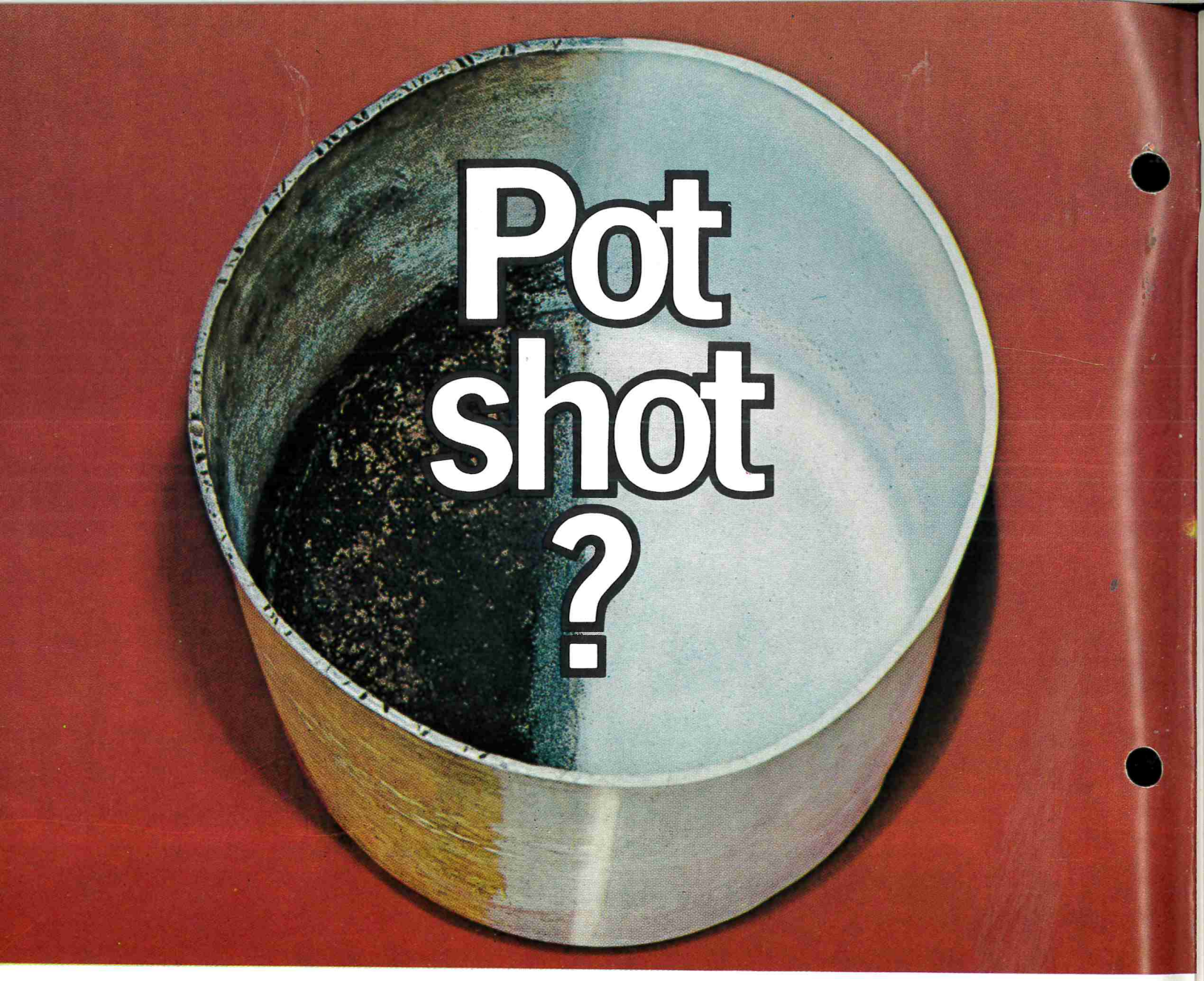
AUGUST, 1971
Vol. 34 P. 389-422 No. 8

Journal of

MILK and FOOD TECHNOLOGY

Official Publication

International Association of Milk, Food and Environmental Sanitarians, Inc.



A food processor we know had just about given up on his aluminum bean pots. Accumulated food stains were uncleanable. Or so he thought until a Pennwalt representative showed him what Pennwalt pot cleaners could do. A quick soak and rinse did the job.

Do you have a cleaning problem with plant utensils and equipment? Chances are Pennwalt has a cleaning process to solve your problem immediately. And if we don't, your Pennwalt representative will put our chemical research staff to work until we do have the solution. And no halfway measures about it.

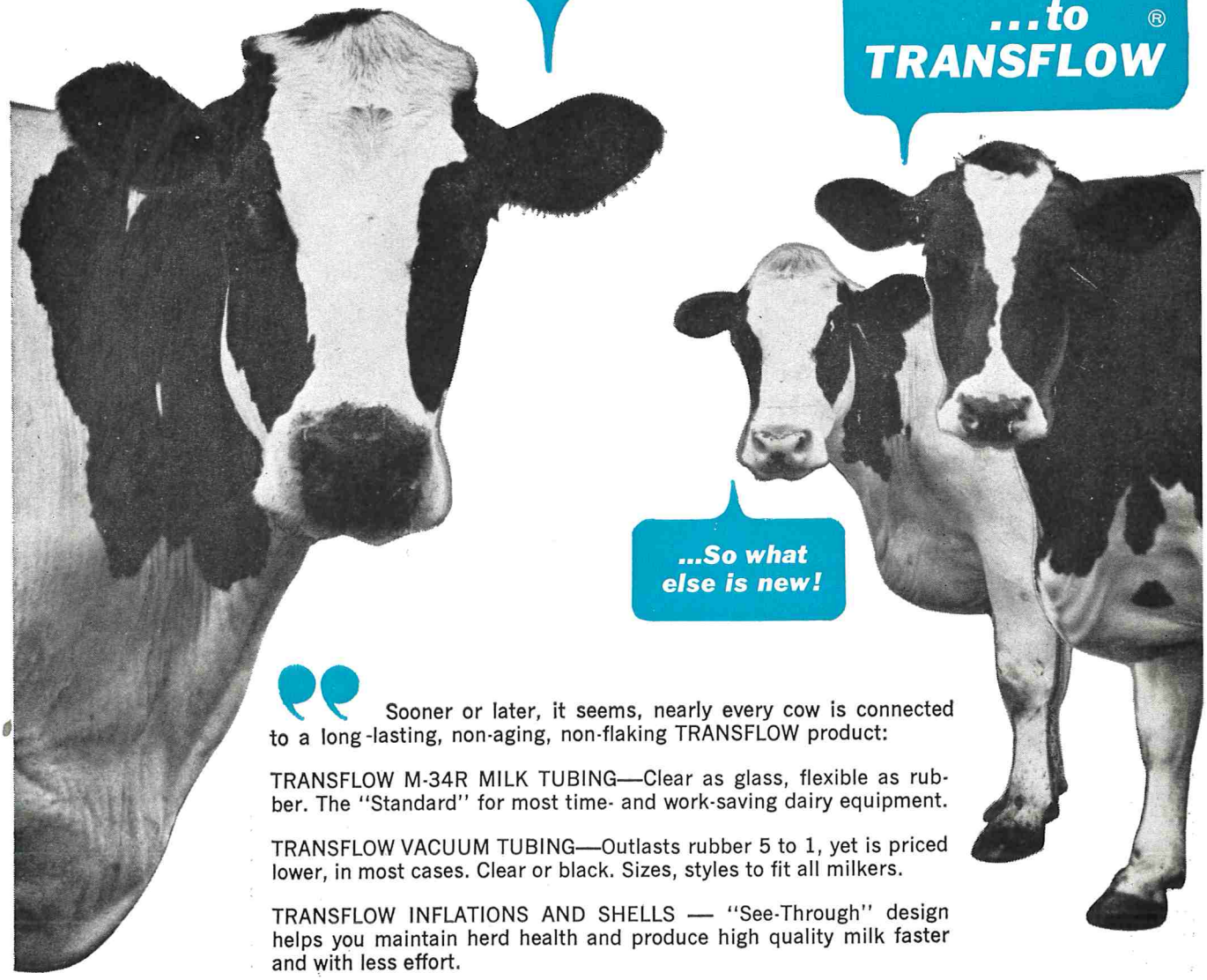
Contact us now. You have nothing to lose but your stains.

Dairy and Food Dept., Pennwalt Corporation
Three Parkway, Philadelphia, Pa. 19102

PENNWALT
CLEANERS & SANITIZERS

Guess what! Daisy's getting hitched!

...to ® TRANSFLOW



...So what else is new!

Sooner or later, it seems, nearly every cow is connected to a long-lasting, non-aging, non-flaking TRANSFLOW product:

TRANSFLOW M-34R MILK TUBING—Clear as glass, flexible as rubber. The "Standard" for most time- and work-saving dairy equipment.

TRANSFLOW VACUUM TUBING—Outlasts rubber 5 to 1, yet is priced lower, in most cases. Clear or black. Sizes, styles to fit all milkers.

TRANSFLOW INFLATIONS AND SHELLS — "See-Through" design helps you maintain herd health and produce high quality milk faster and with less effort.

Of course, TRANSFLOW products meet all Food and Drug Administration requirements as well as all criteria in the 3-A* Plastics Standard.

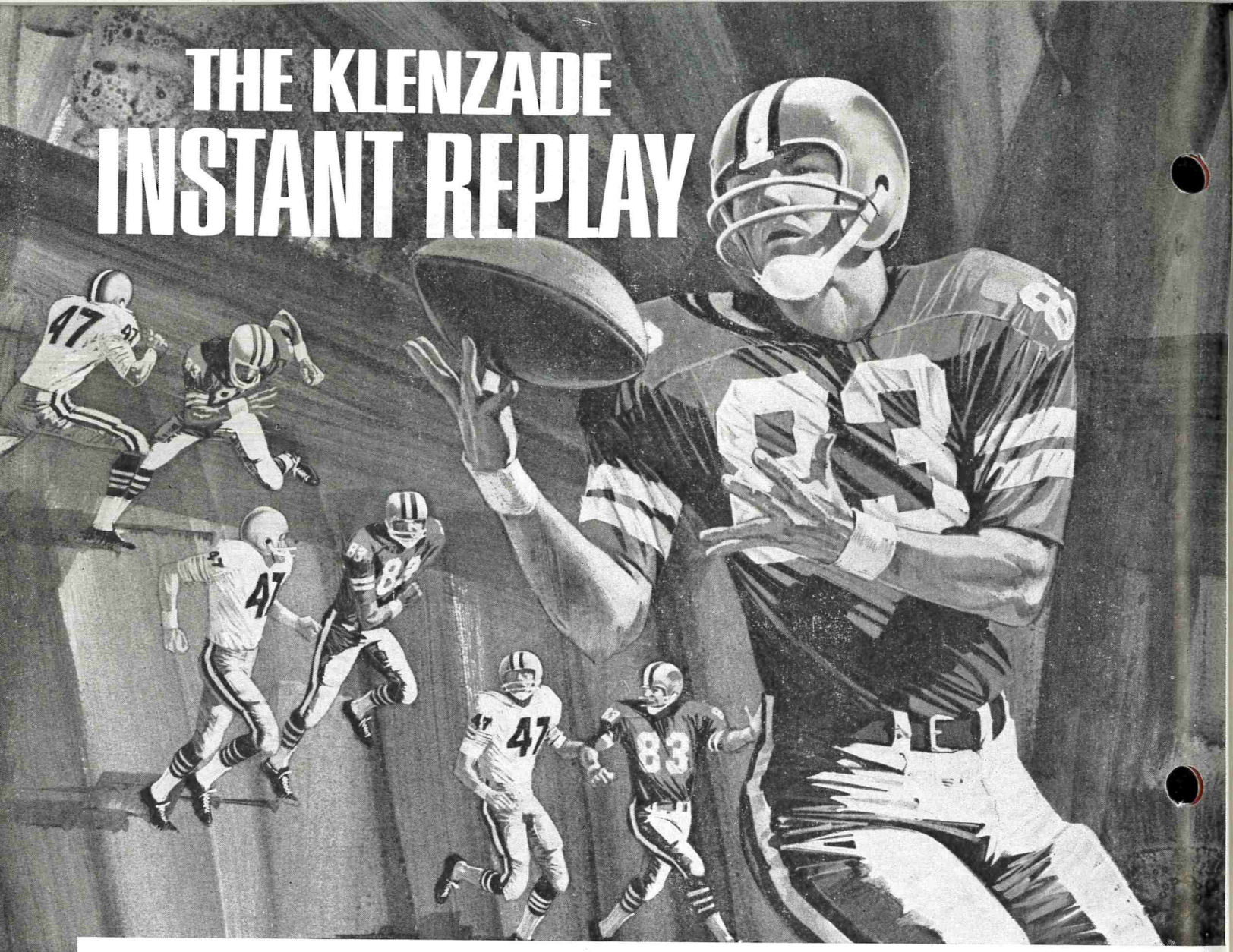
For complete information about any TRANSFLOW product, see your dealer or write Norton Plastics and Synthetics Division, P. O. Box 350, Akron, Ohio 44309.

*International Association of Milk, Food and Environmental Sanitarians; U. S. Public Health Service; The Dairy Industry Committee.

NORTON PLASTICS AND SYNTHETICS DIVISION
FORMERLY U.S. STONEWARE INC. AKRON, OHIO 44309

32-292

THE KLENZADE INSTANT REPLAY

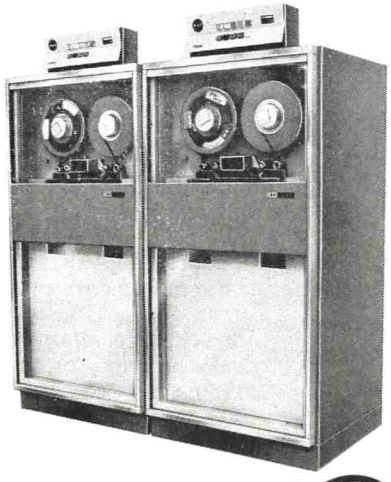


New computer service evaluates cleaning procedures, specifies chemical concentrations, pinpoints all costs.

Klenzade's Computer Cost Survey Analysis instantly evaluates every dairy cleaning job, from pasteurizer circuits to conveyor chains to transport tankers.

Plant sanitation information is transferred to punch cards and fed into Klenzade's computer. All variables involving detergent concentrations, water hardness, iron content, water volume and soil conditions are computed according to programmed standards of sanitation effectiveness. The result is a computerized printout of cleaning efficiency based on existing cleaning procedures. It's a printout which tells you what the precise cost of every cleaning variable should be for each cleaning function.

The Computer Cost Survey Analysis is a first in dairy sanitation. So, ask your Klenzade Representative for the full story. He'll be glad to show you how the Clean Team's memory bank can analyze your plant sanitation procedures play by play. Give him the ball soon.



Join the Clean Team KLENZADE

KLENZADE PRODUCTS DIV., ECONOMICS LABORATORY, INC., Dept. 224 , Osborn Bldg., St. Paul, Minn. 55102

The Journal of Milk and Food Technology is issued monthly beginning with the January number. Each volume comprises 12 numbers. Published by the International Association of Milk, Food and Environmental Sanitarians, Inc. with executive offices of the Association, Blue Ridge Rd., P. O. Box 437, Shelbyville, Ind.

2nd Class postage paid at Shelbyville, Indiana 46176.

EDITORIAL OFFICES: Dr. Elmer H. Marth, Dept. of Food Science, University of Wisconsin, Madison, Wis. 53706. H. L. Thomasson, Managing Editor, P. O. Box 437, Shelbyville, Indiana 46176.

Manuscripts: Correspondence regarding manuscripts and other reading material should be addressed to Dr. Elmer H. Marth, Dept. of Food Science, University of Wisconsin, Madison, Wis. 53706.

"Instruction to Contributors" can be obtained from the editor for the use of contributors of papers.

Page Charge: Effective January 1, 1969 a charge of $25.00 per printed page will be made for all research papers which are published See Volume 31, issues 10, 11, or 12 for details.

Business Matters: Correspondence regarding business matters, advertising, subscriptions, orders for single copies, etc., should be addressed to H. L. Thomasson (address above).

Subscription Rates: One volume per year. Individual non-members. Governmental and Commercial Organization subscription.

1 yr. $12.00
Public and Education Institution Libraries, 1 yr. $10.00
Single Copies .....$ 1.00

Orders for Reprints: All orders for reprints should be sent to the executive office of the Association, P. O. Box 437, Shelbyville, Ind.

Membership Dues: Membership in the International Association of Milk, Food and Environmental Sanitarians, Inc., is $10.00 per year, and Special Certified Student membership is $4.00 per year, which includes annual subscription to the **Journal of Milk and Food Technology.** All Correspondence, regarding membership, remittances for dues, failure to receive copies of the **Journal,** changes in address and other such matters should be addressed to the Executive Secretary of the Association, H. L. Thomasson, Box 437, Shelbyville, Indiana 46176.

Journal of

MILK and FOOD TECHNOLOGY

INCLUDING MILK AND FOOD SANITATION

Official Publication

International Association of Milk, Food and Environmental Sanitarians, Inc.

Reg. U. S. Pat. Off.

Vol. 34 August, 1971 No. 8

ISOLATION OF SALMONELLAE FROM NATURALLY CONTAMINATED DRIED MILK PRODUCTS

I. INFLUENCE OF SAMPLING PROCEDURE ON THE ISOLATION OF SALMONELLAE[1]

BIBEK RAY[2], J. J. JEZESKI[3], AND F. F. BUSTA

Department of Food Science and Industries
University of Minnesota
St. Paul 55101

(Received for publication March 29, 1971)

ABSTRACT

Eight plants that were equipped with several types of driers and that produced a variety of dried milk products were surveyed for *Salmonella* contamination. Each plant had a history of *Salmonella* isolations from finished product. Sampling included production fractions as well as tailings and environmentals. Distribution of salmonellae was not random in fractions of product obtained during a day's operation. One-fourth of the 277 samples of the first product through the system (animal feed) contained salmonellae; this was a higher frequency than the 5.6% positive tests observed in 963 samples of other fractions produced later during daily operation. The incidence of *Salmonella* isolations in these food-grade products also was lower than that observed in 873 tailings samples (10.5%), in 1125 air filter samples (23.6%), and in 763 environmental samples (21.1%). However, plants with *Salmonella*-positive air filters or environmentals were able, on occasion, to manufacture product free of salmonellae. Results of this study indicated that the first product out of the system during daily operation provides the best sample for evaluation of product contamination by salmonellae.

The sampling procedure may be an influential factor in obtaining information about the probable *Salmonella* contamination of dried milk products. In designing an effective procedure, factors such as type of product, size of the production lot, distribution of contaminant, and level of contamination within the lot should be considered *(3)*. *Salmonella* distribution in contaminated egg product was considered to be homogeneous. However, salmonellae in both fish meal and bone meal were reported to be distributed very unevenly *(6)*. In most non-liquid food products *Salmonella* contamination is not homogeneous and the level of contamination is usually low. Thus, a limited number of tests from a large production lot provide little assurance that the lot is not contaminated *(7)*. In the majority of instances, the number of samples is less than the actual number required to verify absence of salmonellae. For dried milk products, the testing of a statistically selected number of samples representing about 10% of the units of a lot was recommended for detection of *Salmonella* contamination *(3)*. If the distribution of contamination of salmonellae is random, i. e., if there is an equal chance for contamination to occur at any stage of the operation, a regular sampling procedure will detect the organisms. However, if contamination is non-random and limited to a certain segment of time during the production of a lot, there is no assurance of obtaining a contaminated sample unless the influence of time is known *(2)*.

In this work, samples from various dry milk plants were tested to determine influence of time on contamination of the product, to establish the possible route of product contamination, and to determine which of the samples provided the best material to evaluate for possible presence of salmonellae in the product as well as in the plant environment.

MATERIALS AND METHODS

Samples

Different types of samples were obtained from dry milk plants with a history of product contamination with salmonellae. Samples consisting of first product out of the system (animal feed), portions from the first, middle, and last bag of food-grade products and tailings from the daily line run were obtained routinely in quantities of at least 100 g. Samples of air filter materials from the drier intake and powder cooler also were obtained regularly. Environmental samples consisting of dry milk residues and swabs were received on selected occasions. The samples were obtained in polyethylene bags. The product, tailings, and filter materials were obtained within 2 to 7 days after manufacture, whereas the environmental samples were received within 24 hr after collection. In most instances, the samples were pre-enriched within 24 hr after they were received in the laboratory. During the interval between receiving and testing, the former groups of samples were stored at room temperature, whereas the environmental samples were stored at 4 C.

Methods of testing

Different fractions of product samples, including animal feed, were pre-enriched in one or more media including

---

[1]Scientific Journal Series Paper No. 7559, Minnesota Agricultural Experiment Station, St. Paul.

[2]Present address: Department of Food Science, North Carolina State University, Raleigh, N. C. 27607.

[3]Present address: Department of Botany and Microbiology, Montana State University, Bozeman, Montana 59715.

TABLE 1. VARIOUS *Salmonella* SEROTYPES ISOLATED FROM DRIED MILK PRODUCTS PRODUCED IN DIFFERENT PLANTS.

Plant No.	Types of products	Types of driers	Serotypes isolated
1	Spray dried skim	Box sweep	*S. typhimurium, S. java and S. blockley,* and non-motile *S. java*
2	Spray dried skim	Box sweep	*S. anatum*
3	Spray dried skim	Box sweep	*S. montevideo*
7	Spray dried skim, instant	Box sweep 1 Vertical 1	*S. oranienberg*
8	Spray dried skim, whole-milk buttermilk, mix.	Box sweep 1 Box manual 1 Horizontal 1	*S. minnesota, S. kentucky and S. tennessee*
9	Spray dried skim	Horizontal 2	*S. anatum, S. derby, S. falkensee, S. kentucky, S. seigberg, S. taksony* and *S. uzaramo*
10	Spray dried skim	Box sweep 1	*S. cubana* and *S. newington*
11	Various products		*S. anatum* (H_2S negative)

0.002% brilliant green water and lactose broth. The other pre-enrichment media used were peptone solution (0.3%), sodium pyruvate solution (0.1%), sterile deionized water, or Ray's medium composed of tryptone, 3.0 g; Soytone (Difco), 3.0 g; yeast extract (Difco), 3.0 g; casamine acids (Difco), 1.0 g; sodium chloride, 1.0 g; dipotassium phosphate, 2.0 g; magnesium sulfate, 0.5 g; and water, 1 liter. Pre-enrichment included incubation of a 100 g sample in 1 liter of medium *(9)*. Tailings in 100 g amounts were pre-enriched in 1 liter of lactose broth and the filter materials were pre-enriched in sterile water (500 ml to 1 liter). The environmental samples also, depending upon the quantities, were pre-enriched in 100 ml to 1 liter lactose broth (to give a final ratio of 1:10, sample to broth). Samples were incubated at 35 C for 24 hr during pre-enrichment, and then 10 ml amounts were enriched in 100 ml tetrathionate broth (with 0.001% brilliant green), plated (by streaking) on Xylose Lysine Deoxycholate Agar plates and then biochemically and serologically tested according to the recommended procedure *(4)*.

