

## INHIBITION OF PROPIONIBACTERIA BY ANTIBIOTIC AND ANTIMICROBIAL AGENTS<sup>1, 2</sup>

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### ABSTRACT

A suitable technique was developed to determine the antibiotic sensitivity of *Propionibacterium*. Then, inhibition patterns of 30 strains of 11 different *Propionibacterium* species against 39 antibiotic or antimicrobial agents were obtained. Propionibacteria were resistant to penicillinase-resistant penicillins such as cloxacillin, nafcillin, oxacillin, and a naphtheridine derivative nalidixic acid. Moderate sensitivity to kanamycin and colistin was shown. Only a few strains of propionibacteria were inhibited by gantrisin (sulfisoxazole), one of nine sulfonamides tested. Determination of sensitivity to antibiotic and antimicrobial agents would not assist in speciation of this genus.

*Propionibacterium* species are used to manufacture Emmental (Swiss) and related cheese varieties. In the past, little attention was given to the effects of antibiotics and antimicrobial agents on this genus except for limited studies using nisin, tylosin, penicillin, and streptomycin. In a review article, Krane (11) mentioned that nisin had little effect on ripening and eye formation in Swiss cheese. Winkler and Fröhlich (13), however, demonstrated that nisin had an inhibitory effect on growth of propionibacteria in Emmental cheese. According to Galesloot (7), propionibacteria were inhibited by nisin. Studies have shown that the antibiotic tylosin is inhibitory to eight strains of propionibacteria (3). Milk containing 0.1 unit of penicillin and 5  $\mu$ g of streptomycin per milliliter totally inhibited growth of *Propionibacterium shermanii*, one of the propionibacterial species used in Swiss cheese manufacture (6).

These investigations, however, were conducted using a limited number of antibiotics and *Propionibacterium* strains. The purpose of this work was to establish the effect of a wide variety of antibiotics and antimicrobial agents on a greater number of strains of *Propionibacterium*. Knowledge of their sensitivity to inhibitory compounds could be useful in understanding the occasional occurrence of a defective product or could assist in the development of a selective medium for isolation or enumeration of propion-

ibacteria. Help in speciation of the genus could be provided and, in general, additional comprehension of this commercially important genus would be gained.

### MATERIALS AND METHODS

#### Bacterial strains

A total of 30 strains representing 11 different species of propionibacteria was included in this study. Sources of strains are listed in Table 1. Test organisms were maintained by weekly transfer in Sodium lactate broth (12). Species identities were confirmed by use of tests outlined in Bergey's Manual of Determinative Bacteriology (2).

Antibiotic and antimicrobial agent sensitivity assays were conducted using Sodium lactate agar (12). For comparative studies on the merits of Sodium lactate agar for determining antibiotic sensitivities, Mueller-Hinton agar (Baltimore Biological Laboratory, Cockeysville, Maryland) also was used.

#### Antibiotics and antimicrobial agents

Commercially available Multidisks, a multitipped sensitivity disc produced by Colab (Colab Laboratories, Inc., Glenwood, Illinois), were used (5). Most of the Multidisks used had nine projecting tips; a majority of the inhibitory agents were applied at two different concentrations.

#### Sensitivity test procedure

Because propionibacteria are relatively slow growing, rather anaerobic and possess specific nutritional requirements, the manufacturer's directions for use of the Multidisk could not be followed. Instead, a more suitable technique was developed: (a) Ten to 12 ml of sterile Sodium lactate agar (pH 7.0) were poured into a level petri dish, solidified, and dried overnight at 37 C. (b) Two-tenths milliliter of actively growing propionibacteria was mixed with 7 ml of sterile, 45 C Sodium lactate agar, and poured onto each dried agar plate. (c) Inoculated agar plates were incubated 18 h at 32 C in candle oats jars (12). (d) Discs impregnated with antibiotic and inhibitory agents were placed on this not yet visible but growing lawn of cells. (e) Plates were then returned to the candle oats jars for an additional 24 h. (f) Zones of growth inhibition were measured from the tip of the antibiotic-impregnated disc to the edge of visible bacterial growth.

Whenever *Escherichia coli* was used, incubation was at 37 C (air) for 16 h. The sensitivity assay procedure followed was as for propionibacteria, except that the antimicrobial agent discs were placed on the lawn of *E. coli* before preincubation.

#### Single agent studies

Sterile, blank, and highly absorbent paper discs (S and S No. 740-E, 12.7 mm diameter, Carl Schleicher and Schuell Company, Keene, New Hampshire) were used whenever studying single agents.

