes that have been isolated from *P. shermanii* to be difficult. Preparations of methylmalonyl-oxaloacetic transcarboxylase, propionyl-CoA transferase, and methylmalonyl isomerase all contained traces of racemase until they were extensively purified.

The dependence on racemase for the coupling of isomerase and transcarboxylase could be demonstrated only with highly purified enzymes. Allen et al. (1) isolated and purified the racemase to the point of homogeneity as judged by sedimentation and electrophoresis. The enzyme has a sedimentation constant of $s_{20,w} = 2.9S$, and evidently has a low molecular weight. The racemase, unlike the isomerase, does not function by transfer of the CoA moiety between carboxyl groups of methylmalonyl-CoA. The enzyme is unusually stable to heat and to perchloric-acid treatment.

Coenzyme A transferase

CoA transferase catalyzes the reversible transfer of coenzyme A from propionyl-CoA or acetyl-CoA to succinate and has the same activity with either acetyl- or propionyl-CoA (3). The reaction can be described by the following mechanism:

Propionyl-CoA + succinate ⇋ succinyl-CoA + propionate

Allen et al. (3) characterized the enzyme from *P. shermanii*. It evidently resembled the transferase found in *Micrococcus lactilyticus* by Whiteley (252) and in *P. pentosaceum* by Delwiche et al. (46), but had a much higher activity than these transferases, even though the ultracentrifuge patterns indicated that the enzyme was still only about 56% pure. Allen et al. (3) determined the following properties of the enzyme: The pH optimum ranged from 6.5 to 7.8; the evident K_m for succinyl-CoA in the transfer to acetate was 1.3×10^{-4} M, and, for the transfer to propionate, it was 6.8×10^{-5} M. The specific activity of the enzyme was 38% higher with acetate than with propionate as the CoA acceptor. The two K_m values for succinyl-CoA agree well, considering that different assays were used. The enzyme did not catalyze the transfer of CoA from acetyl-CoA to methylmalonate, acetoacetate, formate, or fluoroacetate, or from β-hydroxymethylbutryl-CoA to acetate. The enzyme was very stable as evidenced by very little loss of activity when stored at −10 C in 60% ammonium sulfate for 2 years.

Pyruvate dehydrogenase complex (pyruvate dehydrogenase, phosphotransacetylase, and acetyl kinase)

Three enzymes found in propionibacteria are responsible for catalyzing the conversion of pyruvate to acetate and CO_2. Since recent literature groups these three enzymes, found in other microorganisms, into a complex they will be discussed as existing in a like complex in propionibacteria for ease of reporting. Pyruvate dehydrogenase catalyzes the reversible reaction as follows:

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$
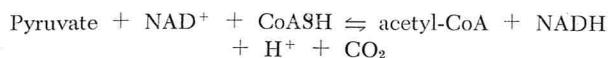
Phosphotransacetylase catalyzes the reversible reaction between acetyl-P and acetyl-CoA. Purification of this enzyme from propionibacteria has been accomplished by Allen et al. (3). A description of its properties as obtained from a preparation of *Clostridium kluyveri* is given by Stadtman (201). Phosphotransacetylase catalyzes the reversible reaction as follows:

$$\text{Acetyl-CoA} + \text{P}_i \rightleftharpoons \text{acetyl-P} + \text{CoASH}$$
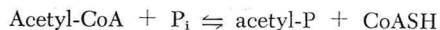
General properties for acetate (propionate) kinase have been described by Pawelkiewicz and Legocki (157). Allen et al. (3) isolated the enzyme in pure and homogeneous form. This enzyme from propionibacteria has equal effect with propionate and acetate, but, in the fermentation, it most likely catalyzes the formation of acetate (see Fig. 1). For this reason it has been given the name acetyl kinase, and designated to catalyze the following reaction:

$$\text{Acetyl-P} + \text{ADP} \rightleftharpoons \text{acetate} + \text{ATP}$$
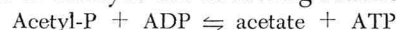
When propionate is the substrate it will catalyze the following reaction with equal rapidity:

$$\text{Propionate} + \text{ATP} \rightleftharpoons \text{propionyl-P} + \text{ADP}$$

Pawelkiewicz and Legocki (157) found that Mg^{++} was essential for the reaction to occur but that concentrations of 10^{-2} M of N-ethylmaleimide and iodoacetate did not inhibit the enzyme. They determined that the optimum temperature for this kinase was 50 C under standard conditions, and that two maxima of enzyme activity occurred at pH 8.1 and 6.7. Wierzbicka et al. (254) reported that the activity of the enzyme is not affected by 1 M concentrations of NaCl, KCl, and NH_4Cl. Using the isolated kinase, they developed an improved procedure for acetate and (or) propionate determination in biological systems.

Information in Fig. 1 and 3 clarifies how the enzymes just discussed participate in propionate fermentation.

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QUALITY CONTROL IN THE CALIFORNIA WINE INDUSTRY[1]

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ABSTRACT

The post-Pasteurian trends in quality control in the wine industry are reviewed. The post-Repeal state and Federal wine standards and their effects are discussed. The contributions of various text books are noted. Specific problems, river disposal of stillage, pond disposal of liquid winery wastes, and tartrate decomposition are mentioned. The activities of the Wine Institute in this field are reviewed, particularly its *Sanitation Guide for Wineries* and its recommendations. The problem of antiseptics, fungicides, and pesticides as they relate to the wine industry are mentioned.

Microbiologists often forget that their Founding Father, Louis Pasteur, started his microbiological studies in the fermentation industries (2). Pasteur's contributions to the wine industry were many. They brought immediate and permanent results—not the least of which was prevention of bacterial spoilage, or if spoilage started, its control by pasteurization. Both of these efforts continue today but the success of the prevention measures (especially in California) has made pasteurization of little importance.

EARLY SANITATION EFFORTS

Pasteur's advocacy of pasteurization had a salutory effect in controlling what was rampant spoilage in the mid-nineteenth century. It was soon recognized, however, that prevention was better than cure. Pure yeast cultures were introduced into the wine industry about 1890 and rapidly were adopted by progressive wine makers as they had been by progressive brew masters. Generally cleanliness and more sanitary procedures were adopted by the better wine makers throughout the world—though one must admit that many primitive practices survived (and in local areas, primarily in Europe, still survive).

The Sanitarian's Founding Father, Harvey Washington Wiley, was especially concerned with this problem. Wiley himself made a special trip to Europe in 1905 to assure himself that alcoholic beverages being imported from Europe met the minimum standards of the then tiny Food and Drug Administration (42, 43). It is interesting that he did not find European practices unduly unsanitary. However, some of the pictures in this report would strongly indicate that sanitation practices of the wineries visited by him (and he certainly did not visit the least sanitary ones!) were below present standards in several European wine districts. He did condemn the excessive use of sulfur dioxide in European wines and the use of water and sugar in making wines in Germany and in eastern United States (a practice which still exists).

Amerine (1) has estimated that as late as 1900 about 20% of the California wine production was spoiled within a few months of the vintage. This resulted from failure to harvest grapes before they became overripe, limited use of pure yeast cultures and sulfur dioxide, and excessively high temperatures during alcoholic fermentation. These practices led to incomplete ("stuck") fermentations and early bacterial spoilage of the slightly sweet new wines. Amerine (1) noted the important roles of chemists and the College of Agriculture staff in Berkeley in devising methods of controlling this spoilage. Prohibition did not, of course, do anything to improve the quality standards of the wine industry in this country.

THE EARLY POST-REPEAL SANITATION PROBLEMS

The early post-Repeal problems of the California wine industry were formidable. The Food and Drug Inspection section of the California Department of Public Health early established minimum chemical standards for commercial wines (9). Mr. Milton P. Duffy (19) had the legal authority to enforce these standards. Wines which exceeded the legal limits could be, and were, "quarantined." In 1935 and 1936 thousands of gallons of California wine were removed from distribution (i.e. quarantined) because of their excessive acetic acid content (volatile acidity) or for other infractions of regulations. The Wine Institute's secretary, H. A. Caddow, wrote a very specific article in 1935 giving details of the need for quality control.

The post-Prohibition California regulations were purposely made more stringent than the federal regulations but were not excessively strict—white table and

dessert wines could not exceed a volatile acidity of 0.110 gr per 100 ml as acetic acid compared to federal standards of 0.120 and red table wine standards of 0.120 and 0.140, respectively (in both instances exclusive of sulfur dioxide). These standards prevented spoiled, or even incipiently spoiled, wines from reaching the market. As a result it is impossible to find a California wine with a vinegary odor on the market—probably the only country in the world, with the possible exceptions of Germany, South Africa, or Australia, where this is true.

There were also minimum and maximum California and United States alcohol standards. These had economic, revenue, and sanitation bases. The minimum alcohol of table wines was fixed at 10%. California wine makers had no difficulty reaching this minimum without the use of added sugar—thanks to our warm climatic conditions. The minimum for dessert wines was fixed at 19.5%. Very few bacteria can develop in wines of this alcohol content even if they are sweet. Recently, thanks to the excellent technological controls of the California wine industry this minimum has been lowered to 18% (17% for sherry).

The text books of this period did not neglect the sanitation problem, though, perhaps, they were not as forceful as they might have been. The circular of Joslyn and Cruess (28) warned wine makers of the dire results of neglect of sanitation as did Cruess's (16) book and the bulletin of Amerine and Joslyn (4), and those of Joslyn and Amerine (25, 26). These bulletins, and the later more extensive books of Amerine and Joslyn (5, 6) and Joslyn and Amerine (27), which succeeded them, have thorough discussions of winery sanitation problems and of quality control in general. There is a particularly thorough treatment of sanitation in the California wine industry in Amerine et al. (3). It is of some interest to note that the dangers of asphyxiation and of excessive use of sulfur dioxide were clearly indicated in these publications. See particularly the report of Russell et al. (35) and Ehlers (23).

Partially as a result of the experimental work and warnings reported in these publications microbial spoilage of wine has disappeared in California—or has become exceedingly rare.

The second legal maneuver after Repeal resulted from the pollution of the Mokelumne River near Lodi. Discharge of distillery wastes into the Mokelumne River resulted in the death of fish because of the excessive BOD of the distillery wastes. A full report was given by Shaw (36). Prompt intervention of government agencies solved this problem. Vaughn and Marsh (38, 39, 40) reviewed the extensive research on this problem in California.