For biochemical tests, reactions on triple sugar iron agar slant, lysine iron agar slant, dulcitol broth, motility medium, and urea broth were observed. For serological confirmation a somatic antigen was determined with specific 'O' group *Salmonella* antisera and a flagellar antigen was determined by the Spicer-Edwards simplified method *(1)*. All media, chemicals, and sera used were obtained from Difco laboratories.

RESULTS

Distribution of Salmonella *serotypes in contaminated dry milk plants*

A number of different *Salmonella* serotypes were isolated from products and environmental samples obtained from eight plants (Table 1). All the plants except plant 11 were producing spray dried products and most plants used box-type or horizontal-type driers. Both single and multiple serotype contaminations were observed in these plants. From samples in plant 9, as many as seven serotypes were isolated. *Salmonella anatum* isolated from plant 11 did not produce hydrogen sulfide (H_2S-negative). Also non-motile *Salmonella java* was isolated from plant 1.

Incidence of Salmonella *contamination in samples from dry milk plants*

Different product fractions, tailings, filter materials, and environmental samples from eight milk drying plants were tested to determine the presence of salmonellae. About 10% of the products including tailings samples and about 22% of the environmental samples including filter materials contained salmonellae (Table 2). In all eight plants, environmental samples showed an incidence of *Salmonella* contamination (range 11.8 to 76.7%) higher than the products samples (range 0.6 to 27.9%). The products samples from plant 9 had a relatively lower percentage of contamination in comparison to the environmental samples (0.6 vs 35.3%).

Data on the frequencies of *Salmonella* isolation from the first product fraction (animal feed), from the first, middle, and last bags of food grade product, from tailings, from filter materials (including air intake and powder coolers), and from environmental samples obtained at six different plants are presented in Table 3. A total of 277 samples of animal feed, and 356, 202, and 405 samples from the first, middle, and last bags, respectively, were tested. The highest incidence of *Salmonella* isolation was obtained from the animal feed fraction. About 25% of the samples tested from this first product fraction were found to contain salmonellae. In comparison, salmonellae could be isolated from only 5.6% of the total food-grade products with highest frequency of isolation observed in the first-bag product and the lowest in the middle-bag product. Relatively high percentages of tailings (10.5%), filter materials (23.6%), and environmental samples (21.1%) were positive for salmonellae. This high incidence of salmonellae in the animal feed fraction compared to the low incidence in the different fractions of food grade products was observed in samples from plants 1, 2, 3, and 7. These four plants also had a high incidence of *Salmonella* contamination in the

TABLE 2. FREQUENCY OF *Salmonella* ISOLATION IN DIFFERENT SAMPLES FROM EIGHT DRY MILK PLANTS[1]

Plant No.	Per cent positive: Products including tailing samples	Environmental samples including filter material
1	18.4	39.6
2	14.0	24.3
3	27.9	76.7
7	7.9	15.3
8	4.4	11.8
9	0.6	35.3
10	10.0	32.8
11	11.1	24.4
Avg.	10.2	21.9

[1]A total of 2342 products including tailings samples and 2134 environmental samples including filter materials were tested and salmonellae were isolated from 240 (10.2%) samples in the former group and from 468 (21.9%) samples in the latter group.

tailings, filter materials, and environmental samples.

In plants 8 and 9, a low incidence or absence of product contamination was observed, although a large number of filter materials and environmental samples contained salmonellae. In plant 8, salmonellae were isolated from 33 of 544 tailings samples.

The relative frequencies of *Salmonella* isolations from various samples from three different driers in plant 8 are presented in Table 4. In all three driers, the incidence of *Salmonella* contamination in the products samples, including the animal feed, was extremely low or negligible. In contrast, salmonellae were isolated from 18 of 202 and 14 of 159 tailings from driers 2 and 3, respectively. Also, a large number of air intake filters from all three driers and the powder cooler filters from drier 3 tested positive for salmonellae.

Environmental samples consisting of dried materials and swabs from the milk drying equipment, dust, and other materials from the roof, various areas inside the plant, vacuum cleaner waste, spilled dry milk, etc. were tested for the presence of salmonellae. Results of such tests from two plants are presented in Table 5. About 27% of the samples from plant 1 and about 46% from plant 9 contained salmonellae. In both plants, the frequency of isolation obtained in the samples from the equipment surface was lower than that observed from the plant surroundings. The incidence of salmonellae was much higher in the samples from the equipment surface from plant 1 (20.3%) than from plant 9 (4.5%). However, the relative number of samples from the two sources in the two plants was different. Note that salmonellae were isolated from 15.3% of the products samples from plant 1, whereas none of the 136 products samples from plant 9 tested positive (Table 3).

DISCUSSION

Samples obtained from dry milk plants contaminated with salmonellae may contain various *Salmonella* serotypes in product or environmental samples, and a single sample may be contaminated with more than one serotype. Some serotypes seemed to occur more frequently. In the present study, *S. anatum* was isolated from three of the eight plants. *Salmonella anatum* was one of the five most common serotypes isolated from contaminated dried milk products in the United States in 1967 *(11)*.

Non-motile *S. java* and H_2S-negative *S. anatum* were isolated from the samples obtained from two plants. Biochemical variants of lactose fermenting *Salmonella newington* have been isolated from nonfat dry milk *(8)*. Lactose fermenting *Salmonella cubana* also was isolated from soybean powder in this laboratory. Thus, in isolating salmonellae from contaminated dried milk products, the presence of possible biochemical variants should be anticipated and tested for on a regular basis.

The incidence of *Salmonella* contamination in dried milk products, even from plants with known *Salmonella* problems was generally low. About 10%

TABLE 3. DISTRIBUTION OF *Solmonella* CONTAMINATION IN VARIOUS SAMPLES FROM SIX PLANTS IN WHICH CONTAMINATION WAS DETECTED.

Type of samples	Number of samples positive for salmonellae/Number of samples tested: Plant 1	Plant 2	Plant 3	Plant 7	Plant 8	Plant 9	Total	%
First products (animal feed)	32/96	10/34	15/25	12/56	0/7	0/59	69/277	24.9
Food grade products								
First bag	14/99	9/77	7/25	1/37	1/97	0/21	32/356	9.0
Middle bag	4/94	1/14	0/19	0/18	0/38	0/19	5/202	2.5
Last bag	9/97	1/45	2/24	0/16	5/185	0/38	17/405	4.1
Total	27/290	11/136	9/68	1/71	6/320	0/78	54/963	5.6
Tailings	30/96	13/72	9/25	6/111	33/544	1/25	92/873	10.5
Filters[1]	83/182	22/65	21/22	10/74	92/649	38/133	266/1125	23.6
Environmentals	23/86	6/50	22/34	60/382	12/129	38/82	161/763	21.1

[1]Filters include both air intake and powder coolers.

TABLE 4. DISTRIBUTION OF *Salmonella* CONTAMINATION IN SAMPLES FROM THREE DRIERS[1] IN PLANT 8

Type of samples	Number positive/Number tested Drier 1	Drier 2	Drier 3	Total
First product (animal feed)	0/0	0/3	0/4	0/7
Food grade products				
First bag	0/25	1/30	0/42	1/97
Middle bag	0/7	0/18	0/13	0/38
Last bag	0/41	4/78	1/66	5/185
Tailings	1/183	18/202	14/159	33/544
Filters				
Drier intake	19/181	15/175	39/171	73/527
Powder cooler	0/1	0/3	19/118	19/122
Environmentals				12/129

[1]Drier 1: Box type, manual removal of product.
Drier 2: Box type, sweep removal of product.
Drier 3: Horizontal type.

TABLE 5. DISTRIBUTION OF CONTAMINATION OF SALMONELLAE IN THE ENVIRONMENTAL SAMPLES OBTAINED FROM DRYING EQUIPMENTS AND FROM PLANTS SURROUNDINGS IN 2 PLANTS[1].

Sources	PLANT 1 Number of samples positive/ Number tested	%	PLANT 9 Number of samples positive/ Number tested	%
Equipment	14/64	20.3	1/22	4.5
Surrounding	9/22	40.1	37/60	63.1
Total	23/86	26.8	38/82	46.3

[1]About 15.3% of the 386 product samples from plant 1 were positive for salmonellae. No salmonellae were isolated from 137 products samples from plant 9.

of the total products and tailings samples from eight plants were positive for salmonellae. A much higher incidence of contamination (about 22%) occurred in the filter materials and environmental samples. These results were much higher than USDA test results in 1968 where only 0.22% of the products (excluding tailings) and about 4.8% of the environmental samples (including tailings) from 210 plants tested positive for salmonellae *(5)*. The high percentage of isolation in this present study could result from testing samples from only the plants known to produce products containing salmonellae, and/or could be a result of testing more than one 100 g portion of sample from many sources because several preenrichment media were used.

Maximum frequency of *Salmonella* contamination among different product fractions from the daily line runs occurred in the first product (animal feed). The frequency of *Salmonella* contamination appeared to be a function of time of operation during the production run; and thus, the distribution of contamination was not random in products produced throughout the day's operation. Statistical sampling procedures, which depend on randomness of distribution, therefore probably are of minimal usefulness. Also, continuous or batch sampling for a composite sample of a day's operation will result in lower recoveries because of dilution of contaminated products with the uncontaminated portions. The testing of a statistically selected number of samples representing about 10% of the units of a lot according to recommended procedure *(3)* thus might not always detect salmonellae from contaminated products. The first product produced (animal feed) provided the best sample for detection of salmonellae, and also offered a better indication about the possible subsequent product contamination. Samples from the first bag of food grade product could also be used along with the animal feed to substantiate potential product contamination because of their close proximity in the time sequence of production (consecutive bags).

In the event of non-random distribution where contamination is limited to a certain segment of the total processing time, sampling should be done during the segment of time of highest possible contamination *(2)*.

Tailings also showed a relatively high incidence of contamination with salmonellae. These were the coarse aggregates of powder that were removed from the product at the sifter. Often tailings containers were not cleaned and sanitized every day or even at times when the powder conveying equipment was cleaned. Also, sweepings and vacuum-cleaner residues were often discarded into the tailings container. Thus, contaminated tailings samples did not necessarily indicate product contamination. In plant 8, from driers 2 and 3, many tailings samples were positive but product contamination was very low (Table 4). Also, in many instances, the presence of salmonellae could not be demonstrated in products samples from the same day's operation where salmonellae were found in tailings. However, tailings may be tested to provide a reliable indication of possible product contamination and the presence of salmonellae in the plant environment.

Air filter materials testing provided an excellent measure of the environment contamination in a dry milk plant. However, contaminated air filters were not necessarily consistently associated with contaminated products. Even when a large number of filter materials tested positive, only a very few products samples contained salmonellae. In a plant where the filter materials were consistently negative, contaminated products were very seldom found. Positive filters were, however, a danger signal that salmonellae were present in the plant surroundings. These results also suggested that air was not the primary source of product contamination.

Environmental samples from different areas of the plant and of the equipment indicated the sani-

tary condition of the plant and sometimes located the focus of contamination. In general, environmental samples showed a high incidence of contamination by salmonellae. Salmonellae often were isolated from environmental samples even when product samples consistently showed the absence of salmonellae. Apparently *Salmonella* problems existed in drying plants only if there was a gross unsanitary condition somewhere in the plant which later could serve as a reservoir to introduce the organism into the drying equipment. A similar hypothesis on the mode of contamination has been suggested previously *(10)*. Once introduced, numerous locations were available where salmonellae could multiply and remain to seed the products over a period of time *(10)*. Our studies in plants producing contaminated products suggested that contamination by salmonellae was associated with moisture accumulation in the equipment and subsequent powder deposit. Moist powder probably provided a substantial opportunity for growth of organisms. Coarse crusty deposits eventually ended up, at least in part, in the tailings; and the shedding of these deposits takes place during the early part of the drying operation. Such a set of circumstances could explain the high incidence of contamination in the first product (animal feed) and the tailings with a gradual reduction in incidence as the operation continued.

For successful implementation of *Salmonella* control programs in dry milk plants, proper sanitation is the single most important factor. Occasional testing of air filter materials, environmental samples, animal feed (first product), and tailings would be useful to detect product contamination potentials. To determine possible product contamination, animal feed (first product) and the first bag from the human food-grade product should be tested.

ACKNOWLEDGMENT

This investigation was supported in part by Food and Drug Administration Contract 67-61 and Contract 68-46 and by a grant from the American Dry Milk Institute. We thank the Consumer and Marketing Service, USDA, for their cooperation in obtaining samples, the Minnesota Department of Health for assistance in serologically typing and identifying isolates, and the local food industries laboratories who assisted in supplying certain samples. We also thank Miss Gretchen Saecker for technical assistance.

We are grateful to a group of people associated with the plants involved, who must remain anonymous; but without whose cooperation and active assistance, these data could not have been collected.

REFERENCES

1. Anon., Difco Technical Information. 1968. Serological identification of the *Salmonella*. p. 2-11.

2. Committee on Salmonella, National Research Council. 1969. An evaluation of the *Salmonella* problem. National Academy of Sciences, Washington, D. C. p. 149-156.

3. Ewing, W. H., J. H. Boring, and M. Galton. 1966. Procedure for examination of dried and liquid milk products for salmonellae. Salmonella Surveillance No. 49. C.D.C., Atlanta, Georgia. p. 6-7.

4. Galton, M. M., G. K. Morris, and W. T. Martin. 1968. Salmonellae in foods and feeds. Review of isolation methods and recommended procedures. U. S. Department of Health, Education and Welfare. Public Health Service, C.D.C., Atlanta, Georgia.

5. Garbe, E. F. 1968. Salmonella surveillance program of dry milk plants. Salmonella Surveillance No. 80. C.D.C., Atlanta, Georgia. p. 2.

6. Jacobs, J., P. A. M. Guinee, E. H. Kampelmacher, and A. van Keulen. 1963. Studies on the incidence of *Salmonella* in imported fish meal. Zentralblatt für Veterinärmedizin 10B: 542-550.

7. Lennington, K. R. 1968. The *Salmonella* problem from an enforcement standpoint. Salmonella Surveillance No. 72. C.D.C., Atlanta, Georgia. p. 2-4.

8. Pickett, G., and G. H. Agate. 1967. Outbreak of salmonellosis due to lactose fermentating variant of *Salmonella newington*. Morbidity and Mortality 16:18.

9. Ray, B., J. J. Jezeski, and F. F. Busta. Isolation of salmonellae from naturally contaminated dried milk products. III. Influence of pre-enrichment conditions on isolation of salmonellae. J. Milk Food Technol. (To be submitted).

10. Salmonella Surveillance Unit. 1966. Progress report-Interstate outbreak of salmonellosis related to nonfat dry milk. Salmonella Surveillance No. 49. C.D.C., Atlanta, Georgia. p. 2-6.

11. Salmonella Surveillance Unit. 1968. Annual Summary 1967. C.D.C., Atlanta, Georgia. p. 6.

GUIDELINES FOR PRODUCTION OF FOOD ADDITIVES AND OTHER CHEMICAL COMMODITIES WITH LEAST CARCINOGENIC POTENTIAL: A BIOCHEMICAL APPROACH[1]

GERALD M. LOWER, JR.[2] AND GEORGE T. BRYAN[3]

The Division of Clinical Oncology
University of Wisconsin Medical School
Madison, Wisconsin 53706

(Received for publication February 15, 1971)

ABSTRACT

An attempt has been made, using a biochemical approach, to construct safety guidelines for synthesis and production of food additives and other chemical commodities with "least carcinogenic potential." In general, chemical carcinogens exist as or are metabolically activated to reactive forms able to interact with biologically-important macromolecules, a process apparently directly related to carcinogenesis by chemicals. A working knowledge of the biochemistry of biologically-foreign compounds is therefore necessary to understand structure-activity relationships observed in chemical carcinogenesis, since it appears that carcinogenic activity can be a consequence of the structure of the chemically-reactive forms of a carcinogen rather than of the structure of the parent compound. Consequently, the biochemistry of foreign compounds, the metabolic activation of various chemical carcinogens, and the nature of the probable active forms of these carcinogens has been briefly reviewed. Utilization of this knowledge by investigators of chemical carcinogenesis has proved rather helpful in the discovery of new chemical carcinogens. By eliminating from consideration those chemicals with carcinogenic potential, there is no reason why this knowledge cannot be successfully utilized as an aid in the discovery of non-carcinogenic chemicals suitable for use as food additives or other chemical commodities.

In recent years, much attention, most of it necessary and justifiable, has been focused on the possible carcinogenic hazards associated with the common use of certain food additives and other chemical commodities. The heated controversy that invariably accompanies and confuses scientific debate over the safety of chemical commodities has had its origins, in part, in conflicting values and objectives and in disparate understandings of the real and potential hazards often associated with use of biologically foreign compounds. The existence of differing viewpoints over the potential carcinogenic hazard of chemicals is understandable since it has been only slightly more than 30 years since a pure chemical, epidemiologically implicated in the human cancer problem, was proven able to induce a similar cancer in experimental animals. Indeed, the concept that chemicals can play an important role in the causation of human cancer has become so very apparent only with the impressive progress made in the study of chemical carcinogenesis in the last decade.

The potential carcinogenic hazard accompanying the use of chemical food additives *(12, 47)*, pesticides *(95)*, and drugs *(94)* has been adequately described as has the importance of safety testing of these chemicals prior to their introduction into the human environment *(11, 101)*. However, this information does little to solve the problems of industry in finding economical means to meet the present minimal safety standards. It should be noted here that a portion of industry's problems in this regard are self-inflicted by the introduction of chemical commodities that have no essential value. For example, food additives that only make a product appear of better quality or food additives used in lieu of good handling and manufacturing practices have little, if any, intrinsic value except in an economic sense. Nevertheless, many food additives and other chemical commodities presently in use appear to be rather essential to the maintenance of existing life styles and, with continuing population growth, may become absolutely essential even to the maintenance of a viable human community.

The need for comprehensive safety testing of food additives prior to their utilization in the food of man has been emphasized previously *(11)*. It should be obvious that arguments for less restrictive food safety regulations can only have a deleterious effect on realizing the goal of providing safe foods for an expanding population. However, one can also sympathize with industry in its search to avoid the expenditure of large sums of capital in safety testing potentially useful food additives only to discover that the compounds often represent potential health hazards. Fortunately, a number of approaches can be and are being investigated to find solutions to this problem without compromising present food safety standards. One approach involves development of more refined

[1]Supported in part by Grants CA-10017, CA-10341, and CA-11946 from the National Cancer Institute, USPHS, and by Grant E-485 from the American Cancer Society.
[2]Predoctoral Fellow of the National Institutes of Health, General Medical Sciences (1 F01 GM 44402-02).
[3]Career Development Awardee of the National Cancer Institute, United States Public Health Service (1 K4 CA-08245).

test systems that can more rapidly and economically be utilized to reliably assess the carcinogenicity of a chemical *(40)*. A second approach involves accumulation of knowledge of the biochemistry of foreign compounds on the premise that this knowledge can be utilized to predict which chemicals might be safe for human use and which chemicals might produce adverse effects.

The present review is intended to serve as an introduction to available knowledge of structure-activity relationships observed in chemical carcinogenesis and to explore how this information might be utilized to more economically and effectively exploit potentially useful chemicals by eliminating from consideration those chemicals with carcinogenic potential.

Basic Aspects of Chemical Carcinogenesis

Pre-, proximate, and ultimate carcinogens

Intensive efforts have been made in the study of the structure-activity relationships observed in chemical carcinogenesis. However, early attempts to correlate chemical structure with carcinogenic activity met with little success because of the seemingly endless variety of organic and inorganic structures of chemicals displaying carcinogenic activity and because of the tacit assumption that these chemical agents were carcinogenic *per se*. Recently, in keeping with the axiomatic concept that carcinogenic agents induce cancer through interactions with tissue components, a vast body of data has become available demonstrating the firm covalent binding of carcinogens (or reactive products therefrom) to biologically important cellular macromolecules *(58, 60, 61)*. These macromolecular targets such as DNA, RNA, and protein play key roles in the processing of information required in maintaining and controlling growth and function of cells. A discussion of possible macromolecular targets critical to initiation of the carcinogenic process is beyond the scope of this review and the reader is referred elsewhere for detailed information on this topic *(33, 35, 58, 60, 68, 100)*.

The interactions between carcinogens and cellular macromolecules often occur *in vivo* even when the administered carcinogen is not itself chemically reactive. In these instances, metabolic activation to a chemically reactive form must occur *in vivo*. Chemical carcinogens, like other compounds, are subject to the enzyme-mediated metabolic processes of the host. In general, metabolism of these foreign compounds appears in most instances to lead to formation of polar, more water-soluble, non-carcinogenic metabolites, and thus constitutes routes of detoxication. However, certain metabolic pathways also may lead to formation of one or more metabolites with carcinogenic activities equal to or, more often, greater than that of the parent compound. Products of this metabolic activation are termed proximate carcinogenic metabolites and the form of the carcinogen which finally reacts with cellular constituents to initiate carcinogenesis is termed an ultimate carcinogenic metabolite. Precarcinogens which require metabolic activation to display carcinogenic activity generally induce tumors distant from the site of administration and require relatively larger doses. Proximate carcinogenic metabolites, however, often induce tumors in greater yield, with a shorter latent period, or both, and often induce tumors near the site of administration.