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TABLE 1. *Propionibacterium* CULTURES USED IN THIS INVESTIGATION

Culture number	Name of culture	Strain designation	Source <sup>a</sup>
1.	<i>P. shermanii</i>	59	A
2.	<i>P. shermanii</i>	E.11.1	B
3.	<i>P. shermanii</i>	6	C
4.	<i>P. shermanii</i>	PF33	D
5.	<i>P. freudenreichii</i>	F13	A
6.	<i>P. freudenreichii</i>	1291	E
7.	<i>P. freudenreichii</i>	F24	A
8.	<i>P. freudenreichii</i>	3 or 5	C
9.	<i>P. peterssonii</i>	1505	E
10.	<i>P. peterssonii</i>	81	D
11.	<i>P. peterssonii</i>	E.5.2 (Demeter str)	B
12.	<i>P. arabinosum</i>	78	D
13.	<i>P. arabinosum</i>	10	C
14.	<i>P. arabinosum</i>	129	D
15.	<i>P. zeae</i>	74	D
16.	<i>P. zeae</i>	86	D
17.	<i>P. pentosaceum</i>	P31C	A
18.	<i>P. pentosaceum</i>	128	D
19.	<i>P. pentosaceum</i>	E214	A
20.	<i>P. fensentii</i>	14	C
21.	<i>P. fensentii</i>	E.1.2 (ATCC 4868)	B
22.	<i>P. fensentii</i>	69	D
23.	<i>P. rubrum</i>	R6	A
24.	<i>P. rubrum</i>	R19	A
25.	<i>P. rubrum</i>	R9611	A
26.	<i>P. thoenii</i>	TH25	A
27.	<i>P. thoenii</i>	TH21	A
28.	<i>P. thoenii</i>	TH20	A
29.	<i>P. technicum</i>	—	D
30.	<i>P. raffinosaceum</i>	—	D

<sup>a</sup>Source: (A) Cornell University, Ithaca, N. Y.; (B) Dr. C. B. Van Niel, Hopkins Marine Station, Pacific Grove, California; (C) Dr. W. Kundrat, University of Munich, Munich, Germany; (D) Iowa State University, Ames, Iowa; (E) Dr. K. W. Sahli, Station Federale D'industrie Laitiere, Liebefeld-Bern, Switzerland.

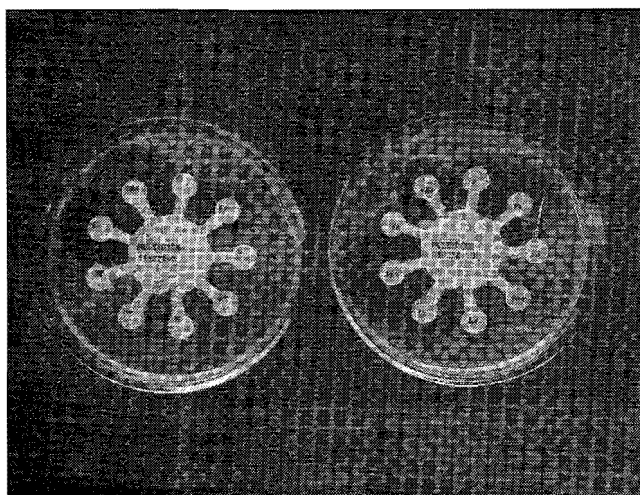


Figure 1. Illustration of inhibitory patterns of antimicrobial agents on lawns of *P. shermanii*.

#### Sulfadiazine standard solution

Aqueous sulfadiazine (Nutritional Biochemicals Corporation, Cleveland, Ohio) solutions were sterilized by Millipore filtration.

#### Electron microscopy

Sulfadiazine-grown (300  $\mu\text{g}/\text{ml}$ ) and sulfadiazine-free propionibacterial cultures were adjusted to pH 7, negatively stained with 2% phosphotungstic acid, and examined with an Hitachi HU-11C electron microscope.

## RESULTS

### Interpretation of test results

Growth reactions to antibiotics when used in two concentrations were reported as: *sensitive*—a zone around the disc tip with the lower concentration of antibiotic; *moderately sensitive*—a zone around the disc tip with the higher concentration only; and *resistant*—no zone around a disc tip with either concentration. Growth reactions obtained by using only a single concentration of antimicrobial agent were reported as: *sensitive*—a zone around the disc; or, *resistant*—no zone around the disc. Figure 1 shows the effect of various agents on a strain of *P. shermanii* and illustrates the differences between sensitive, moderately sensitive, and resistant. The overall results of the action of 30 antibiotics and one antimicrobial agent on 30 strains of 11 different *Propionibacterium* species are summarized in Table 2. Note that in Table 2 the abbreviation "mcg" is used as per the manufacturer's designation instead of the more conventional symbol " $\mu\text{g}$ ".