Prior to World War II the microbial spoilage problem in wines had been largely solved. Also, river disposal of winery wastes had been abolished and pond disposal had taken its place. Pond disposal created problems of soil and air contamination. These problems have still not been completely solved. While they do not contribute directly to deterioration of wine quality they are often a nuisance to down-wind neighbors and constitute poor public relations for the wine industry. The reports of Coast Laboratories (10, 11) and of Cook (12, 13) were directed to this problem. The intermittent irrigation or shallow pond system if used properly will control the odor problem but the problem of soil pollution is not so easily solved, Cook and Cooke (14). Proebsting and Jacob (34) early reported toxicity to plants of stillage when used in place of irrigation. While the soil recovered when stillage disposal ceased, the problem of possible contamination of the underground water supply must be considered. If the Coast Laboratory system using depths not over 4 inches with complete drying between applications is used, underground water contamination from stillage during the short grape distilling season is not a problem.

Industrial sewage disposal is probably the final solution. The seasonal nature of winery waste disposal is an additional problem. Marsh and Vaughn (31) reported that treatment with lime to pH 5 and diluting as much as possible was helpful. Use of trickling filters may be needed to reduce the BOD to a level where municipal sewage plants can handle it. Testimony given at a public hearing of the California Regional Water Control Board (8a) indicated that the shortness of the vintage plus the undesirable settleable solids and high putrescibility of stillage makes it difficult, if not imposible, for municipal sewage plants to assume this loading for a limited time. The expenditure required for extra equipment is impractical as long as there is enough land available for proper shallow intermittent check disposal of untreated stillage.

The trade organization representing the California wine industry is the Wine Institute of San Francisco. It has been concerned since before World War II with the pollution and sanitation problems of the wine industry. The extensive research of the Coast Laboratories just referred to was financed by the Wine Institute.

World War II brought special problems. Demand for tartaric acid was very great since the European sources were not available. This led to programs for extraction of tartaric acid from pressed grapes. Disposal of these wastes and prevention of microbial

spoilage of tartrates constituted especially difficult problems. The reports can be found in Marsh and Vaughn (30), Vaughn and Marsh (37, 38) and Vaughn et al. (41). They recommended drying the tartrates below 5% moisture, use of sulfur dioxide, and observation of sanitary precaution to reduce infection.

Following World War II an additional problem arose. It resulted from the awareness of the U.S. Food and Drug Administration of the sanitation problems of the wine industry. Both M. P. Duffy, Chief of Food and Drug Inspection of the California Department of Public Health (20, 21) and McKay McKinnon, Jr., Chief of the San Francisco district of the Food and Drug Administration, were active in "persuading" the industry to "clean up."

As a direct and positive reaction to the discreet "advice" of the San Francisco office of the Food and Drug Administration, the industry under the Wine Institute started more direct action by employing an industry sanitarian. See Davison (18) for a later Wine Institute response. (He was the first sanitarian of the Wine Institute.)

The most direct response was the Wine Institute *Sanitation Guide for Wineries* in 1946 (44). This was revised in 1957 (45) and brought up-to-date in 1961 and 1963 (46) by the Wine Institute's Sanitarian, Almand D. Davison. In March 1971 a new and revised edition of the *Wine Institute Sanitation Guide for Wineries* was issued (47). As Crawford (15) has noted in many respects this publication constitutes the basis for an "umbrella" GMP (Good Manufacturing Practice) manual for the California wine industry.

The present (47) sanitation guide of the Wine Institute includes sections on qualifications and duties of a winery sanitarian, employee education programs, planning new construction, proper design of equipment and facilities, correct harvesting practices, handling and delivery of wine grapes, general winery sanitation, good housekeeping, control of insects, cleaning, sterilizing and sanitizing agents, pest control, food and drug laws and pertinent references (86 are listed).

The best practices are also summarized by Amerine et al. (3) and by Amerine and Joslyn (6). They emphasized the need of a qualified sanitarian, support of the winery sanitation program by winery management, and the usual basic principles: keeping the winery inside and outside, clean and free of refuse at all times—inspection of the premises and equipment regularly, removal of harmful bacteria, insects and rodents, use of plenty of water for cleaning, choice and use of cleaning and sterilizing agents, and procedures for keeping the equipment in good repair at all times, particularly any parts that come in contact with the wine. There is a special warning that all detergents or sanitizing agents must be removed from containers or equipment by a thorough washing with water before introducing wine. Hypochlorites are especially harmful to the color and flavor of wines. If any hypochlorites remain, wines containing sulfur dioxide will reduce the chlorine to chloride ion. Quaternary ammonium compounds, particularly, must be removed.

Specific instructions for cleaning wooden, concrete, or metal equipment have been developed. Agents for neutralizing, sterilizing, or simple cleaning are listed in the Wine Institute's *Sanitation Guide*. Among the special sanitation problems of wineries are filter cloths, care of crushers, disposal of pomace, cleaning used cooperage, removal of tartrate deposits, treating moldy cooperage, removal of red pigments, storing empty containers, insect and pest control, and health of workers.

The very special problem of the normal or tolerable amount of fungi and insects which can be present in grapes for wine making is a difficult problem. Some fungi, *Botrytis cinerea,* for example under special conditions can have beneficial effects on wine quality. The same fungi under other climatic conditions can have undesirable influences. The state and federal agencies have, therefore, had to compromise on the tolerance levels of infection of grapes. This is closely related to climatic conditions and it is not, perhaps, necessary or desirable to pursue the matter further here. The reports of Berg (7) and of Martini (32) are pertinent to this problem. Their research is the basis of the present inspection program of the California Bureau of Fruit and Vegetable Standardization.

Grower education is very important in improving growing and harvesting practices. As Crawford (15) has noted, the California wine industry is very advanced in this field. As many as 15,000 grower-trucker education letters have been distributed in a single season. They encourage both growers and truckers to use the most sanitary procedures in harvesting and transporting grapes.

The fruit fly, *Drosophila,* problem is a persistent one of wineries in most parts of the world. A number of reports are summarized in the Wine Institute Sanitation Guide (47), in Amerine and Joslyn (6) and in Amerine et al. (3). Clean premises, use of air currents, and discreet applications of approved insecticides can keep them under control.

ANTISEPTICS

The use of antiseptic agents in wine making long precedes Pasteur. The first, and still the most common agent is sulfur dioxide. It can be added as the

liquid or as various salts. It recommends itself to the wine maker not only because of its effectiveness towards yeasts and bacteria, but also because of its anti-oxidative, bleaching, dissolving, acidifying, and clarifying properties. From the quality point of view it prevents spoilage but in excessive amounts it will adversely affect the odor of wine. There is an increasing tendency to lower the maximum limits of sulfur dioxide in wine. The present United States limit is 350 mg per liter for total sulfur dioxide. Very few wines have more than 300. The free sulfur dioxide is more objectionable than the fixed. The usual limit is 70 mg per liter of free sulfur dioxide. It is probable that the wine industry could live with a limit of no more than 50 mg or less for free sulfur dioxide and 250 or even 200 for total sulfur dioxide. The recent research of Lanteaume et al. (29) indicates that neither free nor fixed sulfur dioxide in wine in legal amounts constitute a health hazard—a conclusion which Wiley (42, 43) would probably not agree with, but he was sometimes dealing with wines of 500 mg per liter!

There is some use of sorbic acid and sorbates, neither of which constitute a major health hazard. Unfortunately some people are very sensitive to sorbic acid (or to its breakdown products). Amerine and Joslyn (6) questioned whether it should be used in table wines, particularly those of good quality.

There is also some use of diethylpyrocarbonate (DEPC) as an antiseptic agent. The usual amount used is about 50 to 150 mg per liter. This compound is very toxic to yeasts and rapidly decomposes within 24 hr to carbon dioxide and ethanol in wines. However, about 2 to 3% forms diethyl carbonate and other trade byproducts. No toxicity to humans has been reported in wines at normal pH and treatment levels. Using gas-liquid chromatography and mass spectroscopy, Christensen and Caputi (9a) have been unable to demonstrate any toxic residues in wine.

Fungicides and pesticides seldom constitute a major problem. To control vine mildew sublimed sulfur is used on the vines during the summer. If excessive amounts are sprayed on the vines, free sulfur will be contained in the clusters as they are harvested. In the fermentation this sulfur will be reduced and excessive amounts of hydrogen sulfide and even mercaptans will be formed.

The use of fungicides to control *Botrytis cinerea* is certainly desirable in many cases. However, Curzel and Sandri (17) noted that Phaltan, Difoltan, and Euparen (patented products) applied within three weeks of harvest may result in retardation of fermentation, especially with Difoltan. Dvorak and Schopfen (22) noted that Euparen also caused flavor alterations. Goeldner and Pfannstiel (24) also noted that the retarding effect of Euparen was greater in the cool seasons. Minárik and Rágala (33) noted that Captan not only retarded growth of *Saccharomyces cerevisiae* but tended to increase growth of *Torulopsis*.

As Duffy (20) said "We, and industry, must strive to attain the ultimate objective: clean wine made from sound grapes in a decent environment." In California, at least, this object seems to have been reached in all major aspects.

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3-A ACCEPTED PRACTICES FOR SUPPLYING AIR UNDER PRESSURE IN CONTACT WITH MILK, MILK PRODUCTS AND PRODUCT CONTACT SURFACES

Serial #60403

Formulated by

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

It is the purpose of the IAMFES, USPHS and DIC in connection with the development of the 3-A Sanitary Standards program to allow and encourage full freedom for inventive genius or new developments. Practices for supplying air under pressure heretofore or hereafter developed which so differ in material, fabrication and installation or otherwise as not to conform with the following practices, but which, in the opinion of the operator, manufacturer or fabricator are equivalent or better, may be submitted for the joint consideration of IAMFES, USPHS, and DIC, at any time.

A.

SCOPE

A.1

These 3-A Accepted Practices shall pertain to the equipment used in the supplying of air under pressure which comes in contact with milk or milk products and/or any product contact surface.

A.2

In order to conform with these 3-A Accepted Practices, equipment for supplying air as defined herein shall comply with the following design, material and fabrication criteria and the applicable Special Requirements.

B.

DEFINITIONS

B.1

AIR UNDER PRESSURE: Shall mean air, the pressure of which has been increased by mechanical means to exceed atmospheric pressure, and which is used for agitation of milk and milk products, the movement of milk and milk products, incorporation of air into frozen dessert and frozen dessert mixes, and whipped butter, the automatic opening of containers, the drying of product contact surfaces, and for other purposes where specifically directed at a product contact surfaces.

B.2

LOW PRESSURE AIR: Shall mean air under pressure which does not exceed 300 p.s.i.

B.3

HIGH PRESSURE AIR: Shall mean air under pressure which is in excess of 300 p.s.i.