In contrast, many carcinogens exist essentially in their final reactive forms as administered and are able to participate directly in reactions with target molecules. These important aspects of chemical carcinogenesis are represented diagrammatically in Fig. 1. It is therefore obvious that a working knowledge of the biochemistry of biologically foreign compounds is essential to an understanding of structure-activity relationships in chemical carcinogenesis since it now appears that carcinogenic activity can be a conse-

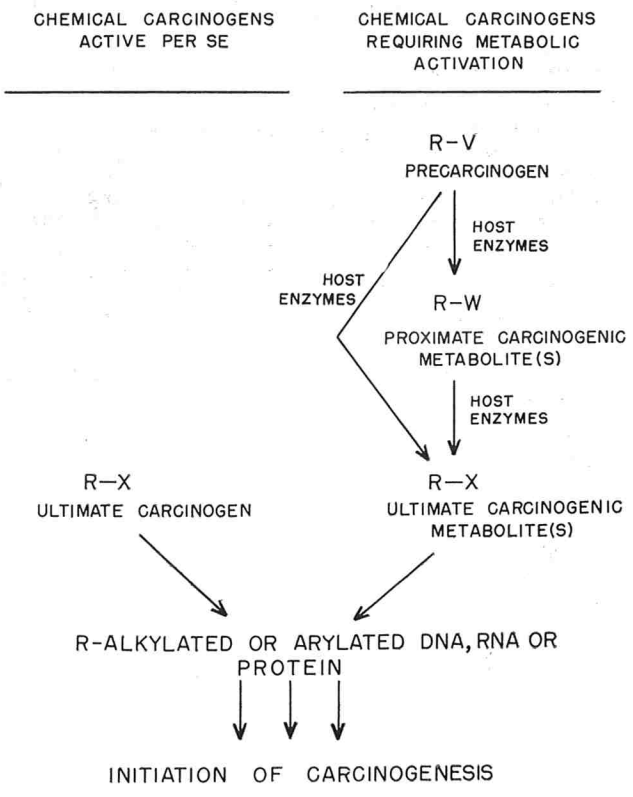


Figure 1. Diagrammatic representation of the concept of ultimate carcinogens and precarcinogens and the need for metabolic activation of the latter.

quence of the structure of the ultimate reactive forms of a carcinogen rather than of the structure of the parent compound *(21)*.

Metabolic transformations of biologically foreign compounds

Enzymes involved and mechanisms by which biologically foreign compounds are metabolized have been the subject of many excellent reviews *(36, 67, 102)*. Consequently, only a brief summary of the metabolic processes pertinent to the theme of this review will be presented.

Oxidation, reduction, hydrolysis, and synthetic or conjugation reactions comprise the four general types of reactions undergone by foreign compounds. The oxidative metabolism of many of these compounds is catalyzed by enzymes associated with the endoplasmic reticulum (microsomal subcellular fraction) of mammalian liver. Oxidative reactions carried out by the hepatic microsomal enzymes require oxygen and NADPH and can be visualized as hydroxylation reactions (Fig. 2), designed to produce derivatives that are less lipid-soluble and hence more readily excreted. Thus, this remarkably nonspecific enzyme system is involved in the hydroxylation of aliphatic and aromatic compounds, dealkylation of O-,N-, and S-alkyl compounds, N-hydroxylation of amines, N-oxidation of tertiary amines, and S-oxidation not to mention epoxidation, oxidative deamination, and desulfuration. In addition, many oxidases and dehydrogenases present in the mitochondrial and soluble fractions of cellular homogenates are able to carry out oxidative deaminations, oxidation of alcohols and aldehydes, and aromatization of alicyclic compounds. Although able to act on certain foreign compounds, these latter enzyme systems, designed to utilize endogenous substrates, tend to be relatively more specific.

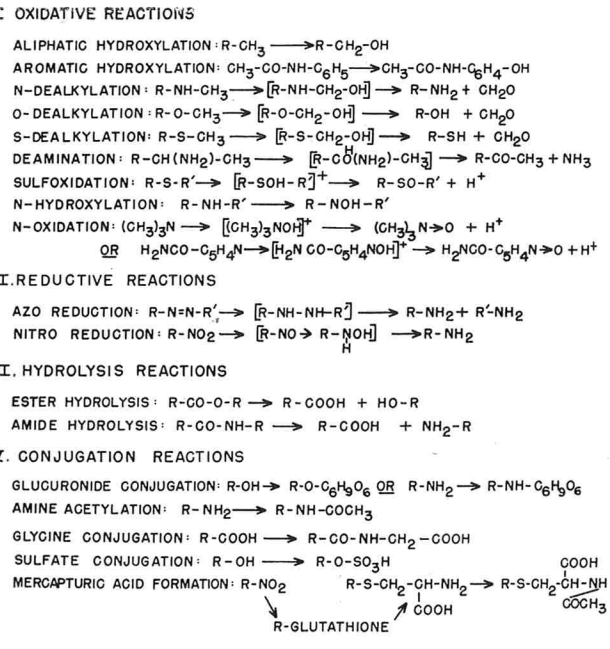


Figure 2. Examples of metabolic transformations observed with biologically-foreign chemicals.

Reductive metabolism of foreign compounds includes reduction of aromatic nitro and azo compounds with the formation of aromatic amines. The nitro reductase enzymes are present in the microsomal and soluble fractions of liver and kidney and require anaerobic conditions and NADPH. The reaction is stimulated by flavins and appears to proceed through the corresponding nitroso and hydroxylamino derivatives (Fig. 2). Likewise, azo reductase catalyzes the reduction of aromatic azo compounds to the corresponding hydrazo derivative and thence via reductive scission to two molecules of the respective aromatic amines.

Foreign compound metabolism by hydrolysis is restricted to esters and amides giving rise to an acid and an alcohol or an acid and an amine, respectively. Esterases and amidases involved are present in blood plasma and soluble fractions of tissue homogenates. Several other soluble enzyme systems also are involved in the conjugation or synthetic reactions so important in the detoxication and elimination of foreign compounds.

Conjugation reactions usually involve attachment of polar moieties to functional groups of suitable substrates as exemplified by conjugation of phenols, alcohols, and hydroxylamines, and carboxylic acids with glucuronic acid. In addition, aromatic amines and thiols occasionally can be converted into labile glucuronic acid conjugates. The transferase enzymes mediating these reactions are found in microsomes of liver and in other tissues and require as cofactor, uridine diphospho-glucuronic acid. A more general pathway for conjugation of many aromatic amines involves acetylation via the action of aryl amine transferases utilizing acetyl coenzyme A as cofactor. A similar mechanism is involved in the formation of glycine conjugates as the result of reaction between glycine and coenzyme A derivatives of aromatic carboxylic acids.

Another important type of synthetic reaction is the sulfate conjugation of phenolic and alcoholic compounds. These reactions, carried out by sulfotransferases in the soluble fraction of mammalian liver, kidney, and intestinal mucosa, involve the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate to a suitable substrate. Other synthetic reactions of importance include O and S methylation by methyltransferases, and formation of mercapturic acids which appears to involve an initial conjugation with

glutathione followed by conversion to the corresponding cysteine derivative and finally by acetylation to yield mercapturic acids. Such reactions occur with certain halogenated aromatic hydrocarbons and with certain aromatic nitro compounds.

It is perhaps ironic that certain of the metabolic pathways designed by Nature to provide a means of detoxifying and eliminating biologically foreign compounds have also, as will become apparent, provided a means of activating chemical precarcinogens to their chemically reactive ultimate forms. However, production of cancer by chemicals is generally characterized in humans and other animals by a long latent period and the symptomatic disease often does not manifest itself until after the reproductive lifespan. Thus, Nature may not have been allowed the opportunity to correct the situation through the process of natural selection.

Nature of reactions between ultimate carcinogens and tissue macromolecules

Alkylation or arylation of tissue constituents by ultimate carcinogenic forms of chemical carcinogens requires that these forms be chemically reactive. An impressive array of data accumulated on the importance and nature of attack of ultimate carcinogenic metabolites on DNA, RNA, and protein has allowed formulation of a basic and unifying concept in chemical carcinogenesis. Thus, as discussed in two classical reviews *(59, 60)*, it appears that most, if not all, chemical carcinogens exist as or are metabolically activated to electrophilic reactants.

Electrophilic reactants are chemicals in which a relatively positive atom in the molecule can combine covalently with relatively negative or electron-rich atoms in target nucleophilic reactants. Such reactions proceed through two distinctly different mechanisms as shown in Fig. 3. The first reaction mechanism is unimolecular and is written as taking place in two steps, the first of which involves a usually slow and reversible dissociation of R-X to R^+ cation and X^- anion. The second step involves a usually fast reaction between R^+ cation and the nucleophile Y^- to yield R-Y. In this type of nucleophilic substitution, the rate limiting factor is the dissociation of R-X which means that the rate of formation of R-Y will depend on the concentration of R-X and not on the concentration of the nucleophile, since Y^- is not utilized except in a fast secondary reaction. Thus, the overall reaction is designated S_N1 for substitution, nucleophilic, unimolecular or first order (Fig. 3).

In the second mechanism, the reaction proceeds in a single bimolecular step. Attack of Y^- occurs simultaneously with loss of X^- or in other words the R-Y bond is formed at the same time that the

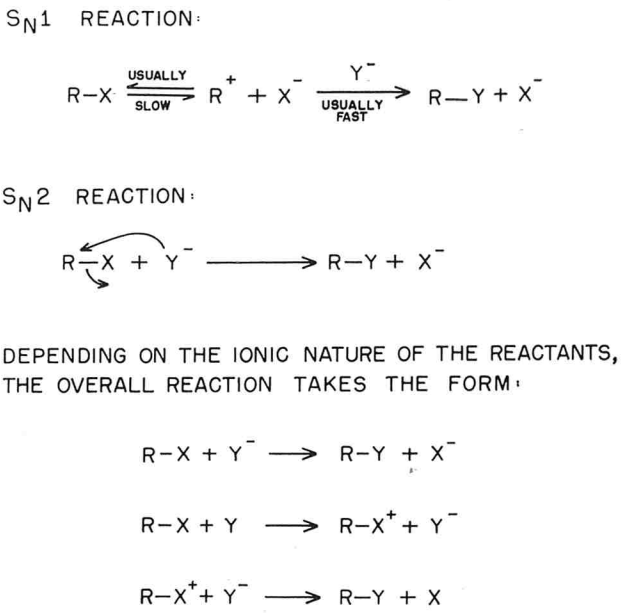


Figure 3. Diagrammatic representation of unimolecular (S_N1) and bimolecular (S_N2) nucleophilic substitution reactions.

R-X bond is broken. This mechanism requires the reaction rate to depend on concentrations of both reactants since the rate-limiting step involves collisions between R-X and Y^-. Thus, the reaction is designated S_N2 for substitution, nucleophilic, bimolecular or second order *(34)*.

The reactivity of a given electrophilic reactant, R-X, in either S_N1 or S_N2 reactions is determined in part by the nature of the leaving group, X^-. In general, there is a reasonable correlation between the reactivity of R-X and the acid strength of H-X, the X groups that correspond to the strongest acids being the best leaving groups. For example, the greater ease of breaking a C-OSO_2R bond than a C-OH or C-OR bond in S_N2 reactions correlates with the greater acid strength of H-OSO_2R in relation to H-OH and H-OR. In fact, hydroxyl and alkoxyl groups generally can be displaced only in a strongly acid medium such that they exist as $-OH_2^+$ and $-OHR^+$.

Steric hindrance also can affect the rates of nucleophilic substitution reactions but are substantially more important in S_N2 reactions because of the crowded transition state involved. In this regard, neighboring group effects of substituents on the electrophilic atom, R, can be very important.

The nucleophilicity of Y roughly parallels basicity. Some nucleophiles observed *in vivo* include electron-rich ring nitrogen atoms in nucleic acid bases, sulfur atoms in sulfhydryl groups of cysteine and methionine in protein, and various phosphate and carboxyl

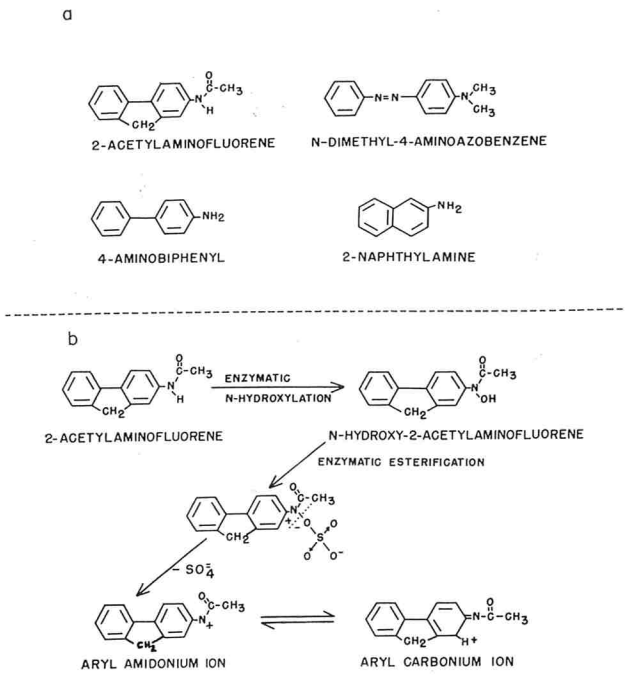


Figure 4. a) Examples of carcinogenic aromatic amine derivatives. b) Proposed metabolic route generally applicable to the activation of carcinogenic aromatic amine derivatives as illustrated with the potent hepatocarcinogen, 2-acetylaminofluorene *(59, 60, 62)*.

compounds, etc. If more than one nucleophile is available for reaction, competition for the electrophilic reactant will result in a mixture of products. This appears to be precisely the *in vivo* situation observed with the ultimate electrophilic forms of chemical carcinogens in that they are observed to react with a variety of low molecular weight tissue nucleophiles and with nucleophilic centers in biologically important macromolecules.

CLASSES OF STRUCTURALLY RELATED CHEMICAL CARCINOGENS AND THEIR POSSIBLE ULTIMATE CARCINOGENIC FORMS

Carcinogenic aromatic amines and amides (Figure 4a)

Metabolic activation of carcinogenic aromatic amines and amides has been the subject of recent reviews *(59, 60, 62)*. Metabolism of aromatic amines *in vivo* includes ring hydroxylation, N-hydroxylation, conjugation of the ring and N-hydroxyl metabolites, and acylation of the amine function. Deacylation and dealkylation also are observed with N-substituted aromatic amine derivatives. The metabolic route generally applicable to activation of carcinogenic aromatic amines and amides appears to involve N-hydroxylation, and is well illustrated by the potent hepatocarcinogen, 2-acetylaminofluorene (Fig. 4b). Thus, N-hydroxy-2-acetylaminofluorene has been demonstrated to be a proximate carcinogenic metabolite more active than the parent compound at usual sites of tumor formation in experimental animals and also active at sites where the parent compound is inactive. Similarly, N-hydroxylation has been shown to occur with 4-acetylaminobiphenyl, 2-acetylaminophenanthrene, 4-acetylaminostilbene, and 1- and 2-naphthylamine, giving rise to proximate carcinogenic metabolites.

Correspondingly, N-hydroxy-2-acetylaminofluorene has been demonstrated to interact covalently with tissue proteins and nucleic acids *in vivo* in much greater yield than does the parent compound. However, failure of N-hydroxy-2-acetylaminofluorene to react non-enzymatically with nucleic acids or other tissue nucleophiles *(62)* to a significant extent *in vitro* suggests the need for further metabolic activation.

A number of studies now indicate that the ultimate carcinogenic and reactive forms of carcinogenic aromatic amides arise through the *in vivo* esterification of the N-hydroxy derivatives. This is supported by the finding that the synthetic N-acetoxy and N-benzoyloxy esters of 2-acetylaminofluorene both induce a greater incidence of tumors and with a shorter latent period than N-hydroxy-2-acetylaminofluorene. Accordingly, these reactive esters, via the arylamidonium or arylcarbonium ions, interact readily *in vitro* at physiological pH with nucleophilic centers in DNA, RNA, and protein and with certain of their component parts such as deoxyguanosine, guanosine, methionine, tryptophan, and cysteine. The order of reactivities of synthetic esters of N-hydroxy-2-acetylaminofluorene is $-OSO_3H > -OCOCH_3$, $OCOCH_2C_6H_5 > -OH$ and, in general, correlates with the acidity of the acid forms of these leaving groups *(62)*.

Neighboring group effects are apparent in that the aryl moiety also plays an important role in determining the chemical reactivity and carcinogenicity of esters of various N-hydroxy-arylacetamides. For example, the N-acetoxy esters of 2-acetylaminofluorene, 2-acetylamino-phenanthrene, 4-acetylaminostilbene, and 4-acetylaminobiphenyl display different chemical reactivities with tissue nucleophiles as well as different carcinogenic activities.

In addition to the aryl moiety and the ester moiety, the acyl group also appears to govern chemical reactivity. Thus, in contrast to N-hydroxy-2-acetylaminofluorene, N-hydroxy-2-aminofluorene reacts non-enzymatically with the guanine bases of nucleic acids *in vitro*. That the reaction takes place more efficiently at a pH lower than 6 is probably related, in part, to the fact that the hydroxyl function, a poor leaving group, would have a greater tendency to

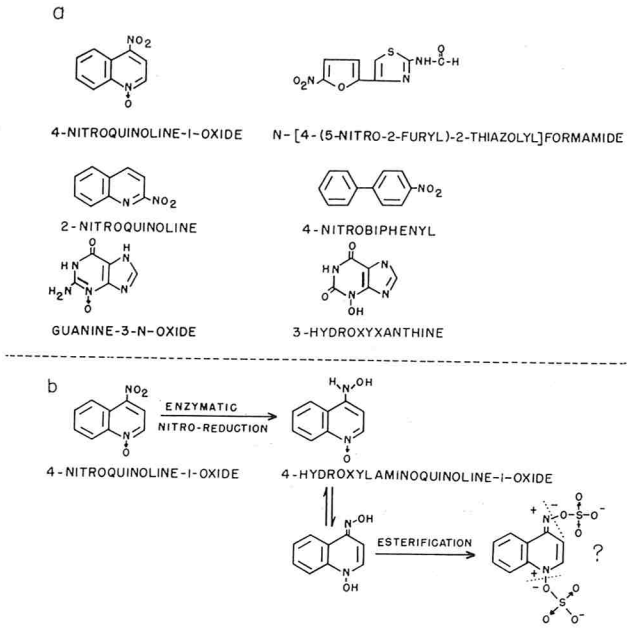


Figure 5. a) Examples of carcinogenic aromatic nitro compounds and heterocyclic N-oxides. b) Proposed metabolic route for the activation of carcinogenic aromatic nitro compounds and heterocyclic N-oxides as illustrated with the potentially bifunctional carcinogen, 4-nitroquinoline-1-oxide *(30, 89)*.

exist as $-OH_2^+$, a more efficient leaving group, in an acidic medium.

The most reactive ester of N-hydroxy-2-acetylaminofluorene, the N-sulfate ester, now appears to be at least one of the ultimate reactive and carcinogenic metabolites of N-hydroxy-2-acetylaminofluorene produced *in vivo (18)*. Thus, incubation of N-hydroxy-2-acetylaminofluorene with nucleic acids in a system compatible with the enzymatic sulfate conjugation of the N-hydroxy function greatly stimulates the covalent interaction of the arylacetamide with nucleic acids. Furthermore, there is a close correlation of N-hydroxy-2-acetylaminofluorene sulfotransferase activity, the enzyme giving rise to the reactive sulfate ester *in vivo*, with susceptibility of experimental animals to hepatocarcinogenesis by N-hydroxy-2-acetylaminofluorene *(18)*.

Similarly, the carcinogenic tertiary aromatic amine, N-dimethyl-4-aminoazobenzene, undergoes N-dealkylation to N-methyl-4-aminoazobenzene prior to conversion to the hydroxylamine derivative which can be activated to a chemically reactive form by esterification. As with the esters of N-hydroxy-2-acetylaminofluorene, the esters of N-hydroxy-N-methyl-4-aminoazobenzene appear to react with tissue nucleophiles via an arylamidonium or arylcarbonium ion.

Determination of the precise role of the aryl moiety *(82)* and the acyl or alkyl moieties as they govern metabolic activation and the nature of the ultimate electrophilic reactant will no doubt lead to meaningful structure-activity relationships in contrast to those studies based on comparisons of the precarcinogenic forms of carcinogenic aromatic amines and amides *(3)*.

Carcinogenic aromatic nitro and heterocyclic N-oxide derivatives (Figure 5a)

Carcinogenic aromatic nitro compounds require metabolic activation to display carcinogenic activity and in this respect the proximate carcinogenic metabolites bear structural similarities to those of carcinogenic aromatic amines and amides. Thus, a proximate carcinogenic metabolite of 4-nitroquinoline-1-oxide is produced by partial reduction of the nitro group giving rise to 4-hydroxylaminoquinoline-1-oxide *(89)* (Fig. 5b). The complete reduction product, 4-aminoquinoline-1-oxide apparently lacks carcinogenic activity.