The results show that all species surveyed are resistant to cloxacillin, nafcillin, and oxacillin all at 1  $\mu\text{g}/\text{disc}$ , and nalidixic acid (30  $\mu\text{g}/\text{disc}$ ). Propionibacteria are moderately sensitive to kanamycin and colistin. Results of the sulfonamide sensitivity test, not presented in Table 2, show that all strains were resistant to both low (30  $\mu\text{g}/\text{disc}$ ) and high (300  $\mu\text{g}/\text{disc}$ ) concentrations of these sulfonamides: gantanol (sulfamethoxazole), madribon (sulfadimethoxine), sulfadiazine, sulfamerazine, elkosin (sulfisomidine), kynex (sulfamethoxypyridazine), sulfathiazole, and thiosulfil (sulfamethizole). The sulfonamide gantarisin (sulfisoxazole) moderately inhibited a few strains as shown in Table 2.

It might be argued that the demonstrated resistance of propionibacteria to sulfonamides could result from a protective effect of Sodium lactate agar. To test this possibility, the known sensitivity of *E. coli* to sulfonamides was used as a standard. *Escherichia coli* growth on Mueller-Hinton agar was determined using the same procedure as outlined for propionibacteria. The response of *E. coli* when grown on Sodium lactate agar also was determined. Propionibacteria were similarly treated. Results are in Fig. 2.



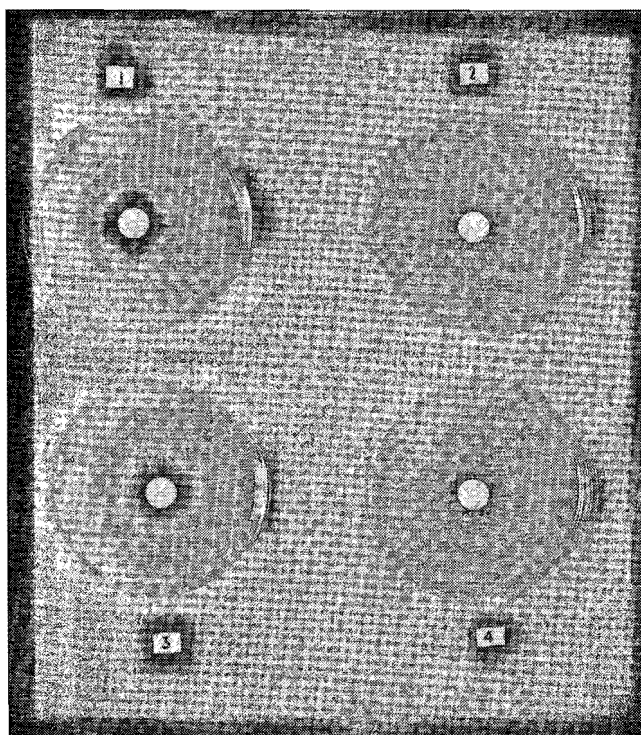


Figure 2. Sulfadiazine (300 µg/disc) inhibitory activity on *E. coli* and *P. shermanii* when grown on Mueller-Hinton agar and Sodium lactate agar. 1. *E. coli* on Sodium lactate agar showing a clear zone of inhibition. 2. *P. shermanii* on Sodium lactate agar. 3. *E. coli* on Mueller-Hinton agar showing a well-defined zone of inhibition. 4. *P. shermanii* on Mueller-Hinton agar exhibiting no zone of inhibition.