B.4

AIR SYSTEMS: Air systems are of two general categories:

B.4.1

CENTRAL SYSTEM: Shall mean those which furnish air to more than one piece of equipment. (See Figure No. 1.) Such systems usually require the use of an air storage tank.

B.4.2

INDIVIDUAL SYSTEM: Shall mean those which furnish air to one piece of equipment, and which may be an integral part of a given piece of equipment. (See Figures No. 2, No. 3, No. 4, and No. 5.)

B.5

PRODUCT: Shall mean milk, milk products, frozen dessert and frozen dessert mixes, and whipped butter.

B.6

PRODUCT CONTACT SURFACE: Shall mean all surfaces that are exposed to the product, or from which liquid may drain, drop, or be drawn into the product.

B.7

NON-PRODUCT CONTACT SURFACE: Shall mean all other exposed surfaces.

C.

MATERIAL

C.1

FILTER MEDIA

Intake and air pipeline filters shall consist of fiberglass with a downstream backing dense enough to prevent fiberglass break off from passing through, cotton flannel, wool flannel, spun metal, sintered metal, activated carbon, activated alumina, non-woven fabric, absorbent cotton fibre, or other suitable materials which, under conditions of intended use, are non-toxic and nonshedding and which do not release toxic volatiles or other contaminants to the air, or volatiles which may im-

part any flavor or odor to the product. Chemical bonding materials contained in the media shall be non-toxic, non-volatile and insoluble under all conditions of use. Disposable media are not intended to be cleaned and re-used.

Note: Electronic air cleaners use electrostatic precipitation principles to collect particulate matter and therefore are not included in the preceding list of acceptable filter media. This does not preclude their use.

C.2

FILTER PERFORMANCE

C.2.1

INTAKE FILTERS: The efficiency of intake filters shall be at least 50% as measured by the National Bureau of Standards "Dust Spot Method",[1] using atmospheric dust as the test aerosol. In an aggravated atmospheric environment, such as industrial districts, prefilters are recommended to prolong the useful life of intake filters.

C.2.2

AIR PIPELINE AND DISPOSABLE FILTERS: The efficiency of either air pipeline filters or disposable filters shall be at least 50% as measured by the DOP[2] test.

C.3

PIPING: Air distribution piping, fittings, and gaskets between the downstream terminal filter and the processing equipment except where the compressing equipment is of the fan or blower type or high pressure type and except as provided in Section H shall conform to "3-A Sanitary Standards for Fittings used on Milk and Milk Products Equipment and Used on Sanitary Lines Conducting Milk and Milk Products, Serial #0809", as amended and supplements thereto, except that where air distribution piping, or fittings and gaskets do not actually contact the product or form a part of the product contact surfaces, transparent plastic tubing may be used.

D.

FABRICATION AND INSTALLATION

D.1

AIR SUPPLY EQUIPMENT

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[1]For a description of this method see: Dill, R. S. — A Test method for air filters. Amer. Soc. Heating and Vent. Eng. Trans., Vol 44, p. 379, 1938.
[2]Dioctylphthalate fog method ("DOP"). For a description of this test see: Military Standard No. 282 (MIL-STD-282, 28 May, 1956) — Method 102.9.1; Naval Supply Depot, 5801 Tabor Avenue, Philadelphia, Pennsylvania 19120.

D.1.1

The air supply shall be taken from a clean space or from relatively clean outer air and shall pass through a filter upstream from the compressing equipment. This filter shall be so located and constructed that it is easily accessible for examination, and the filter media are easily removable for cleaning or replacement. This filter shall be protected from weather, drainage, water, product spillage, and physical damage.

D.1.2

Relatively oil free air may be produced by one of the following known methods or its equivalent:

D.1.2.1

Use of carbon or teflon ring piston, or diaphragm type, or water-lubricated compressors.

D.1.2.2

Use of oil-lubricated compressors with effective provision for removal of oil.

D.1.2.3

Water-lubricated or non-lubricated blowers.

D.1.3

An air tank(s), if used, should meet the requirements of ASME and/or National Board of Underwriters Code for unfired pressure vessels.

D.2

MOISTURE REMOVAL EQUIPMENT: If necessary to cool the compressed air, a liquid-cooled aftercooler shall be installed between the compressor and the air storage tank for the purpose of removing moisture from the compressed air (See Figure No. 1), except that a compressor the design of which incorporates the aftercooling function does not require a separate aftercooler. Other moisture removal equipment may be used downstream from the compressing equipment prior to the final point of application. The resultant condensate from the aftercooler shall flow to a properly trapped outlet and shall be discharged to the atmosphere.

D.3

FILTERS AND MOISTURE TRAPS:

D.3.1

Filters shall be constructed so as to assure effective passage of air through the filter media only.

D.3.2

The air under pressure shall pass through an oil-free filter and moisture trap for removal of solids and liquids. The filter and trap shall be located in the air pipeline downstream from the compressing equipment, and from the air tank, if one is used. (See Figures No. 1 and No. 2.) The

filter shall be readily accessible for examination, cleaning, and for replacing the filter media. The moisture trap shall be equipped with a petcock or other means for draining accumulated water. Air pipeline filters and moisture traps downstream from compressing equipment shall not be required where the compressing equipment is of the fan or blower type. (See Figures No. 3 and No. 4.)

D.3.3

A disposable media filter shall be located in the sanitary air pipeline upstream from and as close as possible to each point of application or ultimate use of the air (See Figures No. 1, No. 2 and No. 5), except that a disposable media filter shall not be required for high pressure air lines or where the compressing equipment is of the fan or blower type (See Figures No. 3 and No. 4).

D.3.4

Filters other than those in D.3.2 shall not be required on high pressure lines.

D.4

AIR PIPING: The requirements of D.4 which follow do not apply where the compressing equipment is of the fan or blower type nor do they apply to high pressure lines (See Section I).

D.4.1

The air piping from the compressing equipment to the filter and moisture trap described under D.3.2 shall be readily drainable.

D.4.2

A product check valve of sanitary design which complies with the criteria set forth in Section E.1 of the "3-A Sanitary Standards for Fittings Used on Milk and Milk Products Equipment and Used on Sanitary Lines Conducting Milk and Milk Products, Serial #0809", as amended and supplements thereto, shall be installed in the air piping downstream from the disposable media filter described in D.3.3 to prevent backflow of product into the air pipeline; except that a check valve shall not be required if the air piping enters the product zone from a point higher than the product overflow level which is open to atmosphere.

E.

SPECIAL REQUIREMENTS FOR AGITATION BY AIR

E.1

Tubing used to introduce air into the product and/or product zone shall be of stainless steel and shall conform to "3-A Sanitary Standards for Fittings Used on Milk and Milk Products Equipment and Used on Sanitary Lines Conducting Milk and Milk Products, Serial #0809", as amended and supplements thereto.

E.2

There shall be no threads on product contact surfaces.

E.3

Where drilled or perforated pipe is used, internal drilling burrs shall be removed and the orifices shall be chamfered on the outer surface of the pipe.

E.4

If the volume of the air from the compressing equipment is in excess of that required for satisfactory agitation, suitable means should be employed to eliminate the excess volume.

E.5

If the product to be agitated is in an enclosed tank, means to allow the air used for agitation to escape should be provided on the tank by a vent or a safety valve as described in F.2.

F.

SPECIAL REQUIREMENTS FOR THE MOVEMENT OF PRODUCTS BY AIR DISPLACEMENT METHOD

F.1

The requirements of E.1, E.2 and E.3 shall also apply to this section.

F.2

A safety (pressure relief) valve should be installed in the air line. This valve should be set to open upon reaching a pressure not greater than the maximum allowable internal working pressure specified by the manufacturer of tank from which the product is to be moved. This safety valve should have ample capacity to freely pass the entire output of the compressor.

F.3

The safe internal working pressure of the tank should be stated on a plate attached to the tank.

F.4

The check valve specified in D.4.2 shall be installed in the air piping wherever air is used for displacement purposes.

G.

SPECIAL REQUIREMENTS FOR AIR WHICH IS TO BE INCORPORATED IN PRODUCTS

An air system in which the air is compressed by a sanitary rotary pump shall require only an intake air filter which shall be of the disposable media type. Non-sanitary air line should be pitched

away from sanitary air inlet pipeline, or a transparent sump shall be provided to collect any moisture or scale that may originate from the nonsanitary air line.

H.

SPECIAL REQUIREMENTS FOR MOVING CONTAINERS FROM ROTATING MANDRELS

H.1

When air under pressure is used for moving containers from rotating mandrel assemblies with integral air passages, the parts forming the air passages shall be of non-toxic, relatively non-absorbent materials.

H.2

A disposable media filter shall be located at the closest possible point upstream from rotating mandrel assembly (See Figure No. 5).

I.

SPECIAL REQUIREMENTS FOR HIGH PRESSURE AIR WHICH IS TO BE INCORPORATED IN PRODUCTS

When high pressure air is to be incorporated in products:

I.1

Stainless steel piping, tubing, and fittings in conformity with applicable ASA Standards for high pressure air should be used downstream from the filter.

I.2

A high pressure stainless steel check valve should be installed upstream from and as near as possible to the point of introduction of air to the product line.

These Practices are effective June 23, 1972, at which time the "3-A Accepted Practices for Supplying Air Under Pressure in Contact with Milk, Milk Products and Product Contact Surfaces," published April 1964, effective July 26, 1964, Serial #60400 and amendments thereto, are rescinded and become null and void.