Early *in vitro* studies suggesting a direct reaction of 4-nitroquinoline-1-oxide with DNA *(55)* are now known to have been misinterpreted *(10)*. However, 4-hydroxylaminoquinoline-1-oxide reacts *in vivo* with nucleic acids *(91)* and also reacts *in vitro* at physio-

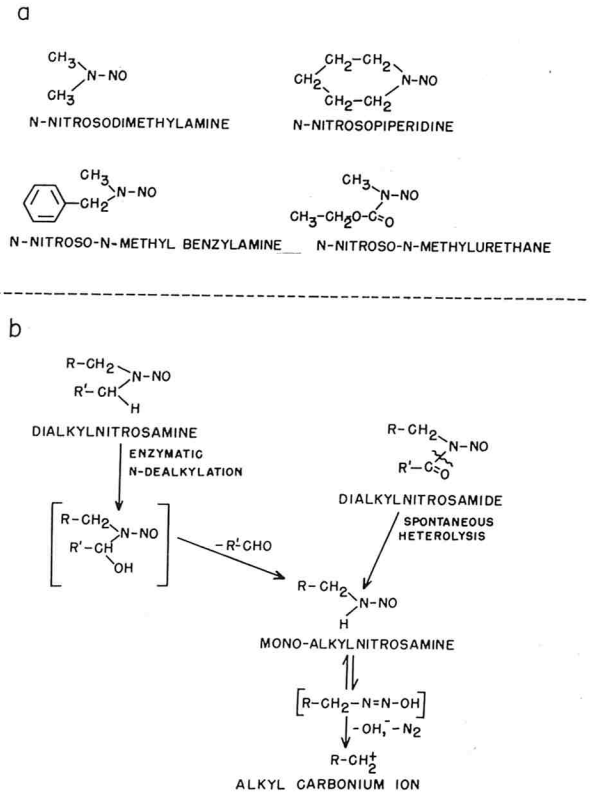


Figure 6. a) Examples of carcinogenic N-nitrosamines and N-nitrosamides. b) Proposed metabolic route generally applicable to the activation of carcinogenic dialkylnitrosamines and its relationship to the spontaneous heterolysis of carcinogenic dialkylnitrosamides *(24, 25, 53, 54)*. See text for a discussion of the special case of aromatic nitrosamine derivatives.

logical pH with sulfhydryl groups of certain proteins *(41) and* with nucleic acids *(30)*. As might be expected, esterification of 4-hydroxylamino-quinoline-1-oxide gives rise to a more chemically reactive electrophile. Indeed, the synthetic 0,0′-diacetyl ester of this carcinogenic hydroxylamine derivative is considerably more reactive toward DNA, RNA, and other tissue nucleophiles *(30)*. As is apparent with many of the carcinogenic aromatic amines, it is possible that enzymatic esterification of the hydroxylamine is involved in the *in vivo* production of an ultimate reactive form of 4-hydroxylaminoquinoline-1-oxide *(60)*. Because hydroxyl, amino, and hydroxylamino substituted heterocyclic N-oxides can exist as the N-hydroxyl tautomers *(44)*, esterification of 4-hydroxylaminoquinoline-1-oxide may lead to a bifunctional electrophile. The possible formation of a reactive ester of the ring nitrogen hydroxyl is similar to the situation observed with the potent carcinogens, 3-hydroxyxanthine and guanine-3-N-oxide *(5, 8)*.

The 3-N-oxide of xanthine, for example, exists primarily as 3-hydroxyxanthine. The synthetic ester of this carcinogen, 3-acetoxyxanthine, reacts *in vitro* with tissue nucleophiles *(104)*. The product of the reaction between 3-acetoxyxanthine and methionine has been detected in the urine of experimental animals administered 3-hydroxyxanthine *(88)* suggesting that metabolic esterification occurs *in vivo*. The very efficient participation of esters of heterocyclic N-oxides in nucleophilic substitution reactions has been studied in detail *(45)* and implies that further studies on the possible carcinogenicity of other heterocyclic N-oxides is in order.

Obviously, proximate carcinogenic hydroxylamine metabolites of carcinogenic aromatic nitro compounds might arise by the complete reduction of the nitro group to an amine which might then be N-hydroxylated. Alternatively, as with 4-nitroquinoline-1-oxide, the hydroxylamine may be produced by partial reduction of the nitro group. This latter route is possibly more important in view of the strong carcinogenic activity of many aromatic nitro compounds. A relatively new and large class of potent chemical carcinogens, the 2-substituted-5-nitrofurans *(14, 31, 64)* is being utilized to study this possibility. For example, the urinary bladder carcinogen, N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide, is rapidly deformylated *in vivo*, probably to 2-amino-4-(5-nitro-2-furyl) thiazole, which is an effective *in vitro* substrate for nitroreductase enzyme systems. The reduced product, probably the corresponding hydroxylamine, reacts with nucleic acids at physiological pH *(52)*. In studies with other 2-substituted-5-nitrofurans *(92)* and related compounds *(37)*, the hydroxylamine derivative appears to be the principal product formed *in vitro* by nitroreductase systems.

That hydroxylamines appear to be proximate carcinogenic metabolites of both carcinogenic aromatic amines and nitro compounds suggests a convergent route of metabolic activation of carcinogenic aromatic amines and their respective nitro derivatives. This concept is supported by the similar carcinogenic activity of 4-nitrobiphenyl and its sister amine, 4-aminobiphenyl *(19)*.

Carcinogenic secondary nitrosamines and nitrosamides (Figure 6a)

A variety of aliphatic, aromatic, and cyclic secondary N-nitrosamines comprise a large class of potent and versatile chemical carcinogens *(24, 25, 53, 54)*. The correlation between carcinogenicity of N-nitrosamines and their transformation *in vivo* into alkylating agents is supported by a considerable volume of experimental evidence. Metabolic activation appears to be initiated by the oxidative N-dealkylation of the dialkylnitrosamine to the hypothetical monoalkylnitrosamine. For this to be possible, there is an apparent requirement for the dialkylnitrosamine to possess at least one hydrogen atom on the alpha carbon. This requirement explains, in part, the non-carcinogenicity of diphenylnitrosamine. The metabolically derived monoalkylnitrosamines, tautomeric with the corresponding alkyldiazohydroxides, are very unstable and readily yield alkyl carbonium ions possibly by virtue of alkylating intermediates such as diazoalkanes, alkyldiazotates, or alkyldiazonium ions *(24)* (Fig. 6b). Recent results suggest that the alkyl groups are transferred intact to nucleophilic targets and consequently the diazoalkanes are not probable candidates for the alkylating intermediate *(39, 51)*.

As a result of metabolic conversion of dialkylnitrosamines to the corresponding alkyl carbonium ions, one observes the *in vivo* alkylation of nucleophilic centers in DNA and RNA involving primarily the N-7 of guanine as well as the N-1 and N-3 of adenine and the N-1 of cytosine *(49, 90)*. Similarly, alkylation of proteins occurs by the attack on the sulfhydryl of cysteine and the N-1 and N-3 positions of histidine *(15)*.

In contrast to the dialkylnitrosamines requiring metabolic activation to display carcinogenic activity, the acylalkylnitrosamines such as N-methylnitrosourea and N-methylnitrosourethane are deacylated by spontaneous heterolysis and consequently represent direct-acting carcinogens. While capable of producing local tumors at the site of administration, these carcinogens also affect various internal organ systems in experimental animals. Because the de-acylation of both N-methylnitrosourea and N-methylnitrosourethane would presumably yield the same monalkyl-

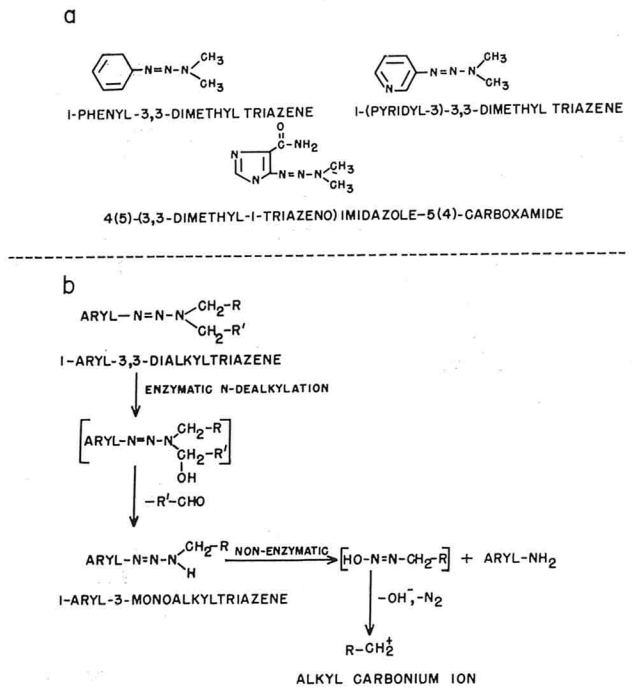


Figure 7. a) Examples of carcinogenic 1-aryl-3,3-dialkyltriazenes. b) Proposed metabolic route generally applicable to the activation of carcinogenic 1-aryl-3,3-dialkyltriazenes *(72, 86)*.

nitrosamine and therefore the same alkylating intermediate, the different organ specificities observed with these two carcinogens is probably attributable to the absorbed and transported forms of the respective intact acylalkylnitrosamines *(26)*. In summary, it appears that the ultimate carcinogenic metabolites of both the alkylnitrosamines and alkylnitrosamides are represented by the corresponding alkyl carbonium ions. However, an exception is observed with the aromatic nitrosamines and nitrosamides. Although N-nitroso-N-methylcyclohexylamine and N-nitroso-N-methylaniline display similar degrees of carcinogenic activity *(38)*, the latter compound is apparently not converted to a methylating agent *in vivo*. However, metabolic dealkylation of N-nitroso-N-methylaniline or the spontaneous heterolysis of N-nitroso-N-phenylurea would give rise to a monoarylnitrosamine, tautomeric with the aryldiazohydroxide. Indeed, N-nitroso-N-phenylurea decomposes with production of the phenyl diazonium ion *(69)* which is possibly the ultimate electrophilic form of this carcinogen.

In leaving this subject, it is noteworthy that certain secondary amines are able to react under *in vivo* conditions with inorganic nitrite to form the corresponding nitrosamine derivatives, the rate of nitrosation increasing as the basicity of the amine decreases. For example, nitrite and methylbenzylamine are not carcinogenic. However, addition of both of these compounds to the diet of experimental animals has led to the formation of tumors, presumably due to the *in vivo* formation of the carcinogenic N-nitroso-N-methyl benzylamine *(79)*. It seems possible, therefore, that certain secondary amines for which an *in vivo* reaction with nitrite is possible and for which the corresponding nitrosamines are carcinogenic, will prove to be undesirable dietary constituents *(50)*. Further studies in this area are certainly warranted with the recent observations *(63)* that human liver tissue is able to efficiently activate carcinogenic N-nitrosamines with the resultant alkylation of cellular nucleic acids, indirect evidence that emphasizes the potential hazard to humans.

Carcinogenic 1-aryl-3,3-dialkyltriazenes (Figure 7a)

The metabolic activation of the carcinogenic 1-aryl-3,3-dialkyltriazenes is very similar to that observed with the carcinogenic N-nitrosamines. Aryldialkyltriazenes are oxidatively N-dealkylated *in vitro* to form arylmonoalkyltriazenes as proximate carcinogenic metabolites *(70, 72, 86)*. The arylmonoalkyltriazenes tested appear to be stronger carcinogens than the parent compounds and produce local tumors at the site of administration.

Arylmonoalkyltriazenes are known to be efficient alkylating agents and readily rearrange to the free arylamine and an alkylating intermediate, possibly the alkydiazohydroxide. In many instances, the arylmonoalkyltriazene appears to be sufficiently stable to allow it to leave the organ in which it was formed and disseminate throughout the body of the host to affect other organs *(84, 86)*.

Administration of aryldialkyltriazenes to experimental animals results in the *in vivo* alkylation of tissue nucleic acids *(87)*. As might be anticipated, the arylmonoalkyltriazenes react *in vitro* with DNA, RNA, and guanosine giving rise to 7-alkylguanine adducts *(71)*. Based on these results, the mechanism of carcinogenesis by aryldialkyltriazenes has been formulated *(72)* as depicted in Fig. 7b.

Carcinogenic hydrazo, azo, and azoxyalkanes (Figure 8a)

Carcinogenesis by hydrazo-, azo-, and azoxyalkanes can be discussed under one heading since they appear to have interrelated pathways of metabolic activation *(46, 70)*. This is suggested by the similar patterns of carcinogenesis of hydrazo-, azo-, and azoxy-derivatives containing a given alkyl residue. For example, 1,2-dimethylhydrazine, azoxymethane, and methylazoxymethanol display nearly identical organ specificity in the production of malignant lesions in experimental animals *(70)*. On the other hand, the three ethyl derivatives, hydrazo-, azo-, and azoxyethane induce similar patterns of malignancies but

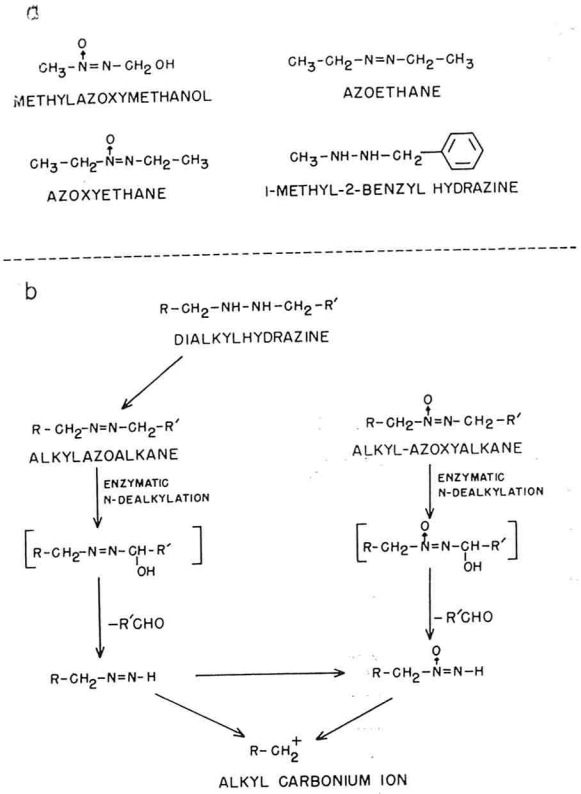


Figure 8. a) Examples of carcinogenic hydrazo-, azo-, and azoxyalkanes. b) Proposed metabolic route generally applicable to the interrelationships and activation of carcinogenic hydrazo-, azo-, and azoxyalkanes *(70)*.

differing completely from those induced by the methyl analogs. Such data suggest a common reactive intermediate with a given alkyl series of these carcinogens.

Hydrazoalkanes are known to be easily oxidized to the corresponding azoalkanes, the transformation being catalyzed by trace amounts of heavy metals. Alkylazoalkanes and alkylazoxyalkanes undergo oxidative N-dealkylation *(103)* to alkylating intermediates, possibly alkyldiimines, alkyldiimine oxides, or alkyldiazohydroxides. Indeed, azoxyalkanes have been postulated as an intermediate in the oxidative dealkylation of azoalkanes, affording an explanation for the similar carcinogenicities of related alkylazoalkanes and alkylazoxyalkanes *(103)*. It is of interest that methylazoxymethanol, a proximate carcinogenic metabolite of cycasin *(56)*, is also identical to the transient alphahydroxylated form of azoxymethane produced during oxidative N-dealkylation. Methylazoxymethanol is easily hydrolyzed to formaldehyde and an alkylating intermediate, possibly methyldiimine oxide, which is tautomeric with methyldiazohydroxide (Fig. 8b). As a consequence, methylazoxymethanol has been observed to methylate nucleic acids *in vitro (56)* and *in vivo (66, 83)*.

Arylolkyl derivatives of hydrazo-, azo-, and azoxycompounds fail to display carcinogenic activity suggesting that both organic residues must be alkyl groups with available alpha hydrogens. For symmetrically substituted hydrazoalkanes, the carcinogenic activity appears to decrease with increasing size of the alkyl residues. However, unsymmetrical hydrazines such as 1-methyl-2-benzylhydrazine display pronounced carcinogenic activity. 1,1-Dialkylhydrazines are virtually non-carcinogenic, suggesting that the hydrazo structure is necessary for activity *(70)*. Correspondingly, 1,1-dialkylhydrazines have no *in vivo* alkylating capabilities *(48)*.

Carcinogenic direct alkylating substances (Figure 9). A variety of diverse types of chemical carcinogens do not require metabolic activation but rather appear to exist essentially in their ultimate reactive form. Such compounds are often locally acting carcinogens and participate in nucleophilic substitution reactions with tissue constituents.

A number of alkylsulfates and sulfonoxyalkanes have displayed carcinogenic activity and the ability to alkylate cellular macromolecules. Examples of these carcinogens are shown in Fig. 9a with dashed lines indicating which bonds are cleaved in the alkylation reactions. Thus, methyl methanesulfonate *(13)*

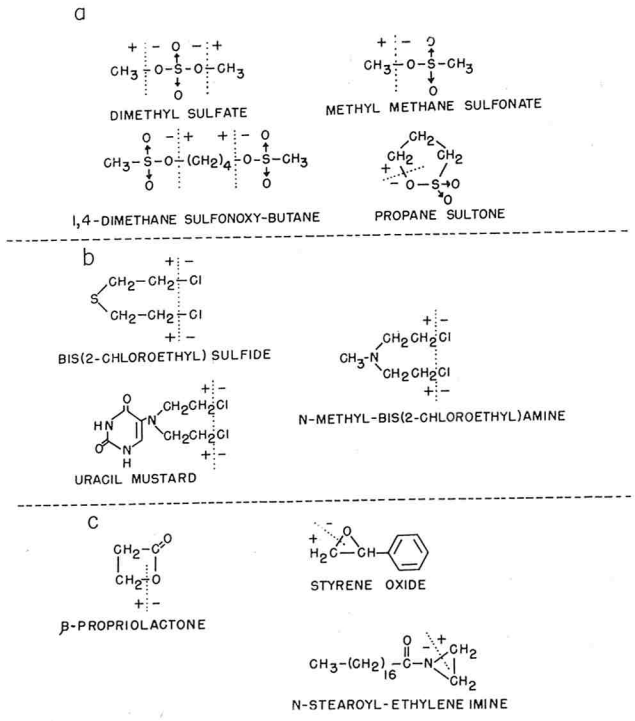


Figure 9. Examples of carcinogenic direct alkylating substances. Dashed lines indicate which bonds are cleaved in the formation of chemically-reactive electrophiles. a) Examples of carcinogenic alkyl sulfates and sulfonoxy alkanes. b) Examples of carcinogenic mustard derivatives. c) Examples of carcinogenic strained ring derivatives.

and dimethylsulfate *(23, 27)* bring about the *in vivo* methylation of nucleic acids *(49, 90)*. Similarly, propanesultone, a potent carcinogen *(22)*, reacts by heterolysis to introduce the propyl sulphonic acid function into target nucleophiles *(65)*. The carcinogenicity of some of these compounds has been difficult to assess because of the rapidity with which they decompose in an aqueous medium. Consequently, it is believed that the very reactive alkylating agents may not escape reaction with water and extracellular materials before gaining access to critical cellular sites. For example, dimethyl sulfate, a methylating agent commonly used in organic syntheses, is inactive as a carcinogen when administered to experimental animals orally or intravenously *(23)*. However, dimethyl sulfate has shown distinct carcinogenic activity when administered by inhalation, and has been associated with cancer of the lung in humans receiving industrial exposure *(27)*. Likewise, a number of mustard derivatives with alkylating capabilities have displayed carcinogenic activity (Fig. 9b). Reactions of the *bis* (2-chloroethyl) amine derivatives with nucleic acids *in vitro* and *in vivo* have been studied in detail *(7, 78)*. In reactions with DNA, the bifunctional nature of these compounds allows a dual alkylation with the subsequent formation of cross-linked DNA strands. Generally, these compounds require multiple large doses at local tissue sites to reveal their carcinogenicity. Again, the high chemical reactivity *(73)* of these agents with water and low molecular weight nucleophiles may explain, in part, why these carcinogens often are not strongly carcinogenic. An exception is noted with uracil mustard which appears to be a potent lung carcinogen in the mouse *(1)*. Furthermore, there is little doubt that certain of these agents represent a hazard for humans, as illustrated by the high incidence of cancer of the lung and upper respiratory tract in workmen receiving industrial exposure to *bis*(2-chloroethyl) sulfide *(99)*.

Finally, a number of strained ring compounds, including certain lactones, ethyleneimines, and epoxides, have shown alkylating as well as carcinogenic activity *(20, 96)*. For example, the carcinogen, β-propiolactone (Fig. 9c), introduces a carboxyethyl moiety into tissue nucleophiles both *in vitro* and *in vivo (6)*. A number of studies correlating the carcinogenicity of these strain ring compounds with alkylating capability *(43)* and with their molecular structures *(97, 98)* are presently available. The possible importance of epoxides in carcinogenesis has recently been discussed *(80)*.

PREDICTION OF CHEMICAL CARCINOGENICITY

Determination of the carcinogenicity of a chemical by present bioassay methods is complicated by a number of variables such as species, sex, age, hormonal status, and dietary factors, etc. In tests of the structurally-related members within the classes of chemical carcinogens, these factors remain as important variables. However, as we have seen, there is an abundance of evidence suggesting that the ultimate carcinogenic forms of chemical carcinogens are electrophilic reactants (alkyl and aryl carbonium ions, arylamidonium ions, etc.) with proximate carcinogenic metabolites of precarcinogens being those metabolites more readily giving rise to the ultimate electrophilic forms. Consequently, lack of or differing carcinogenic activities of closely related chemicals must now be recognized as also being related to one or more of a number of factors including different rates of absorption and excretion, different rates of distribution and intercellular and intracellular transport, different specificities for both activating and detoxifying enzyme systems, different chemical reactivities and stabilities of the ultimate reactive forms, and different affinities of the ultimate forms for the target nucleophiles critical to initiation of carcinogenesis.

Existence of these variables has severely hampered development of useful structure-activity relationships in chemical carcinogenesis and it is likely that structural correlations can be and have often been inadvertently made with the variables associated with carcinogenesis rather than with carcinogenesis itself. For example, it has long been recognized that 2-naphthylamine is more strongly carcinogenic than 1-naphthylamine. This has recently been related to quantitative differences in their *in vivo* conversion to proximate N-hydroxyarylamines *(76)*. It is interesting then that 1-naphthylhydroxylamine appears to be a stronger carcinogen, dose for dose, than 2-naphthylhydroxylamine *(4)*. To explain the differing carcinogenic potencies of the two hydroxylamine derivatives, consideration must again be given to the many variables listed above. It is obvious that the more metabolic intermediates between a precarcinogen and its ultimate carcinogenic metabolites, the more complicated will be the problem of constructing useful structure-activity relationships. The ability to predict with any degree of assuredness the carcinogenicity (or non-carcinogenicity) of a chemical will require a detailed knowledge of how structural features determine rates of absorption, excretion, metabolic activation and detoxication, and the ability of ultimate reactive forms to participate in nucleophilic substitution reactions with biologically important nucleophiles. Unfortunately, this knowledge is rather limited at present, as illustrated by studies with the carcinogenic 2-substituted-5-nitrofuran derivatives. The carcinogenicity and organ specificity

of these carcinogens varies markedly when seemingly minor alterations are made in their molecular structures, a fact which has led to the conclusion that it is virtually impossible, on the basis of structural considerations, to predict which of these compounds will and which will not prove to be carcinogenic *(32)*.