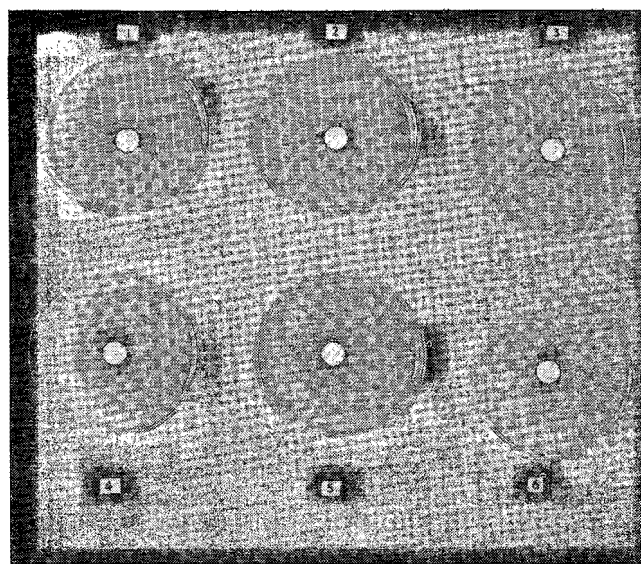


Figure 3. Sulfadiazine (300 µg/disc) inhibitory activity at different pH levels of Sodium lactate agar on *E. coli* and *P. shermanii*. 1. *E. coli* showing a small zone of inhibition at pH 5.5. 2. *E. coli* showing a moderate zone of inhibition at pH 7.0. 3. *E. coli* exhibiting a larger zone of inhibition at pH 8.0. 4, 5, 6. No inhibition of *P. shermanii* at pH 5.0, 7.0, and 8.0.

*Escherichia coli* was inhibited at pH 7.0 both on the Sodium lactate agar and on the Mueller-Hinton agar. Propionibacteria, on the other hand, were not inhibited on either medium. This suggests that Sodium lactate agar did not protect either microorganism against sulfonamides.

Because sulfonamides are more effective at alkaline pH values (8), the pH effect on the sensitivity of propionibacteria to sulfonamides was examined. Sodium lactate agar was adjusted to pH 5.5, 7.0, and 8.0. The testing procedure as previously described was followed. *Escherichia coli* was used as a control. Results are in Fig. 3. It is evident that pH 8.0 enhanced and pH 5.5 greatly reduced the inhibitory effect of sulfadiazine on *E. coli*. Inhibition of *Propionibacterium* did not occur even at pH 8.0. Of 11 different representative propionibacteria included in this experiment, only one strain (PF33) showed slight inhibition at pH 8.0; growth even without sulfadiazine, however, was scanty at this alkaline pH. Therefore, sulfonamide resistance of propionibacteria does not seem to be pH dependent.

To determine the effect of increased concentrations of sulfadiazine on *Propionibacterium*, 250, 500, and 1000 µg per disc were used. Here, also, *E. coli* was used as a control. Results are in Fig. 4. Greater inhibition of *E. coli* occurred as the concentration of sulfadiazine per disc was increased. Propionibacteria, however, were not inhibited even with 1000 µg of sulfadiazine. Obviously, propionibacteria are resistant to sulfonamides.

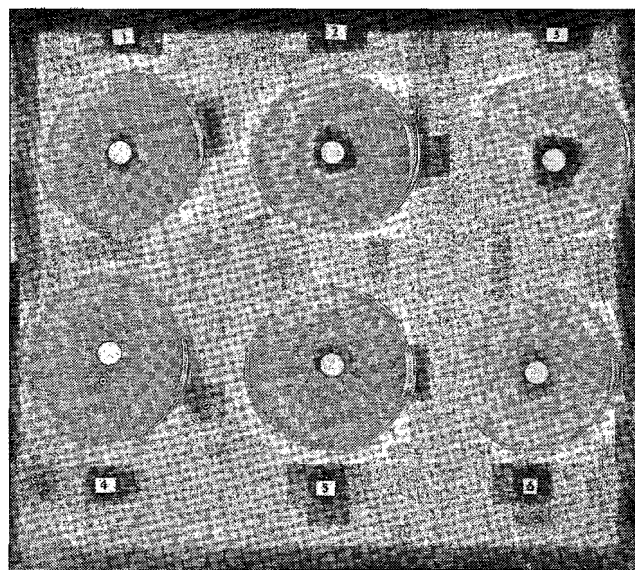


Figure 4. Reactions of *E. coli* and *P. shermanii* to increased concentrations of sulfadiazine (250, 500, and 1000 µg/disc). 1, 2, 3. Inhibition patterns of *E. coli* at 250, 500, and 1000 µg/disc of sulfadiazine correspondingly. 4, 5, 6. *P. shermanii* showing no zone of inhibition at 250, 500, and 1000 µg/disc of sulfadiazine correspondingly.