SELECTED REFERENCE

Friedlander, Silverman, et al. — *Handbook on Air Cleaning.* Atomic Energy Commission, 1952. U. S. Government Printing Office, Washington, D. C. 20402.
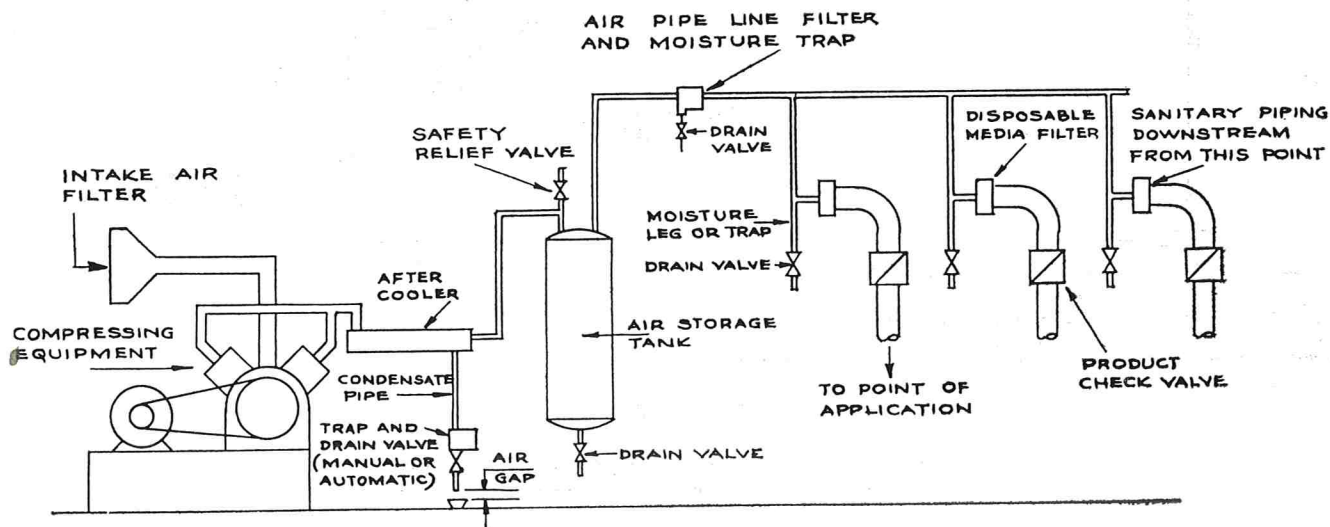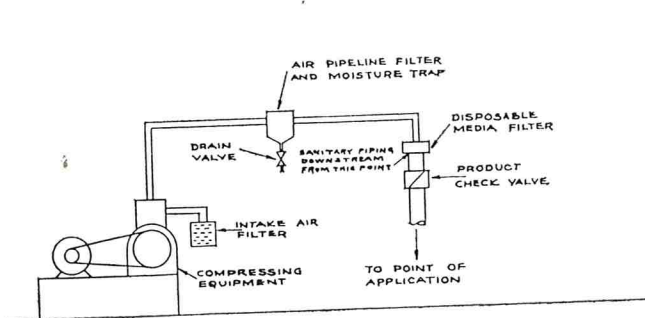

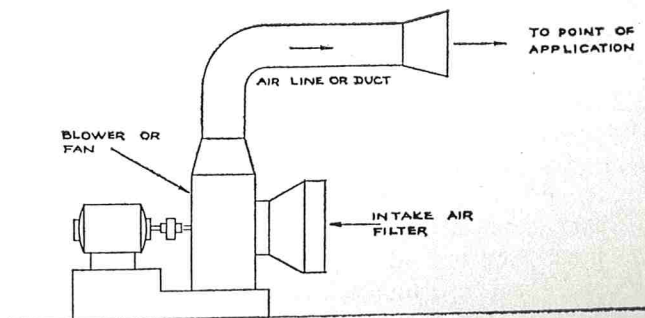FIG. 1   CENTRAL SYSTEM
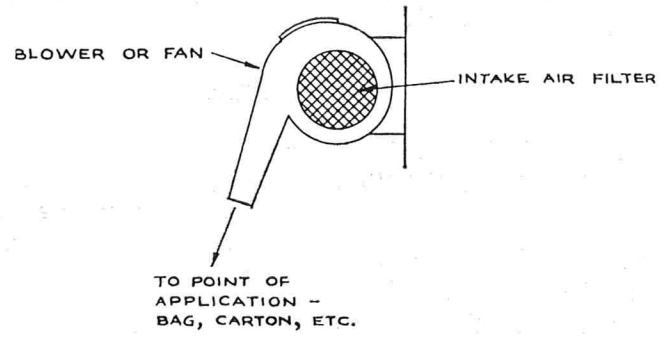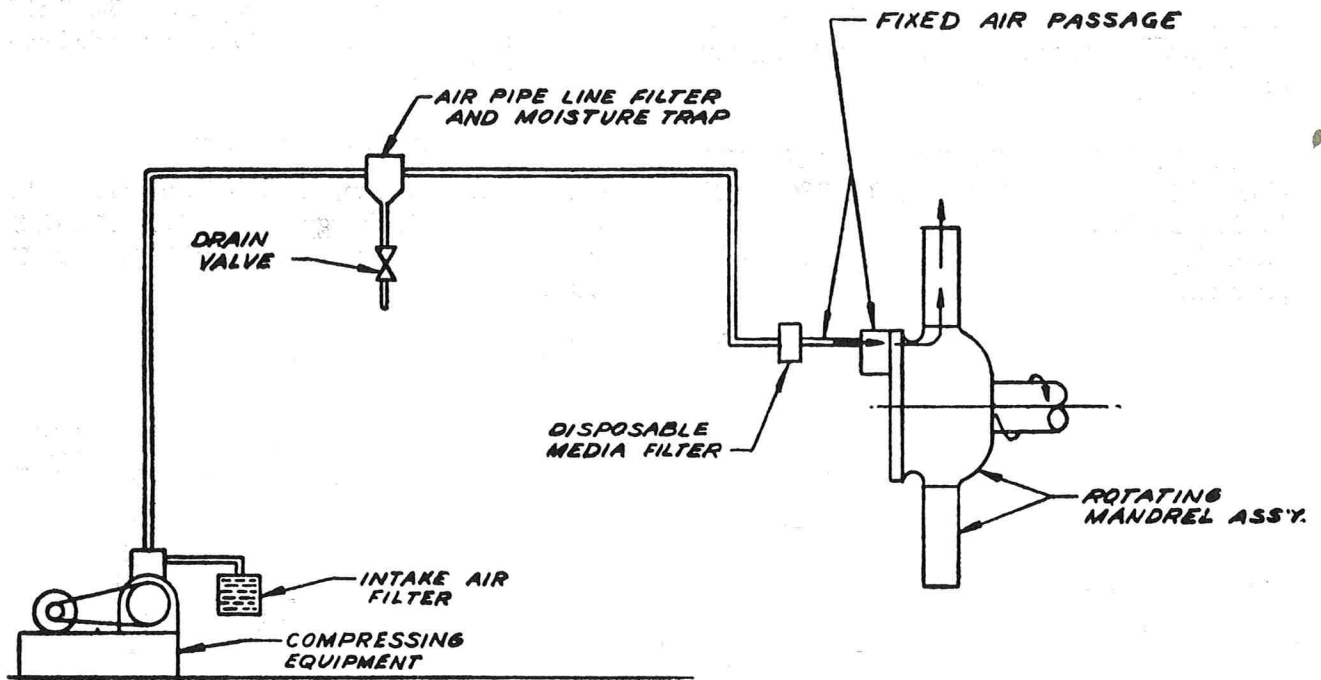

FIG. 2   INDIVIDUAL SYSTEM

FIG. 3   INDIVIDUAL SYSTEM

BLOWER OR FAN

INTAKE AIR FILTER

TO POINT OF
APPLICATION –
BAG, CARTON, ETC.

FIG. 4   INDIVIDUAL SYSTEM

FIXED AIR PASSAGE

AIR PIPE LINE FILTER
AND MOISTURE TRAP

DRAIN
VALVE

DISPOSABLE
MEDIA FILTER

ROTATING
MANDREL ASS'Y.

INTAKE AIR
FILTER

COMPRESSING
EQUIPMENT

FIG. 5

ROTATING MANDREL ASSEMBLY

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A Research Note

RAPID DIAGNOSIS FOR *STREPTOCOCCUS AGALACTIAE* AND *STREPTOCOCCUS UBERIS*

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ABSTRACT

The TKT-ferric citrate medium lends itself well for use in a rapid screening method for *Streptococcus agalactiae* and *Streptococcus uberis* isolation and identification from milk samples in that: (a) nonprofessional technicians can rapidly identify positive colonies as S. *agalactiae* or S. *uberis* on initial isolation; (b) additional confirmatory tests (CAMP, etc.) are usually unnecessary, thereby reducing labor and media costs; and (c) the medium is selective for streptococci and inhibitory to contaminants thereby increasing the number of isolations.

In eradication procedures for the *Streptococcus agalactiae* type of mastitis, it is important that a diagnosis be made as soon as possible. The infected animal can then be treated shortly thereafter, thus limiting the chance of spreading the disease to other animals in the herd. As some *Streptococcus uberis* cultures will give a CAMP (1) reaction, it is necessary to differentiate them from S. *agalactiae*. The labor, time, and material needed to accomplish this diagnosis was too cumbersome, and hence another procedure, described in this report, was devised.

MATERIALS AND METHODS

Milk samples (5-60 ml) were obtained in "whirl-pak" bags from approximately 2,000 cows in herds on the Wisconsin Department of Agriculture Albion Mastitis Control Project. The samples were composite cow samples, aseptically drawn, immediately placed on ice, and delivered to the laboratory the same day.

Two comparisons were made using three media: blood agar (BAP), Bacto Difco Lab blood agar base plus 5% citrated ovine blood; an inhibitory medium (TKT) (2) and then ferric citrate added to the inhibitory medium (TKT-FC) (3). Approximately 1,000 samples were used in each comparison in the following manner:

(a) BAP vs. TKT-FC. Duplicate samples were streaked on BAP and TKT-FC plates divided to accomodate six cow samples. All plates were incubated 24 hr at 37 C.

(b) TKT vs. TKT-FC. Duplicate samples were also streaked here using the same procedure as in comparison study (a).

Positive reactions (clear zones of hemolysis surrounding *Streptococcus* colonies with or without darkening of the medium and colonies) were recorded from the TKT-FC. All TKT-FC plates were reincubated and held another 24 hr for detection of slow-growers or delayed reactions. All suspect *Streptococcus* colonies were picked from the blood agar and TKT and tested for CAMP and aesculin-splitting reactions using a medium containing aesculin and ferric citrate plus blood agar.

The media were compared for accuracy and efficiency in the rapid detection of S. *agalactiae* and CAMP positive S. *uberis*. Special consideration was given to the time-saving aspects of each when culturing milk samples in the laboratory.

RESULTS AND DISCUSSION

Correlations were based on duplicate results whether positive or negative on the plates being compared, while non-correlations were recorded wherever results differed in isolation results. The differences are tabulated below.

In both studies, the TKT-FC showed an advantage in detecting S. *agalactiae* colonies where medium

TABLE 1. COMPARISON OF FOUR MEDIA FOR ISOLATION OF STREPTOCOCCI FROM MILK.