Nevertheless, knowledge of the biochemistry of foreign compounds and presently available information on the nature of ultimate carcinogenic forms of chemical carcinogens does allow judgments to be made regarding the carcinogenic potential of many types of chemicals. This is borne out by past results in testing chemicals for carcinogenic activity. For example, the fact that nearly one of every four chemicals tested in experimental animals is judged to be carcinogenic is related, in part, to a bias in testing "compounds likely to be carcinogenic" *(85)*. Correcting for this bias, it has been estimated that possibly one out of six chemicals tested will display carcinogenic activity. These results point out the feasibility of predicting, if not those chemicals definitely carcinogenic, at least those chemicals with carcinogenic potential. Thus, while it is not possible at present to predict the carcinogenicity of individual 2-substituted-5-nitrofurans, it is safe to say that there is a rather strong likelihood that a given 2-substituted-5-nitrofuran will prove to be carcinogenic. Such a judgment is of perhaps more than passing interest to industrial concerns considering the commercial exploitation of such compounds. Again, looking to past results, one can appreciate how this approach has been and might have been utilized.

Industrial chemicals

The discovery that occupational exposure to 2-naphthylamine led to production of bladder cancer in workmen of certain chemical industries and that 2-naphthylamine was able to induce similar malignancies in experimental animals was followed by the discovery, aided in part by obvious structural similarities, of a number of carcinogenic aromatic amines. 2-naphthylamine moiety should have ever received The warnings that were sounded about the possible hazards associated with the industrial use of aromatic amines such as benzidine (Fig. 10) unfortunately went unheeded for many years *(42)*.

Pharmaceuticals

Likewise, N,N-*bis*(2-chloroethyl)-2-naphthylamine has induced bladder cancer in a number of polycythemia patients treated with this drug *(42)*. In retrospect, it seems rather amazing that a drug containing both the potentially carcinogenic *bis*(2-chloroethyl)amine moiety and the potentially carcinogenic serious consideration as a drug for treatment of human disease.

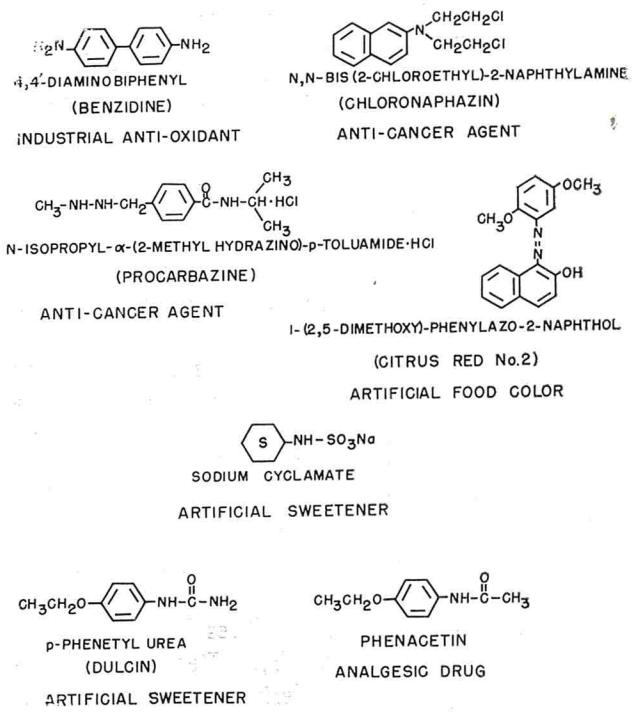


Figure 10. Examples of carcinogenic chemical commodities used by man.

As another example, the *in vivo* alkylation of nucleic acids by the anti-neoplastic agent, 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide, and its obvious structural resemblance to carcinogenic 1-aryl-3,3-dimethyltriazenes (Fig. 7) were primary considerations leading to the discovery that this drug displayed potent carcinogenic activity in experimental animals *(87)*. Another anti-neoplastic agent, N-isopropyl-α-(2-methylhydrazino)-p-toluamide·HC 1, has also displayed strong carcinogenic activity *(46)*. As with other carcinogenic unsymmetrical hydrazines (Fig. 8), this compound is subject to metabolic N-dealkylation with the apparent formation of an alkylating intermediate *(103)*. A number of other anti-neoplastic agents based on the sulfonoxyalkane or *bis*(2-chloroethyl)amine moieties also are carcinogenic. The fact that many chemicals used in the chemotherapy of human malignancies are also able to produce cancerous lesions brings out a point important to the theme of this review. Namely, in some instances, the desired biological activity of a chemical may depend on the same structural characteristics responsible for carcinogenic activity. This appears to be true with certain anti-neoplastic alkylating agents which are designed to alkylate tissue constituents with the goal of inducing cellular death. In the palliative therapy of terminal cancer patients, the cautious use of potentially carcinogenic drugs might be justifiable. In contrast, chemical commodities such as food addi-

tives are not administered to humans on an individual basis and the use of potentially carcinogenic chemicals for such purposes is in no instance justifiable, particularly in view of the large scale human exposure to such agents.

Food additives

A variety of azo dyes previously and presently used to color foodstuffs have displayed carcinogenic activity for the liver, bladder, or lymphoid system of experimental animals *(42, 47)*. For example, 1-2, 5-dimethoxyphenylazo)-2-naphthol (Citrus Red No. 2) (Fig. 10), used in the artificial coloration of oranges, has elicited tumors of the urinary bladder in rats and mice *(16)*. As might be anticipated, such azo dyes can act as substrates for azo reductase systems and undergo reductive scission to the two corresponding aromatic amines. Thus, with Citrus Red No. 2, the initial products would be 2, 5-dimethoxyaniline and 1-amino-2-naphthol. In experimental animals administered Citrus Red No. 2, the latter metabolite has been found present in the urine as the sulfate conjugate, a urinary metabolite also present after administration of 1-naphthylamine *(75)*. Whereas ring-hydroxylated metabolites of carcinogenic aromatic amines often represent detoxified forms of the parent carcinogen, this may not always be true. Indeed, a possible candidate for a proximate carcinogenic form of 2-naphthylamine appears to be a metabolite in which ortho-ring hydroxylation and N-hydroxylation mechanisms have both participated *(93)*. In addition, disubstituted anilines have been observed to display weak to moderate carcinogenic activity in experimental animals *(3)*. It is also commonly noted that ortho methyl or methoxy substituted derivatives of carcinogenic aromatic amines often retain potent carcinogenic activity. These metabolic and structural considerations suggest that certain of the azo dye food colorings are in need of reevaluation and it is not surprising that they have been the subject of recent criticism *(42)*.

Metabolic and structural considerations can be and have been important in the evaluation of certain artificial sweeteners. For example, the artificial sweetener, *p*-phenetylurea (Dulcin) (Fig. 10), was restricted from commercial use with the finding that it was able to induce tumors of the bladder and liver in rats. The wisdom of this regulatory action can now be appreciated. Dulcin is a close structural analog of *p*-acetophenetidin (Phenacetin) (Fig. 10), a chemical which has found common use as an analgesic drug. Although Phenacetin has apparently failed to display carcinogenic activity in experimental animals, the ability of this drug to produce renal damage has been known for some time and warning labels are required on analgesic preparations containing this compound. It is of great interest, then, that recent epidemiological studies have demonstrated a very high incidence of cancer of the kidney and bladder in humans chronically ingesting phenacetin-containing analgesic preparations *(2)*. It is perhaps very fortunate that there has been no need for epidemiological studies to determine the possible carcinogenicity of Dulcin in humans. The chronic ingestion of artificial sweeteners by a large segment of the population obviously requires that these food additives receive comprehensive safety evaluations.

As a more recent example, metabolic and structural considerations have proved important in the evaluation of the cyclamates, chemicals which also have found use as artificial sweetening agents. While the recent restriction of cyclamates by the Secretary of Health, Education, and Welfare *(28)* was based solely on the finding that cyclamates were carcinogenic for the urinary bladder of experimental animals *(9, 74)*, other research findings also were important. The discovery that humans and other animals were able to metabolically convert cyclamates to the more toxic cyclohexylamine was a signal to many investigators that further studies on the safety of cyclamates were warranted. That cyclohexylamine may be converted *in vivo* to N-hydroxycyclohexylamine *(29)* suggests a pathway for the metabolism of the cyclamates that parallels that observed for the metabolic activation of aromatic amines displaying carcinogenic activity for the urinary bladder *(76)*. The metabolic transformations observed with the cyclamates indicate the need for a chronic feeding study in which the urinary bladder of the test animals would be examined most carefully. Studies in which this was done *(28, 74))* have repeatedly demonstrated the carcinogenicity of the cyclamates whereas studies in which the urinary bladders were not examined histologically *(77)* are, for all intents and purposes, meaningless.

It seems apparent that attempts to produce non-carcinogenic artificial sweeteners by exploitation of various amine derivatives have met with little success. In view of the prominent position of aromatic amine derivatives in the classes of chemical carcinogens, it would seem advisable to avoid such chemicals in the search for new food additives. It should be added, parenthetically, that aside from safety evaluations, there is also an obvious need to evaluate the efficacy of new food additives before they are introduced into common use. With artificial food coloring agents, their efficacy in allowing the production of foods that are pleasing to the eye is easily determined, although the so-called "demand" for such esthetically pleasing foods is probably expli-

cable, in part, as a consumer response to "successful" advertising.

The situation is quite different, however, with the artificial sweeteners which have been widely promoted as a useful component of diet foods. Recent studies suggest that cyclamates stimulate the appetite of experimental animals, causing a greater gain in weight and more efficient utilization of food *(17)*. Indeed, a 1969 report from the Food and Drug Administration, published just prior to the confused debate over restriction of cyclamates, stated that "none of the few controlled studies reported to date have established a useful role for nonnutritive sweeteners as weight-reducing aids except under the most carefully controlled conditions" *(81)*. While further studies will be required to determine whether or not artificial sweeteners have any useful role in diet foods, artificial sweeteners would be of definite value to those people who, for medical reasons, must restrict their intake of sugar. In this regard, reports that an amino acid dipeptide may be useful as an artificial sweetener are very exciting *(57)*. The search for new food additives should certainly continue but, in many instances, perhaps this search should continue along avenues different from those used in the past and with motivations different from those used in the past.

CONCLUSIONS

The need for determining potential hazards associated with use of biologically-foreign chemical commodities has long been recognized. With food additives, the individual has little control over the number or amount of these substances he consumes. Indeed, food additives may be consumed almost daily over much of the life-span of the individual and the amount consumed, which is extremely difficult to regulate, may vary considerably from individual to individual. Consequently, the possibility of long-term hazards such as carcinogenesis is a very important consideration *(11)*. Fortunately, most chemical commodities are subjected to chronic toxicity tests in experimental animals in an attempt to spare the human population unnecessary exposure to potential chemical carcinogens. This is of utmost importance in view of the numerous examples of chemical carcinogens which by one means or another have managed to escape detection prior to their introduction into the human environment.

From an industrial point of view, however, early detection of potential chemical carcinogens would also seem most desirable as a means of avoiding a possibly wasted expenditure of capital in further research and development. Furthermore, it would seem even more desirable to be able to predict those chemicals with carcinogenic potential so that they might be eliminated from consideration as a possible chemical commodities.

It should be apparent that, at present, metabolic and structural considerations cannot be used as proof of the carcinogenicity (or non-carcinogenicity) of a chemical. While the present review is necessarily not a comprehensive treatment of this subject, an attempt has been made in the above examples, to demonstrate that such considerations can often provide important clues in the safety evaluation of chemicals. Metabolic studies can often be utilized as an aid in the design of chronic toxicity tests in experimental animals and can often be utilized to support the interpretation of results from such tests. In this sense, metabolic and structural considerations might serve as a useful adjuvant in the commercial exploitation of chemicals.

In addition, metabolic and structural considerations also might prove important in making judgments regarding the potential carcinogenicity of chemicals. Utilization of this approach by investigators of chemical carcinogenesis has been rather successful in the discovery of new chemical carcinogens. There is no reason why this approach cannot be successfully utilized to help discover non-carcinogenic chemicals. Thus, avoidance of attempts to exploit chemicals structurally-related or metabolically related to known carcinogens and their proximate forms would seem indicated. Numerous recent examples point out the feasibility of this approach.

Finally, elucidation of metabolic pathways generally applicable to activation of individual classes of chemical carcinogens may permit prediction of the carcinogenicity of foreign molecules from measures of their *in vivo* and *in vitro* conversion to chemically-reactive electrophiles.

Production of chemical commodities would ideally begin with exploitation of chemicals with *proven efficacy* in meeting some *essential use*. With this honest starting point, subjection of the chemical to comprehensive safety evaluations would hopefully, in addition to preventing human exposure to potentially carcinogenic chemicals, allow detection of potentially hazardous chemical commodities before they become firmly entrenched in existing life styles and before they become the economic basis for the existence of large industries. As with many of the other serious problems facing modern man, solutions to problems of safety and adequately feeding a growing human population will require knowledge from diverse disciplines. It is likely that continued research in the mechanisms of chemical carcinogenesis will provide knowledge needed for a more efficient exploitation of safe and useful chemical commodities.

If the present review has stimulated interest in this approach, it will have served its purpose.

ACKNOWLEDGMENT

The authors thank Mrs. Kathleen J. Deighton and Mrs. Susan Pope for their assistance in preparing the manuscript.

REFERENCES

1. Abell, C. W., H. L. Falk, M. B. Shimkin, E. K. Weisburger, and J. H. Weisburger. 1965. Uracil mustard: A potent inducer of lung tumors in mice. Sci. 147:1443-1444.

2. Angervall, L., U. Bengtsson, C. G. Zetterluno, and M. Zsigmond. 1969. Renal pelvic carcinoma in a Swedish district with abuse of a Phenacetin-containing drug. Brit. J. Urol. 41:401-405.

3. Arcos, J. C., and M. F. Argus. 1968. Molecular geometry and carcinogenic activity of aromatic compounds. New perspectives. Advances Cancer Res. 11:305-471.

4. Belman, S., W. Troll, G. Teebor, and F. Mukai. 1968. The carcinogenic and mutagenic properties of N-hydroxyaminonaphthalenes. Cancer Res. 28:535-542.

5. Bickel, M. H. 1969. The pharmacology and biochemistry of N-oxides. Pharmacology Rev. 21:325-355.

6. Boutwell, R. K., N. H. Colburn, and C. C. Muckerman. 1969. *In vivo* reactions β-propiolactone. Ann. N. Y. Acad. Sci. 163:751-764.

7. Brookes, P., and P. D. Lawley. 1960. The reaction of mustard gas with nucleic acids *in vitro* and *in vivo*. Biochem. J. 77:478-484.

8. Brown, G. B. 1968. Purine-N-oxides and cancer. Progress Nucleic Acid Res. 8:209-255.

9. Bryan, G. T., and E. Ertürk. 1970. Production of mouse urinary bladder carcinomas by sodium cyclamate. Sci. 167:996-998.

10. Bryan, G. T., and G. M. Lower, Jr. 1967. Thin layer chromatography: A nonspecific method for demonstrating carcinogen-DNA interactions. J. Chromatogr. 31:384-389.

11. Bryan, G. T., and G. M. Lower, Jr. 1970. Diverse origins of ubiquitous environmental carcinogenic hazards and the importance of safety testing. J. Milk Food Technol. 33:506-515.

12. Carter, H. E. (Chairman). 1961. Problems in the evaluation of carcinogenic hazard from use of food additives. Subcommittee on Carcinogenesis, Food Protection Committee. Cancer Res. 21:429-456.

13. Clapp, N. K., A. W. Craig, and R. E. Toya, Sr. 1968. Oncogenicity by methyl methanesulfonate in male RF mice. Sci. 161:913-914.

14. Cohen, S. M., E. Ertürk, J. M. Price, and G. T. Bryan. 1970. Comparative carcinogenicity in the rat of Z-hydrazinothiazoles with nitrofuryl, nitrophenyl, or aminophenyl substituents in the 4-position. Cancer Res. 30:897-901.

15. Craddock, V. M. 1965. Reaction of the carcinogen dimethylnitrosamine with proteins and with thiol compounds in the intact animal. Biochem. J. 94:323-330.

16. Dacre, J. C. 1965. Chronic toxicity and carcinogenicity studies on Citrus Red No. 2. Proc. Univ. Otago Med. School 43:31-33.

17. Dalderup, L. M., and W. Visser. 1969. Effects of sodium cyclamate on the growth of rats compared with other variations in the diet. Nature 221:91-92.

18. Debaun, J. R., E. C. Miller, and J. A. Miller. 1970. N-hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. Cancer Res. 30:577-595.

19. Deichmann, W. B., and J. L. Radomski. 1963. The carcinogenicity and metabolism of 2-naphthylamine and related compounds. Indus. Med. Surg. 32:161-166.

20. Dickens, F. 1964. Carcinogenic lactones and related substances. Brit. Med. Bull. 20:96-101.

21. Dipple, A., P. D. Lawley, and P. Brookes. 1968. Theory of tumor initiation by chemical carcinogens: Dependence of activity on structure of ultimate carcinogen. European J. Cancer 4:493-506.

22. Druckrey, H., H. Kruse, and R. Preussmann. 1968. Propanesultone, a potent carcinogen. Die Naturwiss. 55:449.

23. Druckrey, H., H. Kruse, R. Preussmann, S. Ivankovic, and C. Landschütz. 1970. Carcinogene alkylierende Substanzen III. Alkyl-halogenide,-sulfate,-sulfonate und ringgespannte Heterocyclen. Z. Krebsforsch. 74:241-270.

24. Druckrey, H., R. Preussmann, and S. Ivankovic. 1969. N-nitroso compounds in organotropic and transplacental carcinogenesis. Ann. N. Y. Acad. Sci. 163:676-696.

25. Druckrey, H., R. Preussmann, S. Ivankovic, and D. Schmähl. 1967. Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitrosoverbindungen an BD-Ratten. Z. Krebsforsch. 69:103-201.

26. Druckrey, H., R. Preussmann, S. Ivankovic, B. T. So, C. H. Schmidt, and J. Bücheler. 1966. Zur Erzeugung subcutaner Sarkome an Ratten. Z. Krebsforsch. 68:87-102.

27. Druckrey, H., R. Preussmann, N. Nashed, and S. Ivankovic. 1966. Carcinogene alkylierende Substanzen. I. Dimethylsulfat, carcinogene Wirkung an Ratten und wahrscheinliche Ursache von Berufskrebs. Z. Krebsforsch. 68:103-111.

28. Egeberg, R. O., J. L. Steinfeld, I. Frantz, G. C. Griffith, H. Knowles, Jr., E. Rosenow, H. Sebrell, and T. V. Itallie. 1970. Report to the Secretary of HEW from the Medical Advisory Group on Cyclamates. J. Amer. Med. Ass. 211: 1358-1361.

29. Elliot, T. H., N. Y. Lee-Yoong, and R. C. C. Tao. 1968. The metabolism of cyclohexylamine in rabbits. Biochem. J. 109: 11 p.

30. Enomoto, M., K. Sato, E. C. Miller, and J. A. Miller. 1968. Reactivity of the diacetyl derivative of the carcinogen 4-hydroxyaminoquinoline-1-oxide with DNA, RNA, and other nucleophiles. Life Sci. 7:1025-1032.

31. Ertürk, E., S. M. Cohen, J. M. Price, and G. T. Bryan. 1969. Pathogenesis, histology, and transplantability of urinary bladder carcinomas induced in albino rats by oral administration of N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide. Cancer Res. 29:2219-2228.

32. Ertürk, E., J. E. Morris, S. M. Cohen, J. M. Price, and G. T. Bryan. 1970. Transplantable rat mammary tumors induced by 5-nitro-2-furaldehyde semicarbazone and by formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide. Cancer Res. 30:1409-1412.

33. Farber, E. 1968. Biochemistry of carcinogenesis. Cancer Res. 28:1859-1869.

34. Feiser, L. F., and M. Feiser. 1962. Advanced organic chemistry. Reinhold Publishing Co., New York, p. 315-337.

35. Gelboin, H. V. 1967. Carcinogens, enzyme induction, and gene action. Advances Cancer Res. 10:1-81.

36. Gillete, J. R. 1966. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Advances Pharmacology 4:219-261.

37. Gillete, J. R. 1969. Significance of mixed oxygenases and nitroreductase in drug metabolism. Ann. N. Y. Acad. Sci. 160:558-570.

38. Goodall, C. M., W. Lijinsky, L. Tomatis, and C. E. M. Wenyon. 1970. Toxicity and oncogenicity of nitrosomethyl-

aniline and nitrosomethylcyclohexylamine. Toxicol. Appl. Pharm. 17:426-432.

39. Haerlin, R., R. Süssmuth, and F. Lingens. 1970. Mechanism of mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). V. Methylation of DNA by N-trideuteriomethyl-N'-nitro-N-nitroso-guanidine(D_3-MNNG). Fed. Exp. Biol. Soc. Letters 9:175-176.

40. Homburger, F., and J. R. Baker. 1969. Accelerated carcinogen testing. Progress Exp. Tumor Res. 11:384-394.

41. Hozumi, M. 1968. Reaction of the carcinogen 4-hydroxyamino-quinoline-1-oxide with sulfhydryl groups of proteins. Biochem. Pharmacology 17:769-777.

42. Hueper, W. C. 1969. Occupational and environmental cancers of the urinary system. Yale Univ. Press, New Haven. 465 p.

43. Jones, J. B., and J. M. Young. 1968. Carcinogenicity of lactones III. The reactions of unsaturated γ-lactones with L-cysteine. J. Med. Chem. 11:1176-1182.

44. Katritzky, A. R. 1970. The prototropic tautomerism of heteroaromatic compounds. Chimia 24:134-146.

45. Katritzky, A. R., and E. Lunt. 1969. N-oxides and related compounds. XXXV. Reactions of N-alkoxy-pyridinium and quinolinium cations with nucleophiles. Tetrahedron 25: 4291-4305.

46. Kelly, M. G., R. W. O'Gara, S. T. Yancey, K. Gadekar, C. Botkin, and V. T. Oliverio. 1969. Comparative carcinogenicity of N-isopropyl-α-(2-methylhydrazino)-p-toluamide·HCl (procarbazine hydrochloride), its degradation products, other hydrazines, and isonicotinic acid hydrazide. J. Nat. Cancer Inst. 42:337-344.