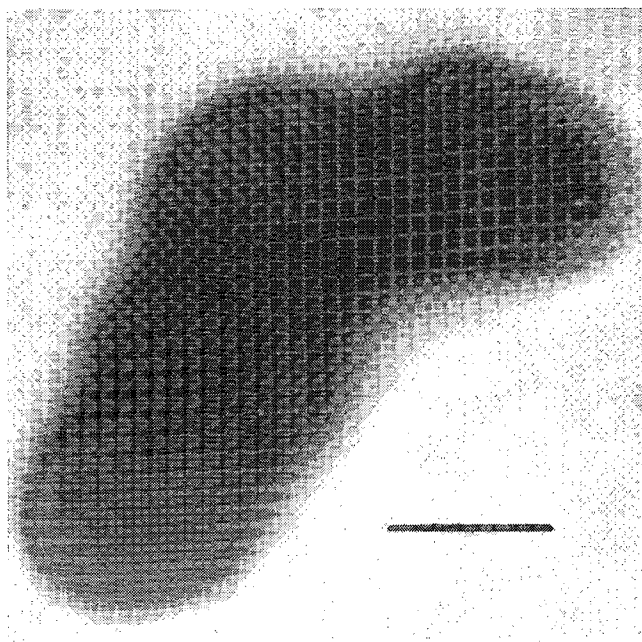


Figure 5. *Propionibacterium jensenii* grown in the presence of 300 µg/ml of sulfadiazine. Line scale equals 0.5 µm.

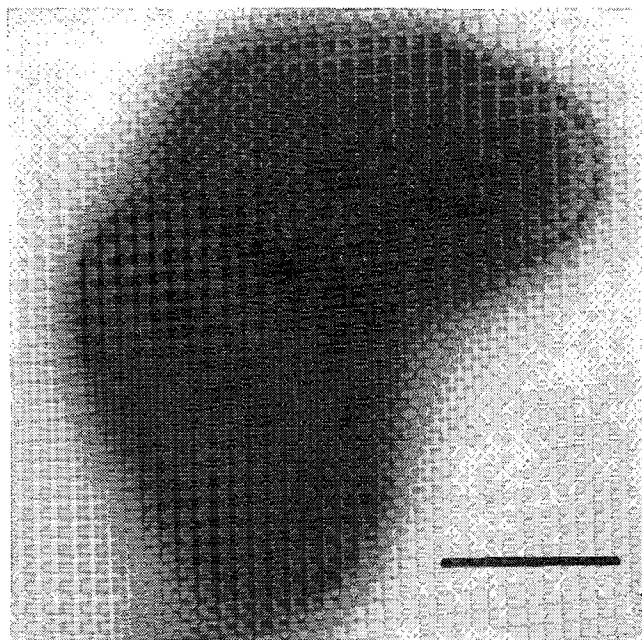


Figure 6. *Propionibacterium jensenii* grown in the absence of sulfadiazine. Line scale equals 0.5 µm.

Electron micrographs of both sulfadiazine-grown (300 µg/ml) and control *P. jensenii* are shown in Fig. 5 and 6. No significant changes in surface structure were observed in this strain of sulfadiazine-grown propionibacteria.

#### DISCUSSION

Sulfonamides as a group, with the exception of gantrisin, had no inhibitory effect on *Propionibacterium*. Generally, the sulfonamides exert a toxic ef-

fect on microorganisms by interfering with folic acid biosynthesis (1). Investigations are now in progress to determine the exact mechanism of sulfonamide resistance in *Propionibacterium*.

*Escherichia coli* grew better on Sodium lactate agar than on Mueller-Hinton agar. This could be part of the reason for the decreased zone of inhibition of *E. coli* on Sodium lactate agar than on Mueller-Hinton agar. In any event, Sodium lactate agar was found suitable for testing the sensitivity of *Propionibacterium*. The sulfonamide resistance of *Propionibacterium* does not seem to be pH dependent.

Klainer and Perkins (9) observed, on sulfamethoxazole-grown staphylococci and *E. coli*, surface changes similar to those induced by penicillin or cephalothin. They stated that kanamycin, chloramphenicol, and sulfamethoxazole, whose site of action is thought to be intracellular, may cause morphological alterations similar to those induced by cell-wall active drugs (penicillin, cephalothin). In our investigation, however, sulfadiazine (300 µg/ml) did not induce any major observable surface changes in *P. jensenii* (Fig. 5 and 6). This may further explain the intrinsic capacity of *Propionibacterium* to resist sulfonamides.

All *Propionibacterium* spp. investigated were resistant, at use level, to most of the penicillinase-resistant, semi-synthetic penicillin drugs, such as oxacillin, nafcillin, and cloxacillin with the exception of methicillin. The parent compound penicillin G, however, caused complete inhibition.