Total tests	Study (a)—BAP vs. TKT-FC						
	Correlation		Noncorrelation				
			S. *agalactiae*		S.		
	Negative	Positive	BAP	TKT-FC	BAP	TKT-FC	
1,087	715	291	11	57	13	0	
	1,006 (92.5%)			81 (7.5%)			

Total tests	Study (b)—TKT-FC vs. TKT						
	Correlation		Noncorrelation				
			S. *agalactiae*		S. *uberis*		
	Negative	Positive	TKT	TKT-FC	TKT	TKT-FC	
	831	112	14	17	4	8	
986	943 (95.6%)			43 (4.4%)			

was not in agreement.

The BAP in study (a) required that 680 colonies be checked for CAMP and aesculin-splitting reactions. It meant a 24-hr delay of diagnosis. Recognition of *Streptococcus* colonies on BAP requires a more experienced technician; more colonies must be selected and tested per sample and other contaminating organisms frequently overgrow the staphylococci and streptococci on this noninhibitory medium.

Since the TKT-FC is selective as well as inhibitory, showing the CAMP and aesculin-splitting reactions immediately, it naturally saves time and increases positive isolations making it most valuable for use in the culturing of milk samples submitted to the laboratory.

The TKT does not show the aesculin-splitting reaction; 135 CAMP tests were necessarily made on aesculin BAP's to differentiate *S. agalactiae* and *S. uberis* isolates. Thus, incorporation of ferric citrate in the TKT medium would seem beneficial.

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1. Christie, R., N. E. Atkins, and E. Munch-Petersen. 1944. A note on a lytic phenomonon shown by group B. streptococci. Austral. J. Exp. Biol. Med. Sci. 22:197-200.
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NEW MILK STORAGE
STANDARD IS ADOPTED

A new sanitary standard that will make it possible to safely store unlimited volumes of cold, raw milk on the farm prior to transporting it to market has been developed by the 3-A Sanitary Standards Committees, a voluntary group of industry, government and regulatory representatives.

The new standard sets guidelines for the cleanability of large tanks with greatly increased storage capacities to assure proper protection of pre-cooled stored milk. "This standard is highly significant in an era of increasingly large dairy herds, in which large milk volumes must be effectively handled prior to tank loading," said Donald H. Williams, secretary of the committee, who is technical director of Dairy and Food Industries Supply Association.

Until the new code was approved at the 3-A's spring meeting May 23-25, 1972, at Louisville, Ky., existing 3-A standards had applied only to smaller, conventional farm tanks suitable for combined cooling and storage.

The new guideline is expected to facilitate the already rapidly changing operations in milk marketing and the trend to farm storage tanks with capacities in excess of 2,000 gallons.

In other action, the 3-A committees completed an amendment to the basic standard for fittings by setting criteria for fittings and components to be used in systems for aseptic processing. The amendment is important in applications requiring that sterilization of the equipment be achieved as well as maintained during entire processing operations. The amended standard will also provide the basis for aseptic applications of pumps and other equipment used in high temperature environments.

Other tentative standards which were reviewed and passed on to appropriate action groups for further study and revision include drafts on scraped surface heat exchangers, uninsulated storage tanks, and revisions in published 3-A standards for instruments, pumps, and fillers and sealers of single service containers.

The meeting marked the beginning of the 27th year of operation of the joint 3-A Sanitary Standards Committees. About 65 health officials, equipment manufacturers and dairy processors attended.

The 3-A program for dairy equipment is the result of cooperation among three groups. (1) dairy processors, the users of dairy equipment; (2) dairy industrial suppliers and equippers, the manufacturers and sellers of dairy equipment; and (3) public health officials and sanitarians, the regulatory officials under whose jurisdiction the equipment is installed and used.

Voluntarily supported by the national trade associations in the dairy processing industry, the program has resulted in the adoption of a total of 32 standards and practices for dairy industrial equipment. Equipment complying with the standards may carry the 3-A Symbol, provided its manufacturer received authorization to do so from the 3-A Symbol Council.

In general, 3-A Standards and practices are acceptable in public health jurisdictions in nearly every town, city and state in the U.S. The 3-A Sanitary Standards and Practices are cited in the recommended Grade "A" Pasteurized Milk Ordinance of the U. S. Public Health Service.

CHANGES IN THE MICROFLORA OF WILD RICE DURING CURING BY FERMENTATION[1]

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ABSTRACT

Freshly harvested wild rice piled 18 inches deep was allowed to ferment for 34 days at ambient temperature or at 10 C (50 F). Some rice at ambient temperature and all rice at 10 C was moistened daily (1 gal water/100 lb. rice). Samples were taken at intervals during the fermentation and tested for numbers and kinds of microorganisms. Each time samples were taken some rice of each lot was parched (dried) at approximately 79 C (175 F) for 1 hr and then was tested microbiologically. The initial total count (gram-negative rods predominated) of freshly harvested wild rice ranged from $1.7\text{-}16.0 \times 10^8$ and increased during fermentation regardless of storage conditions. Molds ($1.8\text{-}5.0 \times 10^5/\text{g}$ initially) also increased in number with less growth evident at the lower storage temperature. Growth of psychrotrophs (mainly *Pseudomonas* spp.) was greatest when wild rice was fermented at 10 C. Some growth of coliforms was evident but results were erratic. *Escherichia coli, Enterobacter areogenes,* and intermediate forms were isolated. Growth of fecal streptococci during fermentation was minimal; *Streptococcus faecalis* was recovered from some samples and *Streptococcus faecium* from the same or other samples. Predominant molds were *Mucor* spp., aflatoxinogenic and nontoxigenic *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* sp.

Bacillus spp. predominated in parched wild rice regardless of how long the rice was allowed to ferment before it was heated. Coliforms, pseudomonads, fecal streptococci, and molds also were recovered but only from a few samples. Microbial numbers in parched rice generally were <1.0% of those in the unheated product.

Wild rice (*Zizania aquatica, Zizania palustris*) is an aquatic wild grass which grows in Canada and in the Great Lakes region of the United States. The plant grows best in slowly moving water which is several inches to 2 or 3 ft deep. Wild rice produces a long (0.5-0.75 inch), slender, nearly cylindrical, purplish-black kernel which is contained within a bristly brown hull that elongates into a long, stiff, twisted, barbed awn (3). Newly harvested wild rice grains are not only covered with the hull but are moist and flexible. The grain must be dried and allowed to harden and the hulls and other debris must be removed before wild rice is suitable for consumption. This is accomplished by a process which involves curing (fermentation), parching (drying), hulling (threshing), and separating (winnowing) the grain from chaff (12).

Harvesting of wild rice has been described previously (6). Such methods often result in yields no greater than 50 lb. wild rice per acre. Use of mechanical harvesters can increase the yield to 300 to 500 lb. per acre, whereas improved strains of wild rice can produce up to 1000 lb. per acre (1).

Freshly harvested full-moisture wild rice is generally stored in small piles in the open so that a natural fermentation can proceed. After fermentation, wild rice is parched and otherwise processed to make it suitable for consumption.

Curing (fermentation) of wild rice is an art which is not well understood. The process is thought to affect not only the microbiological quality of wild rice but also its chemical quality as reflected by flavor and cooking characteristics. Changes in microbial quality of grain are known to affect its viability, storage quality, nutritive value, edibility, and industrial usefulness (11). Deterioration of rice bran during storage has been attributed to hydrolysis of glycerides in bran oil with attendant formation of free fatty acids (10). Microorganisms could be responsible for such deterioration if necessary conditions prevail. This investigation was conducted to determine some of the microbiological changes which occur in wild rice during fermentation under several different conditions similar to those in commercial practice. An earlier report described some of the microbiological changes which occurred when fermented wild rice was parched and cooked (6).

MATERIALS AND METHODS

Fermentation of wild rice

Freshly harvested natural stand, wild rice, obtained from a commercial Northern Wisconsin operation, was fermented using three different storage procedures which were selected because they simulated commercial practices. Three lots of 500 lb. wild rice each received one of the following treatments: *(a)* a layer of wild rice approximately 18 inches deep on a concrete platform was held at ambient temperature (ap-

TABLE 1. CHANGES IN THE MICROBIOLOGICAL CONDITION OF FRESHLY HARVESTED WILD RICE DURING FERMENTATION.

Microbiological test	Treatment of wild rice[a]	Days of fermentation (No./g $\times 10^5$)					
		0	6	13	20	27	34
Total count	1	16,000	17,000	26,000	32,000	23,000	38,000
	2	14,000	37,000	43,000	18,000	38,000	20,000
	3	1,700	7,000	10,000	13,000	12,000	13,000
Psychrotrophic count	1	9.9	76	41	28	—[b]	6.6
	2	120	11	79	65	—	27
	3	41	960	530	360	—	1,100
Coliform count	1	50	540	26	500	64	59
	2	350	1,000	32	110	73	75
	3	30	56	32	370	150	49
Streptococcus count	1	120	86	82	71	40	77
	2	42	370	550	110	87	96
	3	17	15	9.2	18	15	37
Mold count	1	5.0	30	50	39	55	70
	2	2.2	35	3.3	3.4	26	7.5
	3	1.8	6.9	3.0	4.4	6.1	4.0

[a]Treatment 1: 500 lb. raw wild rice, 18 inches deep on concrete platform, held at ambient temperature for 5 weeks, and turned daily.
Treatment 2: 500 lb. raw wild rice treated as in 1 except that 1 gal water/100 lb. rice was added daily.
Treatment 3: 500 lb. raw wild rice treated as in 2 except that it was held at 50 F.
[b]Dash = No data.

proximate daily range 50-70 F) for 5 weeks and was turned daily to minimize heating of the product, (b) the same treatment as described in (a) except that 1 gal of water per 100 lb. of wild rice was added daily, and (c) the same treatment as described in (b) except that the wild rice was held at 10 C (50 F).

Parching

One-hundred-pound quantities of wild rice were removed from each lot of fermenting rice at weekly intervals and then were parched. A commercial gas-fired, vat-type batch parcher equipped with paddle-type stirrers was employed to heat the rice at a bed temperature of approximately 79 C (175 F) until the final moisture content of the product was 6-8% (approximately 1 hr). Because of equipment design, the temperature of some rice kernels exceeded that of the bed when inefficient stirring allowed excess direct contact residence with heated surfaces. This caused slight scorching of some kernel surfaces.

Sampling

Samples of each lot of wild rice (unheated and heated) were taken aseptically at weekly intervals and were placed in Whirl-pak bags. Bagged samples were iced, transported to the laboratory, and tested within a few hours after they were received.