47. Kraybill, H. F. 1963. Carcinogenesis associated with foods, food additives, food degradation products, and related dietary factors. Clin. Pharmacology Therapy 4:73-87.

48. Krüger, F. W., M. Wiessler, and U. Rücker. 1970. Investigation of the alkylating action of 1,1-dimethylhydrazine. Biochem. Pharm. 19:1825.

49. Lawley, P. D., and P. Brookes. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem. J. 89:127-138.

50. Lijinsky, W., and S. S. Epstein. 1970. Nitrosamines as environmental carcinogens. Nature 225:21-23.

51. Lijinsky, W., J. Loo, and A. E. Ross. 1968. Mechanism of alkylation of nucleic acids by nitrosodimethylamine. Nature 218:1174-1175.

52. Lower, G. M., and G. T. Bryan. 1971. Metabolic activation of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. Amer. Ass. Cancer Res. (Abstract) (In press).

53. Magee, P. N. 1969. *In vivo* reactions of nitroso compounds. Ann. N. Y. Acad. Sci. 163:717-730.

54. Magee, P. N., and J. M. Barnes. 1967. Carcinogenic nitroso compounds. Advances Cancer Res. 10:163-246.

55. Malkin, M. F., and A. C. Zahalsky. 1966. Interaction of the water-soluble carcinogen, 4-nitroquinoline N-oxide with DNA. Sci. 154:1665-1667.

56. Matsumoto, H., and H. H. Higa. 1966. Studies on methylazoxymethanol, the aglycone of cycasin: methylation of nucleic acids *in vitro*. Biochem. J. 98:20C-22C.

57. Mazur, R. H., J. M. Schlatter, and L. Jokay. 1969. New sugar substitute. Annu. Meeting Inst. Food Technol. No. 24, Chicago, Ill. Discussed by K. O. Herz, 1969. Food Technol. 24:14-16.

58. Miller, E. C., and J. A. Miller. 1966. Mechanisms of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. Pharmacology Rev. 18:805-838.

59. Miller, E. C., and J. A. Miller. 1969. Studies on the mechanism of activation of aromatic amine and amide carcinogens to ultimate carcinogenic electrophilic reactants. Ann. N. Y. Acad. Sci. 163:731-750.

60. Miller, J. A. 1970. Carcinogenesis by chemicals: An overview. G. H. A. Clowes Memorial Lecture. Cancer Res. 30:559-576.

61. Miller, J. A., and E. C. Miller. 1966. A survey of molecular aspects of chemical carcinogenesis. Lab. Invest. 15:217-242.

62. Miller, J. A., and E. C. Miller. 1969. The metabolic activation of carcinogenic aromatic amines and amides. Progress Exp. Tumor Res. 11:273-301.

63. Montesano, R., and P. N. Magee. 1970. Metabolism of dimethylnitrosamine by human liver slices *in vitro*. Nature 228:173-174.

64. Morris, J. E., J. M. Price, J. J. Lalich, and R. J. Stein. 1969. The carcinogenic activity of some 5-nitrofuran derivatives in the rat. Cancer Res. 29:2145-2156.

65. Mustafa, A. 1954. The chemistry of sultones and sultams. Chem. Rev. 54:195-223.

66. Nagata, Y., and H. Matsumoto. 1969. Studies on methylazoxymethanol: Methylation of nucleic acids in the fetal rat brain. Proc. Soc. Exp. Biol. Med. 132:383-385.

67. Parke, Dennis V. 1968. The biochemistry of foreign compounds. Pergamon Press. 269 p.

68. Pitot, H. C., and C. Heidelberger. 1963. Metabolic regulatory circuits and carcinogenesis. Cancer Res. 23:1694-1700.

69. Preussman, R., H. Druckrey, and J. Bücheler. 1968. Carcinogene Wirkung von Phenyl-nitroso-harnstoff. Z. Krebsforsch. 71:63-65.

70. Preussmann, R., H. Druckrey, S. Ivankovic, and A. von Hodenberg. 1969. Chemical structure and carcinogenicity of aliphatic hydrazo, azo, and azoxy compounds and of triazenes, potential *in vivo* alkylating agents. Ann. N. Y. Acad. Sci. 163:697-716.

71. Preussmann, R., and A. von Hodenberg. 1970. Mechanism of carcinogenesis with 1-aryl-3,3-dialkyltriazenes. II. *In vitro* alkylation of guanosine, RNA and DNA with arylmonoalkyltriazenes to form 7-alkylguanine. Biochem. Pharmacology 19:1505-1508.

72. Preussmann, R., A. von Hodenberg, and H. Hengy. 1969. Mechanism of carcinogenesis with 1-aryl-3,3-dialkyltriazenes. Enzymatic dealkylation by rat liver microsomal fraction *in vitro*. Biochem. Pharmacology 18:1-13.

73. Price, C. C., G. M. Gaucher, P. Koneru, R. Shibakawa, J. R. Sowa, and M. Yamaguchi. 1969. Mechanism of action of alkylating agents. Ann. N. Y. Acad. Sci. 163:593-600.

74. Price, J. M., C. G. Biava, B. L. Oser, E. E. Vogin, J. Steinfeld, and H. L. Ley. 1970. Bladder tumors in rats fed cyclohexylamine or high doses of a mixture of cyclamate and saccharin. Sci. 167: 1131-1132.

75. Radomski, J. L. 1961. The absorption, fate and excretion of Citrus Red No. 2 (2,5-dimethoxyphenyl-azo-2-naphthol) and external D&C Red No. 14 (1-xylyazo-2-naphthol). J. Pharm. Exp. Therapeutics 134:100-109.

76. Radomski, J. L., and E. Brill. 1970. Bladder cancer induction by aromatic amines: Role of N-hydroxy metabolites. Sci. 167:992-993.

77. Roe, F. J. C., L. S. Levy, and R. L. Carter. 1970. Feeding studies on sodium cyclamate, saccharin and sucrose for carcinogenic and tumour-promoting activity. Food Cosmetics Toxicol. 8:135-145.

78. Rutman, R. J., E. H. L. Chun, and J. Jones. 1969. Observations on the mechanism of the alkylation reaction between nitrogen mustard and DNA. Biochim. Biophys. Acta 174:663-673.

79. Sander, V. J., F. Schweinsberg, and H. P. Menz. 1968.

Untersuchungen über die Entstehung cancerogene Nitrosamine im Magen. Hoppe-Zeylers Z. Physiol. Chem. 349:1691-1697.

80. Schoental, R. 1970. Hepatotoxic activity of retrorsine, senkirkine and hydroxysenkirkine in newborn rats, and the role of epoxides in carcinogenesis by pyrrolizidine alkaloids and aflatoxins. Nature 227:401-402.

81. Schrogie, J. J. 1969. Artificial sweeteners. FDA Papers 3:12-13.

82. Scribner, J. D., J. A. Miller, and E. C. Miller. 1970. Nucleophilic substitution on carcinogenic N-acetoxy-N-arylacetamides. Cancer Res. 30:1570-1579.

83. Shank, R. C., and P. N. Magee. 1967. Similarities between the biochemical actions of cycasin and dimethylnitrosamine. Biochem. J. 105:521-527.

84. Shealy, Y. F., and C. A. Krauth. 1966. Imidazoles. II. 5(or 4)-(monosubstituted triazeno)imidazole-4 (or 5)-carboxamides. J. Med. Chem. 9:34-38.

85. Shubik, P. 1970. Symposium on the evaluation of the safety of food additives and chemical residues: III. The role of the chronic study in the laboratory animal for evaluation of safety. Toxicol. Appl. Pharmacology 16:507-512.

86. Skibba, J. L., D. Beal, G. Rameriz, and G. T. Bryan. 1970. N-demethylation of the antineoplastic agent 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide by rats and man. Cancer Res. 30:147-150.

87. Skibba, J. L., E. Ertürk, and G. T. Bryan. 1970. Induction of thymic lymphosarcoma and mammary adenocarcinomas in rats by oral administration of the antitumor agent. 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide. Cancer 26:1000-1005.

88. Stöhrer, G., and G. B. Brown. 1970. Oncogenic purine derivatives: Evidence for a possible proximate oncogen. Sci. 167:1622-1624.

89. Sugimura, T., K. Okabe, and M. Nagao. 1966. The metabolism of 4-nitroquinoline-1-oxide, a carcinogen. III. An enzyme catalyzing the conversion of 4-nitroquinoline-1-oxide to 4-hydroxyaminoquinoline-1-oxide in rat liver and hepatomas. Cancer Res. 26:1717-1721.

90. Swann, P. F., and P. N. Magee. 1968. Nitrosamine-induced carcinogenesis. The alkylation of nucleic acids of the rat by N-methyl-N-nitrosourea, dimethylnitrosamine, dimethylsulphate, and methyl methanesulphonate. Biochem. J. 110:39-47.

91. Tada, M., M. Tada, and T. Takahashi. 1967. Interaction of a carcinogen, 4-hydroxyaminoquinoline-1-oxide with nucleic acids. Biochem. Biophys. Res. Commun. 29:469-477.

92. Taylor, J. D., H. E. Paul, and M. F. Paul. 1951. Metabolism of the nitrofurans. III. Studies with xanthine oxidase *in vitro*. J. Biol. Chem. 191:223-231.

93. Troll, W., S. Belman, and F. Mukai. 1969. Studies on the nature of the proximal bladder carcinogens. J. Nat. Cancer Inst. 43:283-286.

94. Truhart, R. (ed.) 1967. Potential carcinogenic hazards from drugs. UICC Monograph Series Vol. 7: 249 p.

95. United States Department of Health, Education, and Welfare. 1969. Report of the Secretary's Commission on Pesticides and their Relationship to Environmental Health, Parts I and II. U.S. Government Printing Office, Washington, D. C. 677 p.

96. Van Duuren, B. L. 1969. Carcinogenic epoxides, lactones, and halo-ethers and their mode of action. Ann. N. Y. Acad. Sci. 163:633-651.

97. Van Duuren, B. L., L. Langseth, L. Orris, M. Baden, and M. Kuschner. 1967. Carcinogencity of epoxides, lactones, and peroxy compounds. V. Subcutaneous injection in rats. J. Nat. Cancer Inst. 39:1213-1216.

98. Van Duuren, B. L., L. Langseth, B. M. Goldschmidt, and L. Orris. 1967. Carcinogenicity of epoxides, lactones, and peroxy compounds. VI. Structure and carcinogenic activity. J. Nat. Cancer Inst. 39:1217-1228.

99. Wada, S., M. Miyanishi, Y. Nishimoto, S. Kambe, and R. W. Miller. 1968. Mustard gas as a cause of respiratory neoplasia in man. The Lancet 1:1161-1163.

100. Weinstein, I. B. 1968. A possible role of transfer RNA in the mechanism of carcinogenesis. Cancer Res. 28: 1871-1874.

101. Weisburger, J. H., and E. K. Weisburger. 1968. Food additives and chemical carcinogens: On the concept of zero tolerance. Food Cosmetics Toxicol. 6:235-242.

102. Williams, R. T. 1959. Detoxication mechanisms. John Wiley and Sons, Inc., New York. 2nd Ed. 796 p.

103. Wittkop, J. A., R. A. Prough, and D. J. Reed. 1969. Oxidative demethylation of N-methylhydrazines by rat liver microsomes. Arch. Biochem. Biophys. 134:308-315.

104. Wölcke, U., N. J. M. Birdsall, and G. B. Brown. 1969. Purine N-oxides. XXIV. The preparation of 8-substituted xanthines and guanines by a nucleophilic displacement of a 3-substituent. Tetrahedron Letters 10:785-788.

MICROORGANISMS AS INDUCERS OF OVIPOSITION FOR THE CHEESE SKIPPER, *PIOPHILA CASEI* (L) DIPTERA

BILLY RAY JONES, PAUL P. GRAHAM, AND R. F. KELLY

Department of Food Science and Technology
Virginia Polytechnic Institute
Blacksburg, Virginia 24061

(Received for publication March 17, 1971)

ABSTRACT

A technique was developed to isolate the bacteria associated with the cheese skipper, *Piophila casei* (L) Diptera, and to study their influence on oviposition. No bacteria were isolated from the alimentary tract of newly emerged female flies. However, 41 isolates were obtained both from the alimentary tract and from tubes inoculated by 3 day-old flies. The 41 isolates were grouped according to their morphological and physiological characteristics and separately offered on inoculated ham to 50 adult female cheese skippers using five ham treatments per group. Flies deposited eggs on the untreated cured ham sample in all trials, with the exception of one group of isolates. Flies deposited numerous eggs on the sterilized inoculated sample. This isolate was then identified through a series of biochemical tests as *Proteus vulgaris*. Repeated trials using a known culture of *P. vulgaris* as the test inoculum yielded identical results.

Two other species, *Proteus mirabilis* and *Proteus morganii*, also were exposed to 50 flies using identical ham treatments. Although flies showed some tendency to deposit eggs on sterile ham inoculated with these species of *Proteus*, neither produced conditions more favorable than the cured ham itself.

Seven genera of bacteria, isolated in predominant numbers from the cured ham also were offered to the cheese skipper using the five ham treatments. (Although four of these genera produced proteolytic enzymes, flies always deposited eggs on the untreated cured ham sample.)

Production of a high quality country ham requires aging at a temperature of 21 C with a relative humidity of 65%. According to Christian *(5)*, these conditions produce a ham with no mold, a minimum amount of shrinkage, and most important, a uniform product throughout the year. Because of the temperature and relative humidity requirements for aging, combined with the fact that aging may take place in a building not designed to eliminate insects, the cured ham is the host of a number of insect pests. *Piophila casei* or "The Cheese Skipper," is now considered the principal insect species that attacks cured meat.

Control of *P. casei* has been shown to be difficult. Fulton *(9)*, Gerhardt and Lindgren *(10)*, and Kelly and Graham *(17)*, have investigated the usefulness of certain wrapping materials in prevention of insect infestation. From their work, it appears that neither kraft paper bags, certain dips or other wrappers, nor polycarbonate film were effective when high populations of *P. casei* were present.

Since the early 1940's chemical fumigants have proven to be the best method for control of *P. casei*. However, a number of these chemicals have been removed from the market because they leave high residues in certain food products *(9, 18, 20)*. Kelly and Graham *(17)* discovered that fumigation of cured hams with methyl bromide resulted in bromide residue levels far above the Food and Drug Administration (FDA) tolerance levels (125 ppm) in meat products.

Although the symbiotes of the cheese skipper have not been studied in detail, a number of investigators have shown relationships to exist between other insects and microorganisms *(1, 13, 18, 19, 21)*. Microorganisms are also important in the digestive system of many insects and probably aid the enzyme system of insects to produce materials required for their nutritional well being *(2, 5, 8, 11, 13)*.

Since a widely varying microbial flora is known to exist on dry cured ham *(12, 15)*, and since bacteria do play an important role in the hatching, growth, and development of many insects *(14, 21, 23)*, the bacteria associated with *P. casei* and their influence on oviposition were investigated.

PRELIMINARY PROCEDURES AND RESULTS

A preliminary study was undertaken to validate the reports of early workers *(9, 22)*. They noted that the cheese skipper was very temperamental about where it laid its eggs, and that cured meat was preferred over fresh meat as an oviposition site.

Fifty adult skippers were selected at random from the rearing cage and released in an experimental cage. Two ham samples, one fresh and one cured, were blended in separate blendors at 3450 rpm for 5 min, after which a 50-g sample of each was taken. The two 50-g samples were then exposed to the 50 flies for a period of 2 hr, during which time observations on oviposition were made. Following the 2-hr time interval, the samples were removed and examined microscopically for eggs. This study was repeated four times using the same flies. In all four trials, flies deposited eggs only on the cured ham samples during the 2-hr exposure period.

EXPERIMENTAL PROCEDURE

Isolation of bacteria associated with the cheese skipper

The bacteria associated with the cheese skipper were isolated in three separate phases. The first phase was to isolate the

bacteria from the three main parts of the alimentary tract, the foregut, midgut, and hindgut of the newly emerged female. Fifteen pupae were selected and placed in separate, sterile petri dishes. Immediately upon emergence from the pupa, each imago was sexed and all males discarded. The first four females to emerge were used for the isolation procedure which was conducted in the following manner: flies were temporarily exposed to refrigeration (0-5 C) to facilitate handling; then, using a dissecting microscope and sterile insect dissecting equipment, each fly was opened ventrally through the abdomen and thorax. Sterile Ringer's solution was applied to the tissue to prevent drying. After removing the surrounding tissue, the alimentary tract was exposed. The entire tract was removed, opened, and its contents streaked on four sterile Tryptone Glucose Extract agar (TGEA) (Difco) *(7)* agar plates. This procedure was repeated on all four flies, yielding a total of 16 plates. The TGE plates were then incubated at 37 C for 48 hr.

The second phase of the isolation procedure was executed in the same manner as phase one, with the exception of the following: the four female flies in this phase were selected at random from the rearing cage, and had been exposed to feed, water, and male flies, and were approximately 3 days old. The 16 TGE plates obtained in this phase were also incubated at 37 C for 48 hr.

In phase three, 50 flies were selected at random from the rearing cage and released into the experimental cage. Twelve tubes of chopped meat medium, four each of 6 ml chopped meat medium plus 6.5% salt; 6 ml chopped meat; and 6 ml chopped meat plus 5% glucose, were selected for this phase of the experiment. Two tubes of each were exposed to the 50 flies. Within 7 hr, flies were observed in all tubes. After 10 hr, all tubes were assumed inoculated. The six tubes were removed and incubated at 37 C for 24 hr. Following the incubation period, 3 ml aliquots of each of the inoculated tubes plus a 3 ml control tube of each were exposed to 50 additional flies. After 6 hr, all tubes were removed and the following mixed cultures prepared: *(a)* chopped meat (6.5% salt) inoculated on chopped meat (6.5% salt), *(b)* chopped meat medium inoculated on chopped meat (6.5% salt), *(c)* chopped meat medium inoculated on chopped meat medium, and *(d)* chopped meat (6.5% salt) inoculated on chopped meat medium. After incubation of the four mixed cultures, 3 ml aliquots of each of the cultures were again exposed to the flies for 6 hr, after which time duplicates of tubes C and D were used for the isolation procedure.

Separation, grouping, and tentative identification of bacteria

Colonies were picked from all streaked plates, according to their morphological characteristics, and restreaked on sterile TGE plates. After 24 hr incubation, isolates were again picked and a gram stain of each was made. Colonies were again restreaked to obtain isolated colonies. This procedure was repeated to assure purification of isolates. Each isolate was then transferred to two brain heart infusion agar (Difco) slants to be held for further use. A total of 24 isolates was obtained from the previously described procedure. All isolates were grouped and some tentative identifications were made using the procedure of Cowan and Steel *(6)*.

Separation, grouping, and tentative identification of the bacteria from phase three were conducted using the procedure of Cato et al. *(4)*. Duplicates of the two inoculated tubes were streaked on egg yolk plates (EYP) and blood agar plates (BAP). One set was incubated aerobically and the other anaerobically. Brain heart infusion agar—roll streak (BHIA-RS) tubes also were made from each tube. BHIA-RS and aerobic plates were incubated at 37 C until growth was apparent. Anaerobic plates were incubated at 37 C for 48 hr.

Using a dissecting microscope, all plates and streaks were examined and colony types picked. When necessary, some colonies were restreaked to obtain isolated colonies.

Thirteen single colonies were isolated from the aerobic plates and four from the anaerobic plates. Colonies from the anaerobic plates were then transferred to aerobic EYP and BAP to test for obligate anaerobes. Since the four anaerobic colonies also grew aerobically, all 17 single colonies were transferred to chopped meat broth and brain heart infusion agar slants to be held for further use.

Use of isolated bacteria to induce oviposition

The 15 groups of isolates from phases two and three were used in this technique. Since it has been noted by earlier workers that the cheese skipper prefers cured ham to fresh ham as a place for oviposition, it was believed that certain bacteria may play an important role in inducing oviposition.

Each group of isolates, in duplicate, were exposed to 50 flies using five 50-g samples treated under different conditions: *(a)* cured ham control (CHC)—aged 8 months, *(b)* cured ham autoclaved (CHA)—15 min at 121 C and 15 lb. pressure, *(c)* fresh ham inoculated then incubated (FHII)—36 hr at 37 C, *(d)* fresh ham incubated (FHI)—36 hr at 37 C, and *(e)* fresh ham autoclaved, inoculated then incubated (FHAI)—36 hr at 37 C.

Treated hams were exposed to 50 flies at room temperature for 2 hr and then examined microscopically for eggs. Identification of isolates inducing oviposition was conducted using the procedure of Cowan and Steel *(6)*.

Following the identification procedure, pure cultures of *Proteus morganii* and *Proteus mirabilis* were obtained from Midwest Culture Service. Three replicates of each culture including *Proteus vulgaris*, were exposed to the ham skippers by inoculating ham treatments as previously described. In addition, seven pure cultures one each of the genera *Bacillus, Micrococcus, Streptococcus, Paracolabactrum,* and *Pseudomonas* and two species of *Staphylococcus* isolated from the cured ham by Graham *(12)* also were exposed in replicates to 50 flies. Ham samples were treated in the same manner as described in the previous experimental procedure.

RESULTS AND DISCUSSION

No growth was obtained on any of the TGE plates streaked from the alimentary tract of the newly emerged female flies in phase one, which is in agreement with the work of Greenburg *(13)* who noted that the adult blowfly emerging from the pupal stage was free of bacteria. Greenburg attributed this to the fact that as the larvae approaches the pupal stage it stops feeding and its gut gradually contracts. Clusters of the bacteria remain sequestered in folds and crypts of the digestive tract, but these clusters are eliminated when the fly molts within its pupal case. In the fly larva the integument that is shed extends into the foregut and hindgut. The emerging fly, as it squirms free, leaves all or nearly all the bacteria behind in the pupal case. Elimination of bacteria at adulthood may be widespread among the diptera.