Propionibacteria were resistant to nalidixic acid (a naphthyridine derivative). They were only moderately sensitive to the polypeptide colistin and the streptomycin group antibiotic kanamycin. This was not unexpected since these three agents are mostly inhibitory to gram-negative bacteria (8).

Differentiation among species of the genus *Propionibacterium* could not be based on sensitivity to antibiotic or antimicrobial agents. Different species and strains, however, did exhibit various degrees of sensitivity to individual antibiotics. It would not be advisable to attempt to ascribe significance to differences in degree of sensitivity between antibiotics.

It should be noted that the inhibitory patterns of individual antibiotics on oxacillin-resistant *Propionibacterium* are in close agreement with sensitivity patterns of oxacillin-resistant staphylococci (4, 10). Both Bulger (4) and Klastersky (10) have reported on synergistic effects of combinations of other antibiotics on the oxacillin-resistant staphylococci. This was not within the scope of this study, but it is possible that the same responses may be shown by propionibacteria.

Until relatively recently, the sulfonamides were incorporated in many preparations for mastitis therapy. It has been shown that only one of the nine



used in this study would have had any bacteriostatic effect on propionibacteria. Contrary to this finding, of the 30 other antibiotic materials, at least 11 could conceivably be used in the treatment of mastitis or other bovine diseases. Only one of these compounds (cloxacillin at 1  $\mu\text{g}/\text{disc}$ ) did not demonstrate some degree of inhibitory activity against the selected propionibacterial strains. Obviously, retention of small amounts of some antibiotics in fermented milk products would seriously interfere with propionibacteria growth. On the other hand, inclusion of materials such as kanamycin, nafcillin, colistin, oxacillin, or nalidixic acid in a growth medium could well enhance the selective growth of propionibacteria.

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## F. J. BABEL RECEIVES TEACHING AWARD

The recipient of the Milk Industry Foundation Teaching Award in Dairy Manufacturing for 1973 has been described by former students as a meticulous organizer and planner resulting in maximizing of the learning experiences. Other students have described the success of this teacher as depending on a personal student-teacher relationship. In 1972 he was selected by the students in Food Science as the Outstanding Professor in the School of Agriculture, the first award so given. To have been chosen as the initial recipient for this campus award truly reflects the merit of his teaching abilities.

During the past 28 years, Dr. F. J. Babel has been faculty advisor for more than 350 undergraduate students and major professor for 18 M.S. and 14 Ph.D degree students. During the past 25 years he has taught two sequential courses in dairy bacteriology, both of which recently have been revised extensively to include training in the broader area of food microbiology. Seven years ago, a new course in food plant sanitation was organized and became a very popular elective. The present enrollment, ranging from 30 to 50 students for each course, speaks for the popularity of this professor.

Babel was born in Traverse City, Michigan. He received the B.S. degree in dairy manufacturing in 1935 from Michigan State University and the M.S. in 1936 from Purdue University. Continuing his graduate studies at Iowa State University, he received a Ph.D. in dairy bacteriology in 1939. After serving one year as research director for North American Creameries, Inc., he returned to Iowa State University for

6 years as a research associate professor. In 1947 he joined the faculty at Purdue as an associate professor and was promoted to professor in 1951.

Dr. Babel has been active in teaching-related activities, being deeply involved as advisor to the Dairy Science Club for several years. Since 1947 he has served as secretary and program chairman for the Indianapolis Dairy Technology Club. In 1960 he was selected as a member of the Dairy Advisory Committee of the Indiana State Board of Health and served as its chairman in 1967. He currently serves as a member of the Indiana State Mastitis Committee. Since 1962 he has participated in three International Dairy Congresses and visited universities and laboratories in Europe, Australia, and New Zealand. In 1967 Babel was invited to present lectures at the London Dairy Show and Dairy Research Institutes in the Netherlands and Belgium. He has presented numerous equally important lectures and seminars during the past 12 years. He also has been honored by election to several professional and honor societies.

This outstanding teacher spends approximately 40% of his time on independent research programs in applied dairy and food microbiology. He is the author or coauthor of 90 research papers, (some have appeared in the *Journal of Milk and Food Technology*), contributor to Volume 4 of *Advances in Applied Microbiology*, coauthor of a chapter in *Developments in Industrial Microbiology*, Volume 7, and coauthor of the textbook, *Dairy Bacteriology*, 4th Edition. He has prepared numerous review papers and trade journal articles.