Bacteriological analysis

Eleven grams of wild rice (freshly harvested, fermented, or parched) and 99 ml of sterile phosphate buffered distilled water were added to a sterile Waring blendor jar and then were blended at low speed for 1 min. Earlier work by Goel et al. (6) indicated that more organisms were recovered when wild rice was blended rather than shaken in the diluent Serial dilutions were made with sterile phosphate-buffered distilled water and were plated in duplicate for: (a) total aerobic count: plate count agar (Difco), incubation at 30 C for 48

hr; (b) psychrotrophic plate count: plate count agar, incubation at 7 C for 10 days; (c) yeast and mold count: potato dextrose agar (Difco) acidified to pH 3.5 with sterile tartaric acid, incubation at 22 C for 3-5 days; (d) coliform count: violet red bile agar (Difco), incubation at 32 C for 24 hr; (e) staphylococcus count: mannitol salt agar (Difco), incubation at 37 C for 48 hr; (f) streptococcus count: KF agar (Difco), incubation at 37 C for 48 hr; and (g) salmonellae: 10 ml of the 1:10 dilution were preenriched in lactose broth at 37 C for 24 hr, enriched in tetrathionate (Difco) and selenite (Difco) broths at 37 C for 24 hr, enrichment broth was then streaked on brilliant green (Difco) and SS (Difco) agars, typical colonies were picked to triple sugar iron (TSI) agar (Difco) slants, TSI-positive isolates were tested for urease and lysine decarboxylase, and finally appropriate isolates were serotyped (done at the Wisconsin State Laboratory of Hygiene).

After plates were incubated as described, colonies were counted, and average values were calculated. Between 50 and 100 colonies were picked from each group of plates which were poured with the same medium. Appropriate tests were done to permit identification of the isolates as to genus or genus and species (2). Molds identified as Penicillium or Aspergillus species were tested for their ability to produce aflatoxin.

Identification of isolates

Isolates obtained from violet red bile agar were tested for gram reaction, ability to ferment lactose, and, when appropriate, reaction to the IMViC series of tests and changes produced on TSI agar slants. Approximately 64% of the colonies picked from violet red bile agar plates proved to be Escherichia coli (7%), Enterobacter aerogenes (14%), or intermediates (43%).

Streptococci obtained from KF agar plates were identified on the basis of gram reaction; catalase test; growth at 10

TABLE 2. KINDS OF MICROORGANISMS AND FREQUENCY OF ISOLATION FROM FERMENTING AND PARCHED WILD RICE.

Source and kind of microorganism	Fermenting wild rice	Parched wild rice
	(No. specific kind/total isolates)	
From plate count agar (30 C)		
Gram-positive rods	5/27	17/25
Gram-negative rods	14/27	6/25
Gram-positive cocci	8/27	2/25
Bacillus species	0/27	15/25
Corynebacterium species	0/27	2/25
Lactobacillus species	2/27	0/27
Leuconostoc species	3/27	0/27
From plate count agar (7 C)		
Pseudomonas species	23[a]	3[a]
Pigmented *Pseudomonas* species	8/23	0/3
From violet red bile agar		
Escherichia coli	2/44	1/6
Enterobacter aerogenes	5/44	1/6
Intermediates	37/44	4/6
From KF agar		
Streptococcus faecium	14/35	7/11
Streptococcus faecalis	6/35	1/11
Other streptococci	15/35	3/11
From acidified potato dextrose agar		
Aspergillus species	5/16	2/10
Penicillium species	2/16	3/10
Mucor species	8/16	5/10
Rhizopus species	1/16	0/16

[a] A total of 70 colonies was isolated from fresh and parched wild rice during fermentation.

TABLE 3. TYPES OF BACTERIA RECOVERED WITH PLATE COUNT AGAR FROM FERMENTING WILD RICE.

Type of bacteria	Treatment of wild rice[a]	Days of fermentation				
		6	13	20	27	34
Gram-positive rods	1	—[b]	—	—	—	+[c]
	2	—	—	—	—	+
	3	—	—	+	—	+
Gram-negative rods	1	+	—	+	+	+
	2	+	+	+	+	+
	3	+	+	+	+	+
Gram-positive cocci	1	—	—	—	—	+
	2	—	—	—	—	—
	3	—	+	+	—	—

[a] For description of treatments see Table 1 or the Materials and Methods Section.
[b] Dash = Not detected
[c] Plus = Detected

and 45 C, in 6.5% sodium chloride, at pH 9.6, and in litmus milk; survival after heating at 60 C for 30 min; and growth in azide dextrose broth (BBL) and ethyl violet azide broth (BBL) *(16)*. *Streptococcus faecalis* was differentiated from *Streptococcus faecium* on the basis of growth at 50 C and growth in 0.04% potassium tellurite. Forty-five percent of the isolates from KF agar were *S. faecium*, 15% were *S.*

faecalis, and the remainder gave variable results.

Isolates thought to be pseudomonads were characterized by gram-reaction, oxidase test, and by oxidative or fermentative metabolism of carbohydrate *(8)*.

Isolates of molds were characterized according to descriptions given by Gilman *(5)* and Hazen and Reed *(7)*.

Production and detection of aflatoxin

Molds to be tested for their ability to produce aflatoxin were grown in a sterilized broth [2% (w/v) yeast extract, 20% (w/v) sucrose, and water] for 7 days at 28 C. The broth was then recovered, filtered (Whatman filter paper No. 1), and extracted with chloroform to recover the aflatoxin *(14)*. Extracts were tested for possible presence of aflatoxin with thin-layer chromatographic procedures as described by Shih and Marth *(13)*.

RESULTS

Microorganisms are present both on the outside of wild rice hulls and the outside of grains, with larger numbers appearing on the outside of hulls *(6)*. No attempt was made to separate hull microflora from grain microflora in these studies since, presumably, the total microflora contributes to characteristics of the finished product. In this study wild rice was subjected to three different treatments during a 34-day fermentation. Portions of rice were removed at weekly intervals from the mass of fermenting rice and were then parched at approximately 79 C. Results obtained from tests on the fermenting wild rice will be considered first.

Fermenting wild rice

Changes in numbers of microorganisms during fermentation of wild rice are reported in Table 1. The total aerobic count of wild rice increased during fermentation regardless of treatment used. However, the rate of increase was affected by the treatment. As might be expected, microbial growth was slowest in rice stored at 10 C rather than at ambient temperatures. Growth of aerobic bacteria was fastest in moistened rice held at ambient temperatures. The total aerobic count of wild rice as recorded in Table 1 compares favorably with values reported earlier *(6)* and with values for wheat as given by James et al. *(9)*. Gram-negative rods predominated among the isolates recovered from total count plates (Table 2). These bacteria, probably coliforms and *Pseudomonas* spp. (Tables 2 and 4), were recovered from the rice during the entire fermentation process, regardless of fermentation conditions (Table 3). Gram-positive cocci were next in frequency of isolation (Table 2) and were recovered sporadically from unmoistened rice and from moistened rice held at 10 C. Fecal streptococci (Table 4), *Leuconostoc* spp. (Table 2), and perhaps other streptococci were the major components of this group of bacteria. Less than 20% of the isolates were gram-positive rods (Table 2) and

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TABLE 4. OCCURRENCE OF DIFFERENT MICROORGANISMS IN WILD RICE DURING FERMENTATION.

Organism	Treatment of wild rice[a]	Days of fermentation					
		0	6	13	20	27	34
Pseudomonas sp.	1	+[b]	−[c]	−	−	+	+
	2	+	−	−	+	+	+
	3	−	−	+	+	+	+
Escherichia coli	1	−	−	−	−	−	−
	2	−	−	−	−	−	−
	3	−	+	−	−	−	−
Enterobacter aerogenes	1	−	−	−	−	−	+
	2	−	−	−	−	−	+
	3	−	−	−	−	−	−
Streptococcus faccium	1	+	−	+	+	+	−
	2	−	−	−	−	+	+
	3	+	+	−	−	−	+
Streptococcus faecalis	1	−	+	−	−	−	−
	2	−	−	−	+	−	−
	3	−	−	−	+	+	−
Molds	1	−	−	M[d]	M	P[g]	M, P
	2	−	−	M	A[f]	A	A
	3	−	−	M, R[e]	M	A	A, M

[a]For description of treatments see Table 1 or the Materials and Methods Section.
[b]Plus = Detected
[c]Dash = Not detected
[d]M = Mucor sp., [e]R = Rhizopus sp., [f]A = Aspergillus sp.,
[g]P = Penicillium sp.

they were the predominant bacteria which survived the parching treatment (Table 2). Use of the high dilutions needed to obtain plates suitable for counting probably accounts for the failure to recover *Bacillus* spp. from fermenting rice.

The number of psychrotrophic bacteria increased most rapidly in the moistened wild rice held at 10 C and more slowly in the unmoistened rice held at ambient temperature (Table 1). Moistened rice at ambient temperature appeared to be unfavorable for growth of these bacteria. Although pigmented and non-pigmented pseudomonads were recovered from plates incubated at 7 C (Table 2), it is possible that other genera might be found if more colonies were isolated. Nevertheless, *Pseudomonas* spp. were certainly the predominant psychrotrophic bacteria present in the fermenting raw wild rice. These organisms were recovered most frequently from moistened rice held at 10 C (Table 4), but they also appeared in rice fermented under the other conditions.

The coliform count of fermenting wild rice was erratic (Table 1). Growth of these bacteria was minimal and may have occurred in only portions of a fermenting lot of rice so that samples did not adequately represent the entire mass of product. As expected, *E. aerogenes* was recovered more often than *E. coli*, but intermediate forms clearly predominated (Table 2). Thomas and Hobson (17) tested ears and panicles of growing cereal crops and recovered intermediate coliform types, *E. aerogenes*, and *Enterobacter cloacae*. Hence, results of this study are in substantial agreement with those of

they appeared at the end of the fermentation in wild rice subjected to all treatments (Table 3). Although *Lactobacillus* spp. were isolated (Table 2), it is probable that *Bacillus* spp. also were present since

TABLE 5. THE MICROBIOLOGICAL CONDITION OF WILD RICE WHICH WAS PARCHED AFTER 6 TO 34 DAYS OF FERMENTATION.