Phase two of the experiment, in which gravid females were used, yielded a total of 24 isolates. These

TABLE 1. FLY RESPONSE[a] TO 15 GROUPS OF ISOLATES USING FIVE HAM TREATMENTS

Group Tested	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
Trial	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Treatments																	10[b]						10[b]							
A - CH	*	*	+	*	+	*	*	*	+	+	*	*	*	*	0	*	50	*	*	*	+	+	0	50	*	*	*	*	*	*
B - CHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C - FHII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D - FHI	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E - FHAI	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0

[a]eggs deposited
[b]10-50 eggs after 2 hr
0 - no eggs after 2 hr
* - 100-200 eggs after 2 hr
+ - eggs too numerous to count after 2 hr
CH - cured ham aged 8 months
CHA - cured ham autoclaved
FHII - fresh ham inoculated and incubated
FHI - fresh ham incubated
FHAI - fresh ham autoclaved, inoculated, and incubated

TABLE 2. IDENTIFICATION OF BACTERIA[a] INDUCING OVIPOSITION OF *Piophila casei*

		Morphological and physiological properties											
	Isolate	Cell morphology	Colony morphology	(H_2S) TSI	Glucose	Lactose	Mannitol	Indole	V-P	Urease	OD	Gelatin	Identification
Group #12	ASC	Short gram- rod	Grayish spreader	+	AG	–	–	+	–	+	–	+	*Proteus vulgaris*
	ISC	Short gram- rod	Grayish spreader	+	AG	–	–	+	–	+	–	+	*Proteus vulgaris*
	ESC	Short gram- rod	Grayish spreader	+	AG	–	–	+	–	+	–	+	*Proteus vulgaris*
	DSCf	Short gram- rod	Grayish spreader	+	AG	–	–	+	–	+	–	+	*Proteus vulgaris*

[a]Cowan and Steel *(6)*
TSI- triple sugar iron
V-P - Voges Proskauer
OD - ornithin decarboxylase
AG - acid and gas

isolates were all gram-negative rods and grew aerobically on sterile TGE plates. All 24 isolates were grouped according to their morphological and physiological characteristics, and some tentative identifications were made. Ten isolates were tentatively identified as *Enterobacter aerogenes* and one as *Escherichia coli*. No identification of the remaining 13 isolates was made.

Phase three of the isolation procedure was arranged to give the flies an opportunity to inoculate three different tubes of chopped meat medium. Subjecting flies to this type of trial gave an opportunity to obtain bacteria associated, both externally and internally, with the cheese skipper. Following assumed inoculation, the first 8 tubes were incubated at 37 C for 24 hr, after which 3 ml aliquots of each inoculated tube plus a control tube of each were again exposed to 50 additional flies. After 6 hr exposure, flies had deposited eggs in the inoculated chopped meat medium and the inoculated chopped meat (6.5% salt) medium and chopped meat mixed with chopped meat (5% glucose) medium or any of the control tubes. From duplicates of the inoculated tubes in which eggs were deposited, four mixed cultures were made. After incubation of the four mixed cultures, 3 ml aliquots of each were again exposed to 50 additional flies. These flies deposited eggs in chopped meat medium plus 6.5% salt mixed with chopped meat medium and chopped meat mixed with chopped meat medium. From duplicates of these tubes, isolation procedures began.

A total of 13 aerobic and four facultative isolates were cultured from the inoculated tubes in this phase. All 17 isolates were grouped according to their morphological and physiological characteristics and tentative identification of these groups was made. Group 10 containing one single colony was identified as *Staphylococcus aureus*, group 11 containing seven

TABLE 3. FLY RESPONSE[a] TO THREE TRIALS OF FIVE HAM TREATMENTS USING *Proteus vulgaris*

Trial	1	2	3
Treatments			
A - CH	*	20-50[b]	0
B - CHA	0	0	0
C - FHII	20-50	0	0
D - FHI	0	0	0
E - FHAI	+	+	+

[a]eggs deposited
[b]20-50 eggs after 2 hr
0 - no eggs after 2 hr
* - 100-200 eggs after 2 hr
+ - eggs too numerous to count after 2 hr
CH - cured ham aged 8 months
CHA - cured ham autoclaved
FHII - fresh ham inoculated and incubated
FHI - fresh ham incubated
FHAI - fresh ham autoclaved, inoculated and incubated

single colonies as *Streptococcus faecalis*, and group 12 containing four single colonies as a *Proteus* species. The remaining five single colonies composed groups 13, 14, 15, and no identification was made.

Fly response to 15 groups of isolates

The isolates from phases two and three were grouped according to their morphological and physiological characteristics to yield 15 groups. Each group of isolates was then separately exposed for 2 hr to 50 flies using the five ham treatments previously described. Table 1 presents the response of the flies to each group of isolates. During the 2-hr exposure period, flies always deposited eggs on the untreated cured ham, with the exception of trials one and two of group 12. In these trials, flies deposited numerous eggs on treatment E and very few eggs on treatment A. The isolates used in this trial were obtained in phase three of the isolation procedure and had been tentatively identified as a *Proteus* species.

Identification of bacteria inducing oviposition

Table 2 presents the morphological and physiological properties of the four isolates in group 12 upon which identification is based. All isolates were gram-negative rods, 0.5 to 2.0 μ in length, occurring singly or in pairs with some chains. Agar colonies were grayish spreaders that produced hydrogen sulfide on TSI and rapid liquefaction. Acid and gas were produced from glucose, but no acid or gas from lactose or mannitol. They were all positive on urease and produced indole, and were negative on Voges-Proskauer and ornithine decarboxylase media. Final identification of all four isolates was *Proteus vulgaris*.

Fly response to three trials of Proteus vulgaris.

Following identification of the isolates in group 12, three replications were conducted using *P. vulgaris* as the test organism. Table 3 shows that within 2 hr flies had deposited numerous eggs on treatment E in all three trials. In trial one, a few eggs were found on treatment C which also was inoculated with the test organism. A few eggs also were found on treatment A of trials one and two. This gave a total of five trials using *P. vulgaris* as the test organism, and each trial produced very similar results. Since *P. vulgaris* produces a putrefactive odor and is commonly isolated from putrid meat, infusions, and abscesses, *(3)*, and since Steinhaus *(24)* noted that the simplest type of relationship between insects and microorganisms is the use of microorganisms or their by-product as food, the results from the five trials are not surprising.

Fly response to P. vulgaris, P. morganii, and P. mirabilis

TABLE 4. FLY RESPONSE[a] TO HAM TREATMENTS INOCULATED WITH *P. vulgaris*, *P. morganii*, AND *P. mirabilis*

Trial	1	2	3	1	2	3	1	2	3
Test organism	*P. vulgaris*	*P. vulgaris*	*P. vulgaris*	*P. morganii*	*P. morganii*	*P. morganii*	*P. mirabilis*	*P. mirabilis*	*P. mirabilis*
Treatments									
A - CH	0	*	0	+	*	*	*	*	0
B - CHA	0	0	0	0	0	0	0	0	0
C - FHII	0	0	0	0	0	0	0	0	0
D - FHI	0	0	0	0	0	0	0	0	0
E - FHAI	+	+	+	*	0	*	*	+	*

[a]eggs deposited
0 - no eggs after 2 hr
* - 100-200 eggs after 2 hr
+ - eggs too numerous to count after 2 hr
CH - cured ham aged 8 months
CHA - cured ham autoclaved
FHII -fresh ham inoculated and incubated
FHI - fresh ham incubated
FHAI - fresh ham autoclaved, inoculated and incubated

Proteus morganii and *P. mirabilis* also were tested in three trials. *Proteus vulgaris* was again offered to the flies in three trials. Table 4 shows that flies had a greater tendency to deposit eggs on *P. mirabilis* than on *P. morganii;* however, in all trials using these bacteria, flies continued to deposit eggs on all cured ham samples. Neither bacterium produced conditions that were more favorable than the cured ham except in trial three using *P. mirabilis*. *Proteus vulgaris* continued to produce conditions more favorable than the cured ham in all three trials.

Eight trials using *P. vulgaris* as the test organism all produced evidence that flies were attracted to sterilized fresh ham that was inoculated with this bacterium. However, in only one trial flies deposited eggs on the unsterilized fresh ham which had been

TABLE 5. FLY RESPONSE[a] TO SEVEN GENERA OF BACTERIA ISOLATED FROM CURED HAM

Trial	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Test organism	*Bacillus*	*Bacillus*	*Micrococcus*	*Micrococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Staphylococcus*	*Streptococcus*	*Streptococcus*	*Paracolobactum*	*Paracolobactum*	*Pseudomonas*	*Pseudomonas*
Treatments														
A - CH	*	+	20-30[c]	*	+	*	+	+	20-50[b]	*	+	+	+	*
B - CHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C - FHII	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D - FHI	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E - FHAI	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]eggs deposited
[b]20 - 50 eggs after 2 hr
[c]20-30 eggs after 2 hr
0 - no eggs after 2 hr
* - 100-200 eggs after 2 hr
+ - eggs too numerous to count after 2 hr
CH - cured ham aged 8 months
CHA - cured ham autoclaved
FHII - fresh ham inoculated and incubated
FHI - fresh ham incubated
FHAI - fresh ham autoclaved, inoculated and incubated

inoculated with the *Proteus* sp. This was trial one presented in Table 3, which shows only a few eggs deposited on sample C. From these results, it is postulated that bacteria already present on the fresh ham may have inhibited growth of *P. vulgaris* and that once the treatment was sterilized, as in treatment E, all bacteria were eliminated with the exception of the inoculum, which allowed faster growth and no competition from other bacteria.

Fly response to seven genera of bacteria isolated from cured ham

Since Hunt *(15)* found the largest percentage of proteolytic bacteria on hams aged from 4 to 18 months, it was thought that certain proteolytic bacteria found on the cured ham may be producing conditions which influence oviposition. Seven genera of bacteria, one proteolytic species of *Bacillus*, a *Micrococcus*, two species of *Staphylococcus*, a *Streptococcus* sp., a proteolytic *Paracolobactrum*, and a proteolytic species of *Pseudomonas*, isolated from cured ham by Graham *(12)*, were used in the final experiment. Table 5 shows fly response to two trials of each of these bacteria. In all trials, flies deposited eggs only on the cured ham samples.

Although organisms of the genus *Proteus* have not been isolated in predominant numbers from either fresh or cured meat products, Jensen *(16)* and Graham *(12)* have reported their presence in relatively low numbers. Although *Proteus* is a spoilage organism, the relative numbers of *Proteus* required to cause spoilage are not known. The *P. vulgaris* isolated in this work was obtained from tubes inoculated by the mature flies and not from the cured ham. Thus, it is possible that the fly itself is the carrier of the *Proteus* species. It also seems likely that once the fly has infested cured ham, the rapid growth and proteinaceous odor produced by *Proteus* continues to attract *P. casei*, either to feed or oviposit. A large number of insect larvae requires the action of microorganisms for their nutritive requirements. Thus, the chemicals inducing oviposition may be correlated with a source of subsistence for the larvae that emerge from the eggs oviposited by the female.

The major impact of these experiments is that microbial symbiotes may, as shown in this work, influence insect oviposition. The metabolic products of these symbiotes *(P. vulgaris)* may be nutritionally important for the normal growth and development of *P. casei*. Therefore, altering the insects' environment in such a way as to destroy its symbiotes presents a possible method of biological control of *P. casei*. This also would provide an extra margin of safety by eliminating the hazard of pesticide residues in cured hams.

REFERENCES

1. Atkin, E. E., and A. Bacot. 1917. The relation between hatching of the eggs and the development of the larvae of *Stegomyia fasciata*. Parasitol. 9:482.

2. Bogdanow, E. A. 1906. Development of the larvae of the flesh fly *(Calliphora vomitomia)* in sterile medium. Arch. Anat. Physiol. 113:97.

3. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed., The Williams and Wilkins Co., Baltimore, Maryland.

4. Cato, E. P., C. S. Cummins, L. V. Holdeman, J. L. Johnson, W. E. C. Moore, R. M. Smibert, and L. DS. Smith. 1969. Outline of clinical methods in anaerobic bacteriology.

The Anaerobic Laboratory, Virginia Polytechnic Institute.

5. Christian, J. A. 1962. Country ham boom in North Carolina. Nat. Provisioner 14:10.

6. Cowan, S. T., and K. J. Steel. 1966. Manual for the identification of medical bacteria. Cambridge University Press, Cambridge, England.

7. Difco Laboratories Incorporated. 1953. Difco manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures. 9th ed., Difco Laboratories, Incorporated, Detroit, Michigan.

8. Friend, W. G., E. H. Salkeld, and I. L. Stevenson. 1959. Nutrition of onion maggots, larvae of *Hylemya antiqua* (Meig.), with reference to other members of the genus *Hylemya*. Ann. N. Y. Acad. Sci. 77:384.

9. Fulton, B. B. 1953. The cheese skipper. North Carolina Agr. Exp. Sta. Bul. 103.

10. Gerhardt, P. D., and D. L. Lindgren. 1954. Penetration of various packaging films by common stored product insects. J. Econ. Entomol. 47:282.

11. Glaser, R. W. 1924. Microorganisms in the nutrition of Diptra. Amer. J. Trop. Med. 4:85.

12. Graham, P. P. 1970. The bacterial flora of prefrozen dry cured ham and its relationship to quality. Ph.D. dissertation, North Carolina State University, Raleigh.

13. Greenburg, B. 1965. Flies and disease. Scientific Amer. 213:92.

14. Hagen, K. S. 1966. Dependence of the olive fly *Dacus oleae* on symbioses with *Pseudomonas savastoni* for utilization of the olive. Nature 209:423.

15. Hunt, W. E. 1939. Qualities of hams and rapidity of aging as affected by curing and aging conditions and processes. Md. Agr. Exp. Sta. Bull. 428:31.

16. Jensen, L. B. 1954. Microbiology of meats. 3rd ed., The Garrard Press, Champaign, Illinois.

17. Kelly, R. F., and P. P. Graham. 1968. Pest prevention in country hams without pesticides. J. Anim. Sci. 27:142.

18. Kraybill, H. F. 1966. Pesticide residues in mammalian tissue, problems, incidences, and controls. Proc. 19th Ann. Recip. Meat Conf., Chicago, Illinois. 9:190.

19. Mansour, K. 1934. The intracellular microorganisms of some Bostrychid beetles. Quart, J. Microscop. Sci. 77: 243.

20. Pfeifer, C. C. 1945. Boric acid ointment. J. Amer. Med. Ass. 128:266.

21. Rozeboom, L. E. 1934. The effect of bacteria on the hatching of mosquito eggs. Amer. J. Hyg. 20:496.

22. Simmons, P. 1927. The cheese skipper as a pest in cured meats. U.S.D.A. Dept. Bul. 1453:1.

23. Steinhaus, E. A. 1949. Intra and extracellular microbiota of healthy insects. Principals of Insect Pathol. McGraw-Hill Book Company, Inc., New York 1:83.

SUPPLEMENT NO. 1 TO THE E-3-A SANITARY STANDARDS FOR INSTRUMENT FITTINGS AND CONNECTIONS USED ON LIQUID EGG PRODUCTS EQUIPMENT

Serial #E-0902

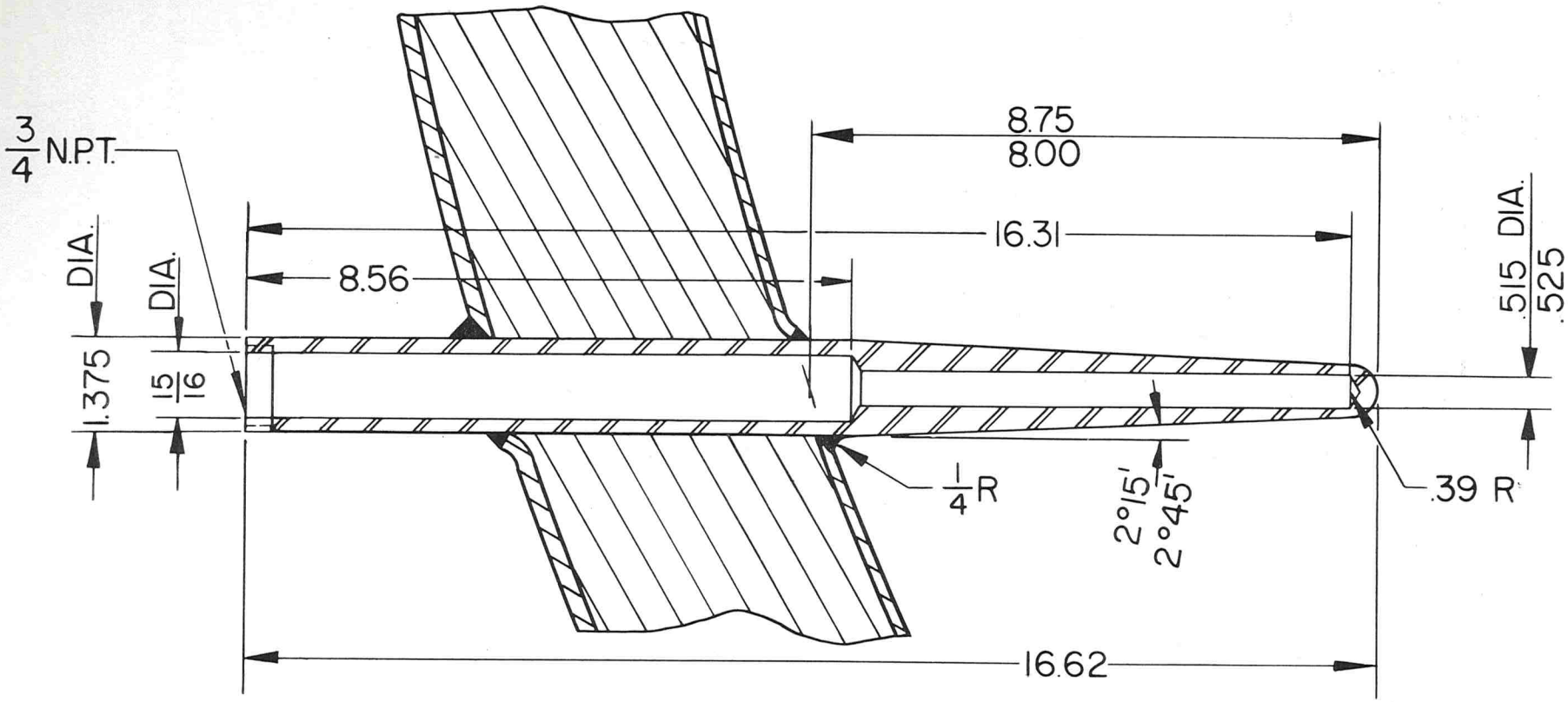
Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Public Health Service
United States Department of Agriculture
Institute of American Poultry Industries
Dairy and Food Industries Supply Association

This supplement incorporates the following fittings into this standard:

	Page No.	E-3-A Drawing No.
3A9 Temperature sensor well (short) for liquid egg and liquid egg product storage tanks	10	3A-101-11
3A10 Temperature sensor well (long) for liquid egg and liquid egg product storage tanks	11	3A-101-12

Note: The temperature sensor portions of the above wells are longer than those of the 3A7 and 3A8 thermometer wells. Other dimensions are different.

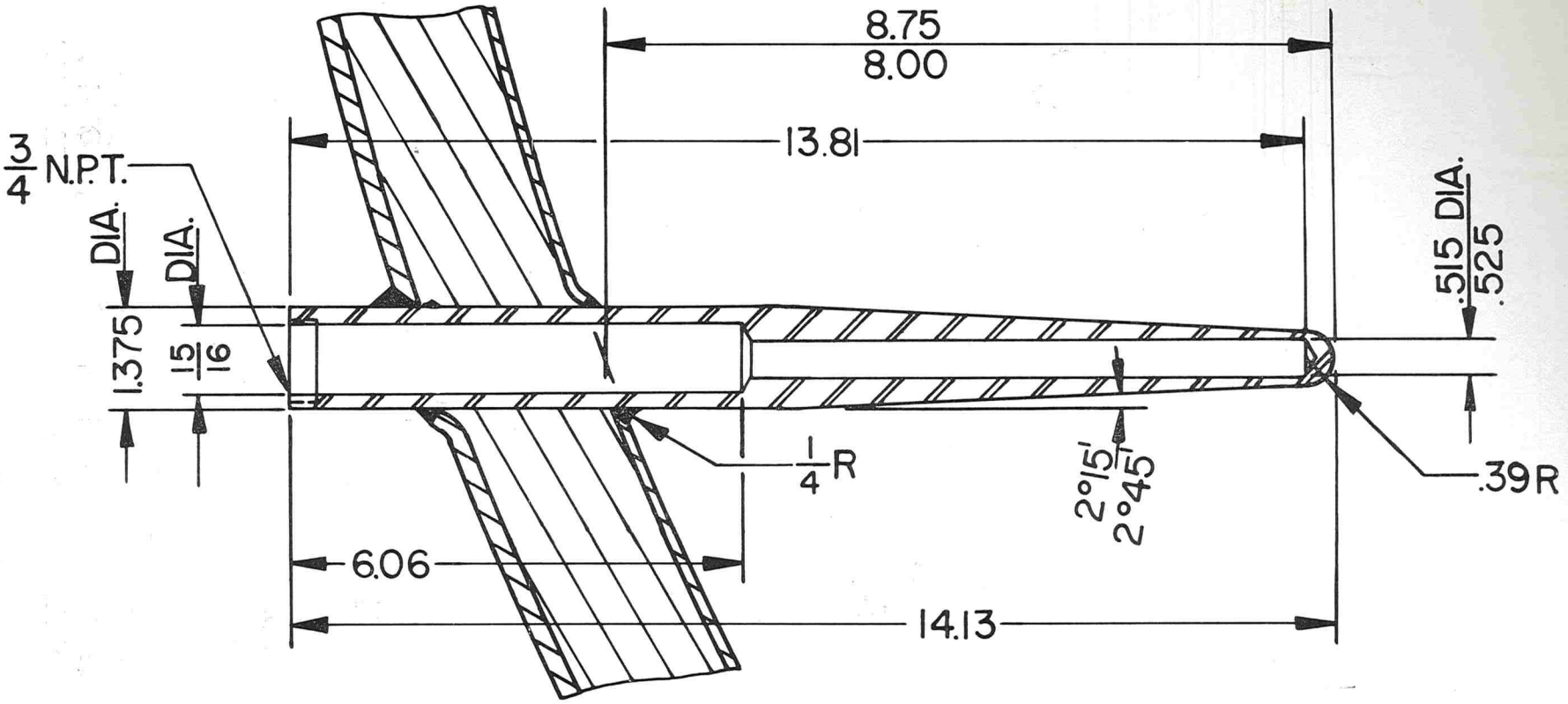
This supplement is effective October 28, 1971.