Microbiological test	Treatment of wild rice[a]	Days of fermentation				
		6	13	20	27	34
Total count	1	3.5×10^6	2.2×10^7	5.6×10^6	3.9×10^6	4.0×10^6
	2	2.2×10^7	4.3×10^6	1.3×10^6	1.4×10^6	4.3×10^5
	3	1.0×10^5	5.8×10^5	1.6×10^7	5.3×10^5	4.0×10^4
Psychrotrophic count	1	1.9×10^6	540	<30	−[b]	150
	2	<1,000	800	<30	−	<30
	3	<1,000	<30	<30	−	<30
Coliform count	1	1.0×10^4	3.2×10^3	<30	220	270
	2	<1,000	<30	90	<30	160
	3	<1,000	470	100	<30	430
Streptococcus count	1	1.0×10^4	2.4×10^3	<30	860	220
	2	1,000	70	220	<30	<30
	3	<1,000	<30	470	<30	<30
Mold count	1	<100	1.7×10^3	<30	200	40
	2	100	100	180	<30	140
	3	<100	80	390	10	20

[a]For description of treatments see Table 1 or the Materials and Methods Section.
[b]Dash = No data.

TABLE 6. TYPES OF BACTERIA RECOVERED WITH PLATE COUNT AGAR FROM WILD RICE WHICH WAS PARCHED AFTER 6 TO 34 DAYS OF FERMENTATION

Type of Bacteria	Treatment of wild rice[a]	Days of fermentation				
		6	13	20	27	34
Gram-positive rods	1	+[b]	+	+	+	—
	2	+	+	+	+	+
	3	—[c]	+	+	+	+
Gram-negative rods	1	—	—	—	+	+
	2	—	+	—	—	+
	3	—	—	+	+	—
Gram-positive cocci	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	+	—	—

[a]For description of treatments see Table 1 or the Materials and Methods Section.
[b]Plus = Detected
[c]Dash = Not detected

Thomas and Hobson (17). Appreciable growth by either *E. coli* or *E. aerogenes* during fermentation and hence more frequent isolation of these bacteria is clearly not indicated (Table 4).

Fecal streptococci apparently failed to grow in unmoistened rice at ambient temperatures (Table 1) but were recovered more often from this rice than from rice which received the other treatments (Table 4). Growth of these bacteria also was not evident in moistened wild rice at 10 C but did occur in the moistened product held at ambient temperatures (Table 1). *Streptococcus faecium* was recovered more often than *S. faecalis* (Table 2).

Coagulase-positive staphylococci and salmonellae were not recovered from wild rice during fermentation. However, *Proteus* spp. were detected when salmonellae were sought. In earlier work, Goel et al. (6) did recover salmonellae from some fermented but otherwise untreated wild rice. Perhaps use of larger samples of wild rice would have resulted in recovery of some salmonellae but even so, substantial numbers were not present.

Mold counts on fermenting wild rice also were erratic (Table 1) and this can be accounted for by variability of mold growth in a given lot of rice. There is evidence of mold growth in all three lots of wild rice but more growth seemed to occur in the two lots held at ambient temperatures than in the lot stored at 50 F. Most growth was evident in the unmoistened rice. Yeasts, if present, could not be counted because of excessive mold growth on plates. Molds in the genera *Mucor* and *Aspergillus* were isolated more frequently than were *Penicillium* spp. or *Rhizopus* spp. (Table 2). *Aspergillus* spp. and *Mucor* spp. were most often isolated from the moistened rice regardless of storage temperature (Table

4), whereas *Penicillium* spp. and *Mucor* spp. were detected in the unmoistened rice.

Since some aspergilli and penicillia can produce aflatoxin (4), isolates from wild rice were tested to see if they were aflatoxinogenic. Aflatoxins B_1, B_2, and G_2 were recovered from some *Aspergillus* and *Penicillium* isolates after growth in laboratory media. Tests were also made on wild rice itself and aflatoxin was not recovered.

Parched wild rice

When wild rice was allowed to ferment for various time intervals and then parched, microbiological results summarized in Table 5 were obtained. The total aerobic count was much lower than that of the fermenting wild rice (Table 1) indicating that parching effectively inactivated most of the organisms present. Total counts of parched moistened rices decreased as the fermentation progressed. This is in marked contrast to results obtained with fermenting wild rice where microbial counts increased during fermentation and suggests a slight decrease in the heat-resistant flora as the fermentation progressed. Parching resulted in a shift in the predominant aerobic organisms which appeared in the wild rice. Gram-positive rather than gram-negative rods predominated (Table 2) and they were recovered during all stages

TABLE 7. OCCURRENCE OF DIFFERENT MICROORGANISMS IN WILD RICE WHICH WAS PARCHED AFTER 13 TO 34 DAYS OF FERMENTATION.

Organism	Treatment of wild rice[a]	Days of fermentation			
		13	20	27	34
Pseudomonas sp.	1	—[b]	—	—	—
	2	+[c]	—	—	+
	3	—	—	—	—
Escherichia coli	1	—	—	—	—
	2	—	—	—	—
	3	—	+	—	—
Enterobacter aerogenes	1	—	—	—	+
	2	—	—	—	—
	3	—	—	—	—
Streptococcus faecium	1	—	—	+	—
	2	+	+	—	—
	3	+	+	—	—
Streptococcus faecalis	1	—	—	—	+
	2	—	—	—	—
	3	—	—	—	—
Molds	1	M[d]	—	P[f]	P
	2	M	M	—	A
	3	M	A[e]	P	—

[a]For description of treatments see Table 1 or the Materials and Methods Section.
[b]Dash = Not Detected
[c]Plus = Detected
[d]M = *Mucor* sp., [e]A = *Aspergillus* sp., [f]P = *Penicillium* sp.

of fermentation (Table 6). *Bacillus* spp. comprised most of the gram-positive flora and this could be expected because their spores are heat resistant.

Psychrotrophic bacteria, coliforms, streptococci, and molds were present in markedly lower numbers (Table 5) after wild rice was parched than in corresponding unparched samples (Table 1). The gram-negative coliforms and pseudomonads were isolated only sporadically (Table 7). Although *S. faecium* was detected in samples of rice subjected to the three conditions of fermentation, the bacterium did not appear regularly (Table 7). *Streptococcus faecalis* was obtained from a single sample (Table 7), whereas coagulase-positive staphylococci and salmonellae were not detected in any samples of fermented parched rice.

Cultures of *Mucor* and *Aspergillus* were recovered from parched rice obtained early in the fermentation and cultures of *Penicillium* and *Aspergillus* were isolated during later stages of the fermentation.

DISCUSSION

Fermentation of freshly harvested wild rice is accompanied by changes in the microbial flora of the product. When the fermentation was carried out at ambient temperatures, there was an increase in the total aerobic count and the mold count. Some growth of psychrotrophic and coliform bacteria was observed when moisture was not added to rice at ambient temperatures but decreases occurred in numbers of these bacteria, particularly toward the end of the fermentation, when moistened rice was examined.

The predominant flora of the wild rice during fermentation consisted of gram-negative rods (*Pseudomonas* spp. and coliforms). Gram-positive cocci (fecal streptococci, *Leuconostoc* sp., and others) were the second most prominent group, and gram-positive rods (*Lactobacillus* spp. and probably *Bacillus* spp.) were isolated least often. Gram-positive cocci and gram-positive rods appeared sporadically and failed to become predominant types during the fermentation. Adding moisture to rice at ambient temperatures had no marked effect on the microflora during fermentation. As expected, storage of moistened rice at 10 C enhanced growth of psychrotrophic bacteria which were mainly pseudomonads. Tests for mesophilic and thermophilic anaerobes were not done and so their behavior during curing of wild rice is unknown. Relationships between microorganisms observed during fermentation and desired organoleptic qualities of rice remain to be determined. If it is established that certain bacteria must grow to produce the desired flavor and other characteristics in wild rice, it might be possible to inoculate parched rice with such bacteria and thus enable use of a controlled fermentation.

Parching markedly reduced number of microorganisms and altered the microflora of the rice so gram-positive rods (*Bacillus* spp.) predominated. Inactivation of microorganisms through parching of wild rice, as observed in these tests, was comparable to results obtained earlier by Goel et al. *(6)*.

Fermentation of wild rice affords molds an opportunity to grow. Under conditions of an extended fermentation, production of aflatoxin or other mycotoxins is a distinct possibility. In fact, some aflatoxinogenic molds were recovered from samples of fermenting rice tested in these studies. Although limited trials on wild rice failed to clearly demonstrate the presence of aflatoxin, further studies are warranted to determine if mycotoxins can be produced in wild rice during extended fermentation and if the toxin can penetrate into the kernel which generally remains within the hull until after parching. Storage of wild rice at 10 C should minimize the chances for mycotoxin formation since at least aflatoxin is seldom produced at that temperature *(15)*. Furthermore, insect infestation during fermentation of raw wild rice is also possible and care must be exercised to minimize or eliminate this potential health hazard. Parched rice contained primarily *Bacillus* spp. It would be worthwhile to determine if food-poisoning strains of *Bacillus cereus* are present in the wild rice and if there is a chance for these bacteria to reach the consumer.

Finally, it must be remembered that fermented wild rice is subjected to further processing which alters the microflora before the product is consumed. Parching serves to reduce the number of microorganisms and hulling separates the high-count outer hull from the low-count kernels *(6)*. Boiling of wild rice in water before consumption serves to eliminate virtually all viable microorganisms *(6)*, but this heat treatment would be inadequate to inactivate some mycotoxins if they were present.

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ASSOCIATION AFFAIRS

MISSOURI ASSOCIATION OF MILK AND FOOD SANITARIANS AWARD—1972

The 1972 Sanitarian's Award recipient is a young, vigorous promoter of outstanding health services in one of the fast growing cities of Missouri. His tenure of service in the sanitation profession spans only eleven years, but his accomplishments suggest half a lifetime of work.

He served as Sanitarian I from 1961 to 1965 when he became Chief Sanitarian for his city health unit. He became supervisor of section for meat, milk, food and environmental sanitation, the health clinic and the animal control unit. He helped "break in" the new food ordinance, revised the milk ordinance in accordance with the 1965 recommendations, started a nursery school sanitation program, improved the program for itinerant restaurants to one very comparable to the regular restaurant program, and initiated revision of the swimming pool ordinance.

He has kept people in the forefront of his thought and actions. He has increasingly stressed professionalism to his staff. He cross-trained them to maximize proficiency. He directed the health clinic to provide more and better services to indigent persons.

His department provides many more services to the city, 50 to 100% more in some areas, than when he became Chief Sanitarian. And surveys show significant improvements in ratings, especially in milk and food control. His practice of working closely in guiding new businesses in construction and installation of equipment has saved many hours for his sanitarians and for business men.

The recipient is former president of the Safety Council, past vice-president and secretary of his Cosmopolitan Club, serves on the board of directors
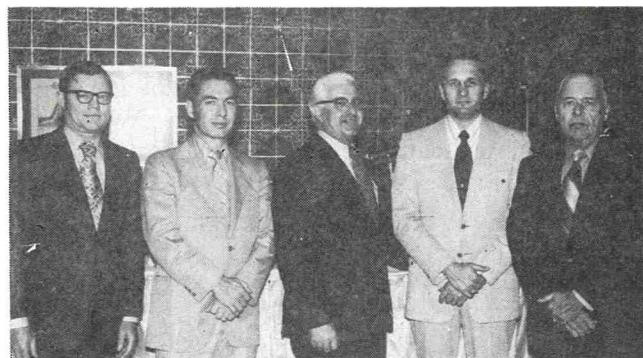
Officers for the 1972 year in photograph from left to right are: Secretary-Treasurer, Erwin Gadd, Director, Bureau of Milk, Food and Drug Control, Missouri Division of Health; Immediate Past President, Robert Gillilan, Hospital Sanitarian, Missouri Division of Health; President, Charles Van Landuyt, Sanitarian, Morgan County Health Department; First Vice-President, Harold Bengsch, Superintendent of Sanitation, Springfield, Missouri Health Department; and Second Vice-President, Jerry Burns, Supervisor of Milk Sanitation, Kansas City Health Department, Kansas City, Missouri.
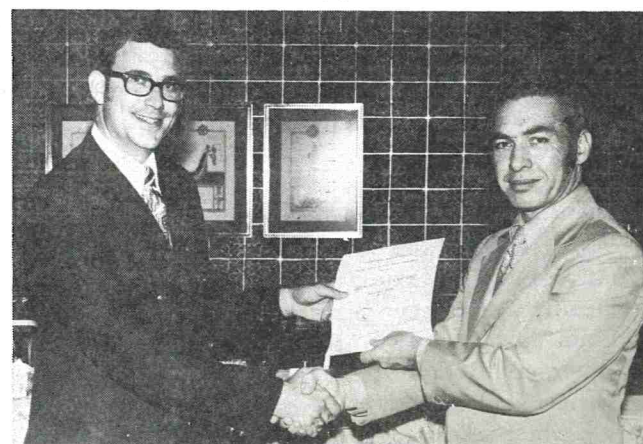
Mr. Michael Sanford receiving the Sanitarian's Award from President Gillilan.

of the Columbia Public Schools School of Practical Nursing, and is a deacon in his church.

A large number of sanitarians supported his nomination. The following are exerpts of their comments: "He approaches various problems with sincerity and enthusiasm which I believe contributes to his success in good program administration." "His advise, experience and consultation have benefited everyone involved in the food sanitation program." "He has done much to create a professional and respected image of sanitarians." "He is a very bright and personable young man and I depend upon him a great deal in the operation of the Health Department." "His ability to take a positive stand on issues involving public health has been a major factor in projecting his leadership capabilities." "Among his greatest assets is his ability to express his views in a way that stimulates your thinking and makes you glad that he was present." "Through his ability as an administrator and leader he has broadened the horizons of the Columbia City Health Department considerably."

Surely there are few who have not guessed that the winner of the 1972 Sanitarian's Award of the Missouri Association is Michael Sanford, Chief Sanitarian, City of Columbia.

———————

SOUTH DAKOTA SANITARIANS MEET

The 24th annual conference of the South Dakota Environmental Health Association was held in Pierre, South Dakota, on May 8-10, at the Falcon Restaurant. An exceptional program was presented to the State, city, county and Reservation sanitarians in attendance.

South Dakota Governor, Richard Kneip, gave the opening keynote address, stressing the importance of each professional's attitude as he works with the public. H. L. "Red" Thomasson followed this with a discussion of sanitarians, are they specialist or generalist. He also stressed achieved professionalism.

Other outstanding speakers included Bert Mitchell, National Park Service Consultant, H.E.W.; R. G. Miner, Vice President, Lystads; Stan Reno, Regional Program Director, National Institute Occupational Safety and Health; Denzil Inman, Food Consultant, P.H.S./F.D.A.; and Jim Brown, Sales Manager, Stewart In-Fra-Red Commissary of Minnesota, Inc. State Health Department personnel appearing on the program included Lyle Randen, Air Quality Control Program, Robert H. Hayes, M.D., State Health Officer and Morris Johnson, Pesticides Consultant.

Two speakers from the P.H.S., Indian Health Service Area Office, Dale Johnson and Thomas Goninion, discussed various aspects of Reservation sanita-
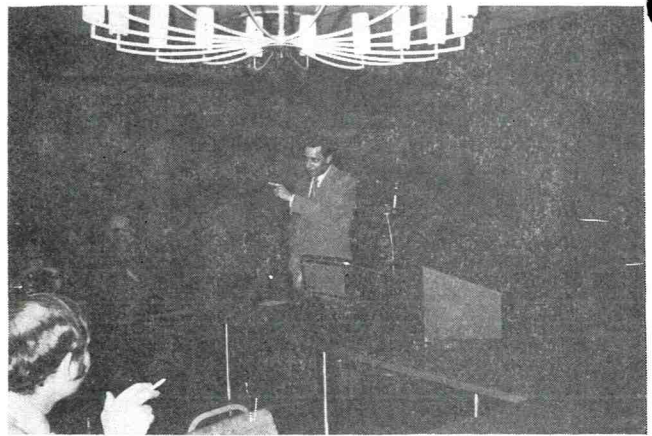
South Dakota Governor, Richard Kneip keynoted the South Dakota Environmental Health Association's 24th annual convention. Pierre Mayor, Clint Gregory (L) gave the official welcome to Pierre.

R. G. Miner, Vice President, Lystads, Inc., discusses pesticides and the role of the P.C.O. in pest control. Seated (L-R) is affiliate secretary-treasurer, Howard Hutchings and Moderator Orville Schneider.

H. L. "Red" Thomasson talks about the sanitarian and his professional role.

tion. Goninion presented a colorful slide story of a recent buffalo slaughter in Wind Cave National Park and discussed the care, utilization and distribution of the meat.

Other speakers were Joe Becker, South Dakota

Tom Goninion could pass as an original buffalo hunter. His topic — "Wind Cave Buffalo Harvest" — with color slides. Also shown are Orville Schneider (L) and Denzil Inman. The cigar smoker is Virgil Meenk.

Bakery Association, F. K. Olsen, South Dakota Lodging Association; and Earl Neumeyer, Chief Inspector, State Plumbing Board. Banquet speaker B. J. Rose used a slide series to illustrate how man and his environment are at odds with one another, especially as it relates to wild game and preservation of scenic areas.

New officers elected for 1972-73 include Robert Wermers, Pennington County Health Department

(Rapid City), president and Edward Michalewicz, South Dakota State University, Brookings, vice president. Howard Hutchings remains as secretary-treasurer. The 1973 convention will be held in the Black Hills.

CORROSION SHORT COURSE

The annual Pacific Northwest NACE Corrosion Short Course will be held at the Rivershore Motor Inn, Richland, Washington on November 9 and 10, 1972. The short course entitled "Corrosion in the Food Industry" will be jointly sponsored by the Inland Empire Section of the National Association of Corrosion Engineers and the Lewis and Clark Section of the Institute of Food Technology in cooperation with the College of Engineering, Washington State University.

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There is no substitute for good cow milking. After the expense and effort of careful breeding and feeding, it just makes good sense to get all the milk the cow produces. Good cow milking includes proper pre-milk stimulation, establishing a favorable routine which makes milking a pleasant experience for the cow and gets her full cooperation. Treat each quarter as an individual and guard against over-milking. Besides the benefit of increased production, good cow milking is also the best defense against the dairyman's worst enemy, mastitis.

A sound mastitis detection and control program makes you money in many ways. It can save on total treatment costs, lets you sell all your milk, helps increase production per cow, produces a higher quality product, and extends the milking life of cows in the herd. The table illustrates the value of the savings which would come by reducing the CMT score on bulk tank milk from 2 to trace. This would mean reducing the leukocyte count from over 2 million to less than 500,000. Although most dairies are now consistently below 1,500,000 on their cell count some are still pushing the limit and bounce into the 2 million cell range from time to time. On the other hand, our best dairies have bulk tank CMT scores of negative to trace and cell counts in the 300,000 to 500,000 range.

The average dairy may have tank milk CMT scores of 1 and cell counts of about 900,000. If such dairies can change the CMT scores to trace and their cell counts to about 400,000 their increased income may be about one half that shown in the table, or $40 to $70 per cow, depending upon per cow capability and other factors.

These figures on loss, or potential profits, still do not include several important economic aspects of mastitis control. For example, labor costs are reduced and less culling permits a better opportunity to select replacement heifers and permits more unneeded heifers to be sold. Cows are permitted to stay in the herd longer and to reach their greatest production potential, further increasing the herd average. Each owner can add such values to those listed in the table.

PROFITABLE DAIRYING MEANS DOING EVERYTHING RIGHT. It is like a relay race. If one runner drops the baton, the others can seldom run fast enough to win the race. It begins with understanding of the cow. Gentle treatment and calm surroundings permit successful completion of all the important steps of good milking.

Begin with observation of milking procedures and a thorough check of the milking system. Sometimes substantial changes are necessary to bring older installations up to modern standards. All milking equipment needs regular maintenance and every dairyman should arrange for a competent person to regularly check his equipment and to make repairs promptly. Preventive maintenance is inexpensive insurance to protect the dairyman's investment and income.

Mastitis control requires management and treatment of the infection problem. Lactation treatment, dry period treatment and dipping of teats after milking all play important roles in mastitis control. Optimum application of these tools will vary depending upon herd conditions and the infections involved. It is here that the veterinarian competent in mastitis control is so valuable to the dairyman.

QUALITY MILK. Many high bacterial counts are due to mastitis organisms and high leukocyte counts are a direct result of mastitis. Control is essential to good milk quality. It protects the dairyman's investment and can substantially enhance profit margins. Mastitis control is possible, and not particularly difficult. *It starts with good cow milking and requires that everything is done right.*

DOLLARS RETURNED BY MASTITIS CONTROL

Potential Production pounds/year	Dollars Received per Cow from			Increased Income	
	Increased Production[1]	Reduced Treatment[2]	Reduced Replacement[3]	Dollars per cow	Dollars per 100 cows
8,000	48	15	15	78	7,800
10,000	60	15	17	92	9,200
12,000	72	15	20	107	10,700
14,000	84	15	22	121	12,100
16,000	96	15	25	136	13,600

[1] By reducing the bulk tank CMT from 2 to trace.
[2] By reducing treatments from 2 cases per week to 1 case per 2 months.
[3] By reducing replacement of low producing cows from 10 to 5 cows per year.