3A10 TEMPERATURE SENSOR WELL (LONG) FOR LIQUID EGG AND LIQUID EGG PRODUCTS STORAGE TANKS

Note: The temperature sensor portion of this well is longer than that of 3A8 Termometer Well. Other dimensions are different.

3A STANDARD
INSTRUMENT
FITTINGS & CONNECTIONS
3A-101-12



3A9 TEMPERATURE SENSOR WELL (SHORT) FOR LIQUID EGG AND LIQUID EGG PRODUCTS STORAGE TANKS

Note: The temperature sensor portion of this well is longer than that of 3A7 Thermometer Well. Other dimensions are different.

3A STANDARD
INSTRUMENT
FITTINGS & CONNECTIONS
3A-101-11

SUPPLEMENT NO. 2

3-A SANITARY STANDARDS FOR FITTINGS USED ON MILK AND MILK PRODUCTS EQUIPMENT AND USED ON SANITARY LINES, CONDUCTING MILK AND MILK PRODUCTS, REVISED

Serial #0812

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

This supplement adds the criteria for boot-seal type valves to Section E. SPECIAL CONSIDERATIONS 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, Revised, Serial #0809." (Reference 3-A Drawings No. 3A-100-33, No. 3A-100-34 and No. 3A-100-35).

These valves shall comply with the applicable provisions of this standard and the following:

E.7.1
The valve assembly shall consist of a boot seal, poppet, helix pin, knob, cap, and body. The boot seal shall separate the product from the working assembly. The valve shall not have a stuffing box.

E.7.2
All product contact surfaces and surfaces which may become product contact surfaces if the boot seal fails in service shall be readily accessible for cleaning and inspection.

E.7.3
The valve shall be of such design as to be self-draining in its installed position. The bodies of boot seal valves shall be permanently marked to show the self-draining angle, when the valve is placed in service and an arrow to indicate direction of product flow.

E.7.4
In the section of the poppet that is covered by the boot seal, there shall be three 3/32 inch holes for detection of leakage. One shall be in the bottom end of the poppet and two 180° apart in the sidewall.

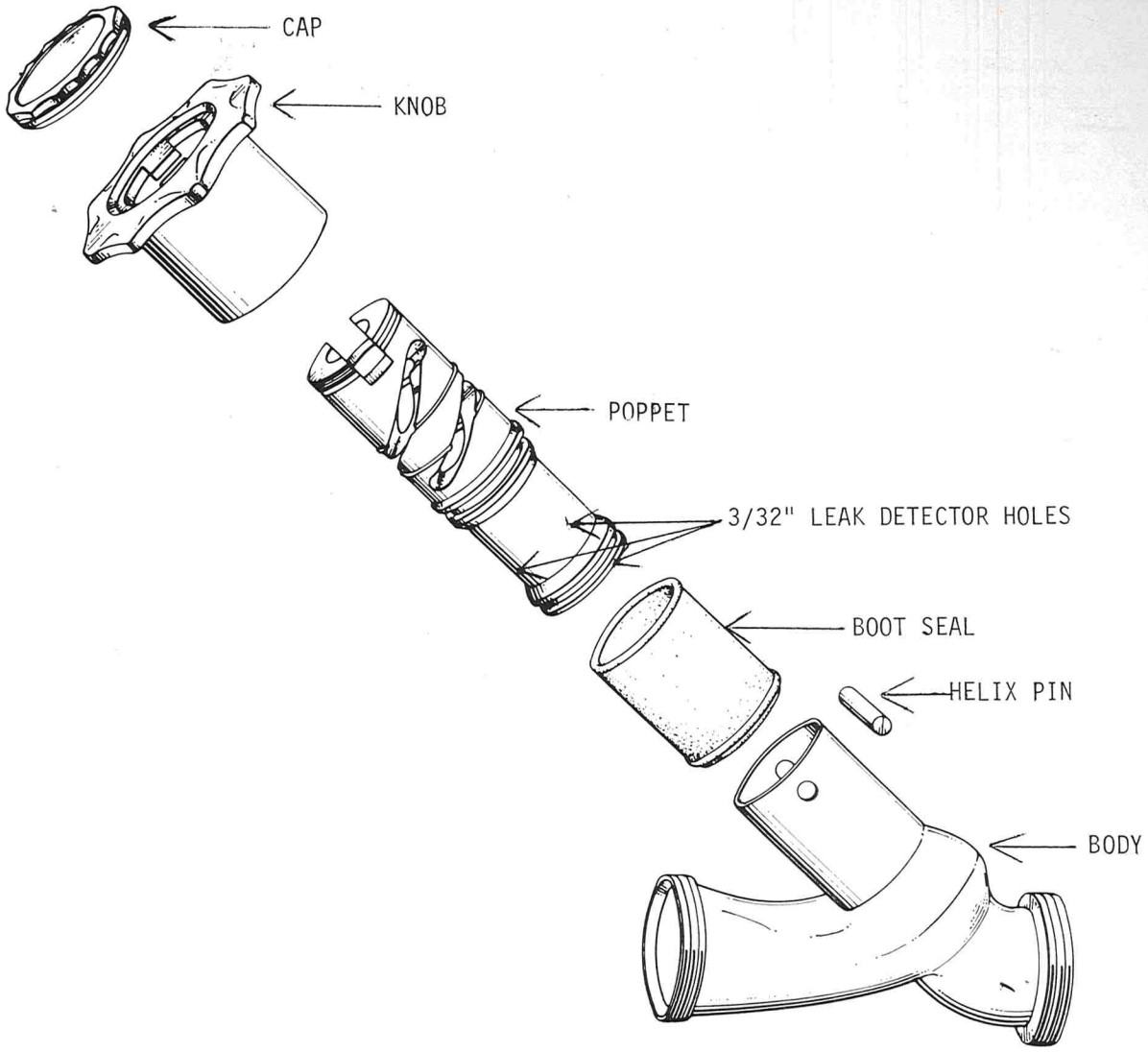
E.7.5
Rubber and rubber-like materials may be used for boot seals and in sealing applications.

E.7.6
Plastic materials may be used for poppets, boot seals, and in sealing applications.

E.7.7
All parts of the valve shall be readily demountable. Add the following to the list of drawings in subsection F.1 of this standard:

Fitting name	Page Number	3A Drawing Number
Boot seal-type valve	27	3A-100-33
	28	3A-100-34
	29	3A-100-35

This supplement is effective November 25, 1971.



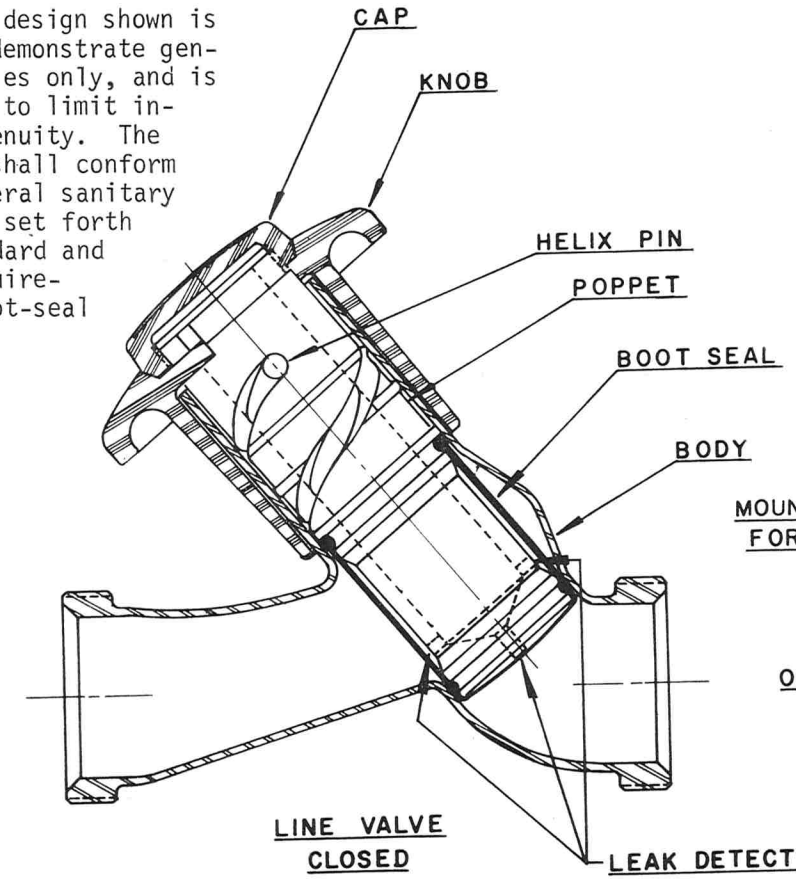
3-A STANDARD

SANITARY FITTINGS

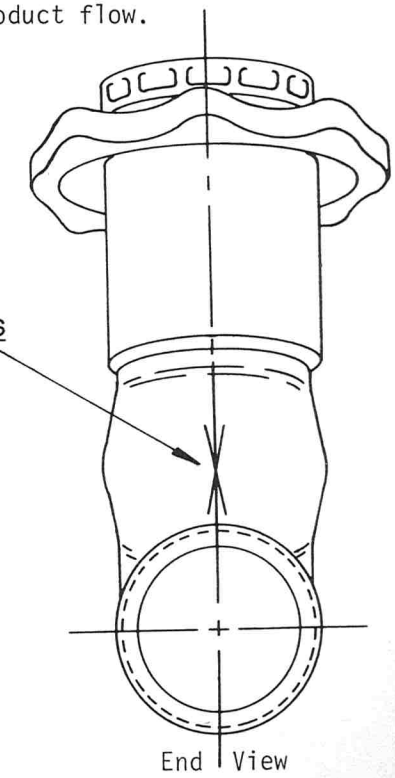
3A-100-33

BOOT SEAL VALVE
Exploded View

Note No. 1:
The internal design shown is intended to demonstrate general principles only, and is not intended to limit individual ingenuity. The design used shall conform with the general sanitary requirements set forth in this standard and specific requirements for boot-seal type valves.



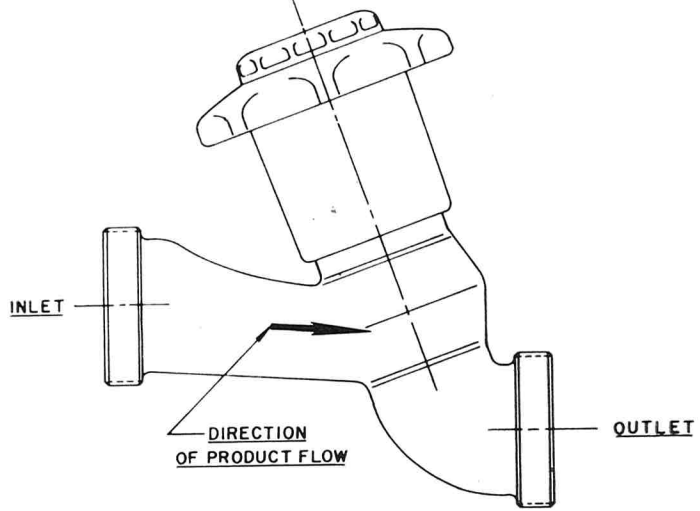
Note No. 2: The valve body is to be marked with a line (s) to show the self draining angle when the valve is placed in service, and an arrow to indicate the direction of product flow.



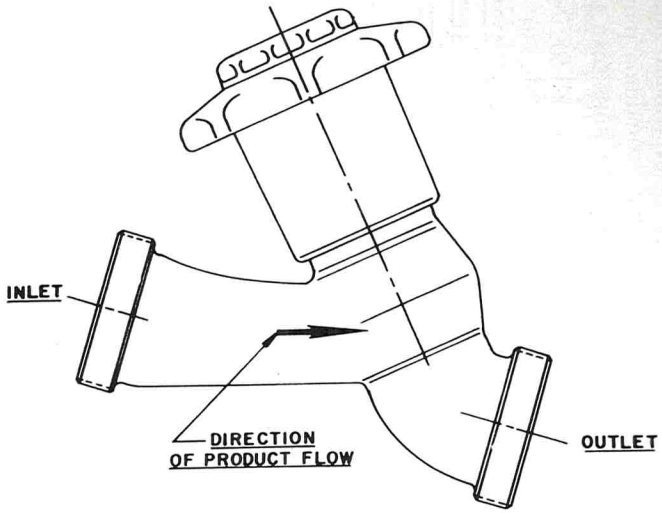
BOOT SEAL VALVE

3-A STANDARD
SANITARY FITTINGS
3A-100-34

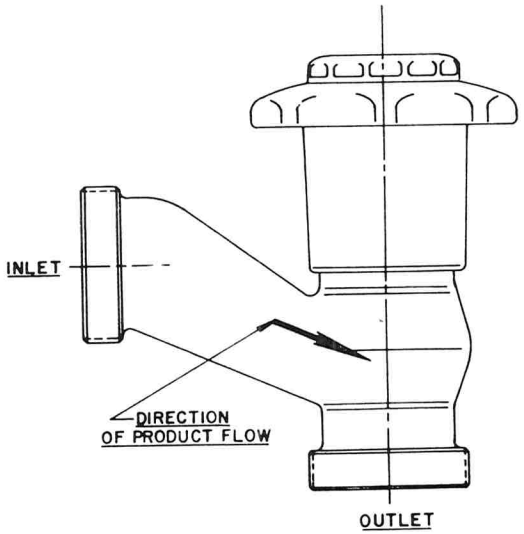
Note No. 3: The connections may be threaded or be of a design to be used with non-threaded sanitary connections (flanged, clamp type, etc.) or may have plain ends for use in welded sanitary pipelines. Threaded connections shall conform to connections on the drawings for other threaded fittings included in this standard. This would include the dimensions and form of the connection and the threads, and the inside diameter. Center-to-face dimensions may vary with valves made by different manufacturers.



GRAVITY VALVE



LINE VALVE



ANGLE VALVE

3-A STANDARD
SANITARY FITTINGS
3A-100-35

BOOT SEAL VALVE
Body Styles

AMENDMENT TO E-3-A SANITARY STANDARDS FOR THERMOMETER FITTINGS AND CONNECTIONS USED ON EGG PRODUCTS EQUIPMENT

Serial #E-0901

Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Public Health Service
United States Department of Agriculture
Institute of American Poultry Industries
Dairy and Food Industries Supply Association

The "E-3-A Sanitary Standards for Thermometer Fittings and Connections Used on Egg Products Equipment, Serial #E-0900" are amended as indicated below.

1. The title is changed to "E-3-A Sanitary Standards for Instrument Fittings and Connections Used on Liquid Egg and Liquid Egg Products Equipment, Serial #E-0900".
2. The word "instrument" shall be substituted for "thermometer" wherever it appears in the text of the standard and on the drawings except where it designates a thermometer or a fitting for a thermometer.

This amendment is effective October 28, 1971.

NEWS & EVENTS

MILK INDUSTRY A HEALTHY INVALID - DRINC CHIEF

"Don't listen to those people who are always crying wolf about the dairy industry", said Dr. Richard Farrar in an address to the Chicago Dairy Technology Society, "if the dairy industry is an invalid then its the healthiest invalid I have ever seen." Dr. Farrar is Executive Vice President of Dairy Research, Inc., better known as "DRINC", a non-profit farmer-supported corporation organized to sponsor research into new uses for dairy products.

"Dairy Research, Inc. was not organized to save the dairy industry", Dr. Farrar went on, "because it does not need saving. However we are dedicated to expanding the industry's horizon's". DRINC is approaching individual food companies to find out what research on new products that could use based ingredients is under way, according to Dr. Farrar. After review, DRINC will offer to subsidize up to 50% of the market research and, if that proves favorable, 50% of the product development costs.

Final marketing costs are the responsibility of the individual company. All patents belong to the final marketing organization. However, any company participating in a DRINC project agrees to pay Dairy Research, Inc. twice DRINC's investment in the project out of sales profits. Don't expect instant results", Dr. Farar said, "because research is like a crop. It has to be planted, nurtured, fertilized and finally harvested. However, you can be sure that the major food companies in the United States readily recognize the superiority of dairy products".

The Chicago Dairy Technology Society includes members from local dairies and dairy-allied companies. The society meets on the second Tuesday of every month at the Furniture Mart, 666 Lake Shore Drive, Chicago.

USDA AWARDS 6 RESEARCH AGREEMENTS

Six research agreements have been signed with public or private agencies for studies on specific agricultural subjects, the U. S. Department of Agriculture reports. The agreements, funded by USDA's Agricultural Research Service, will provide data for a broad, continuing program designed to improve the efficiency of agricultural production and marketing, and protect consumers and the environment.

The agreements signed include:

A 1-year, $23,778 contract with H. B. Maynard and Company, Inc., Pittsburgh, Pa., to evaluate

production costs of selected items on menus in 10 "coffee shop"-type restaurants to learn what improvements are needed and can be made.

A 1-year, $30, 054 contract with A. T. Kearney and Company, Inc., Chicago, Ill., to study advanced mechanized grocery warehouse operations and incorporate all present techniques into a proposed prototype warehouse for 1980.

A 2-year, $25,000 contract with the Kansas Agricultural Experiment Station, Manhattan, to determine the effect of composition and form of various dry blended feeds containing dried cheese whey as a pre-starter for calves. New ways to make beneficial use of this source of protein and to reduce pollution are continually being sought by USDA.

A 2 1/2-year, $111,667 contract to Oklahoma State University, Stillwater, to develop a pilot-plant scale peanut blanching process in which moldy peanuts can be removed by electronic sorters.

Improved insect control in mushroom houses and better ways to prevent serious fly larvae damage to mushrooms will be studied under a 3-year, $74,968 grant awarded to the Pennsylvania Agricultural Experiment Station, University Park.

A $10,800, 1-year contract with Germfree Life Research Center, Fort Lauderdale, Fla., for health-related research in cigarette tobaccos.

NEW EXECUTIVE VICE-PRESIDENT FOR DFISA

John H. Vogt, former executive administrator of Lions International, has joined Dairy and Food Industries Supply Assn. as Executive Vice-President. On July 26, he took over staff responsibilities for the 400-member organization of food equippers and suppliers at the Washington, D.C. headquarters.

Mr. Vogt, who had been asociated with The International Assn. of Lions Clubs since 1957, had complete responsibility for the business operations of the worldwide organization. During his administration of Lions, membership grew from 530,000 to nearly 1,000,000 members, and clubs multiplied from 13,000 to 25,000.

An expert in convention planning, Mr. Vogt annually organized the Lions for the largest service club association convention in the world. More than 30,000 attended the week-long event that included forums, workshops, training seminars, business sessions and social events.

The 52-year-old executive is a gifted publicist, having worked extensively with all types of media in his promotion responsibilities. He was in charge of the Lions monthly magazine, distributed in 17 countries in 10 languages.

Previous to Lions, Mr. Vogt was afiliated with Ford Motor Co., Chicago, for six years in various management positions, ultimately assistant to the general manager. He was also associated with Continental Can Co., Chicago, as senior industrial engineer and National Pressure Cooker Co., Eau Claire, Wisc., as chief standards engineer.

INDEX TO ADVERTISERS

CLASSIFIED ADS

FOR SALE

Single Service milk sample tubes. For further information and a catalogue please write, Dairy Technology Inc., P. O. Box 101, Eugene, Oregon 97401.

Application for Membership

INTERNATIONAL ASSOCIATION OF MILK, FOOD & ENVIRONMENTAL SANITARIANS, INC.

Box 437, Shelbyville, Indiana 46176

Name _____ **Date** _____

Please Print

Address _____ ☐ **Renewal**

_____ **Zip Code** _____ ☐ **New**

Business Affiliation _____ ☐ **Re-instatement**

Direct Member Annual Dues $10.00 ☐ **Check** ☐ **Cash**
Membership Through An Affiliate—$8.00 Plus Affiliate Dues
Student Membership $4.00
(Membership Includes Subscription to Journal of Milk & Food Technology.)

Please Print

Recommended by _____

Shelbyville, Ind.
Box 437

Subscription Order

JOURNAL OF MILK & FOOD TECHNOLOGY

(Monthly Publication)

Name _____ **Date** _____

Please Print

Address _____ ☐ **New**

_____ ☐ **Renewal**

Educational Institution & Public Libraries (Annually) $10.00 ☐ **Check** ☐ **Cash**

Individual Non-Member Subscription (Annually) $12.00
Government Agencies, Commercial Organizations

I.A.M.F.E.S. & J.M.F.T.
Box 437, Shelbyville, Ind.

Change of Address

FROM

Name _____ **Date** _____

Please Print

Address _____

TO

Name _____

Please Print

Address _____

I.A.M.F.E.S. & J.M.F.T.
Box 437, Shelbyville, Ind.

Order for 3A Standards

Name _____ **Date** _____

Please Print

Address _____

( ) **Complete Set @ $5.50 =** _____ ( ) **Complete set bound (durable cover) @ $7.00 =** _____
( ) **Revised HTST Std.—without cover = $1.50 F.O.B. Shelbyville, Ind.**
( ) **Revised HTST Std.—25 or more = $1.00 each F.O.B. Shelbyville, Ind.**
( ) **E-3-A Sanitary Standards—Set Unbound @ $1.25 =** _____

3-A Accepted Practices For Milking Machines
1-100 = 25c ea.; 100-1000 = 20c ea.; 1000 or more = 15c ea.
5 Year Service on Standards as Published = $6.00 additional

Order for Reprints of Articles

Amt. _____ **Title** _____

Schedule of letterpress prices for reprints within 30 days after publication, F.O.B. Shelbyville, Indiana

	1 Page	2 Pages	3 & 4 Pages	6 & 8 Pages	12 Pages	Cover
100 or less	$19.50	$23.40	$32.80	$46.80	$78.00	$33.60
Add'l 100's	2.40	3.00	5.00	6.60	10.80	5.50

Offset Reprint prices add $2.00 per page

THE ONLY *Approved*
SANITARY METHOD OF APPLYING
A U. S. P. LUBRICANT
TO DAIRY & FOOD
PROCESSING EQUIPMENT

Haynes Spray

U. S. P. LIQUID PETROLATUM SPRAY

U.S.P. UNITED STATES PHARMACEUTICAL STANDARDS

CONTAINS NO ANIMAL OR VEGETABLE FATS. ABSOLUTELY NEUTRAL. WILL NOT TURN RANCID — CONTAMINATE OR TAINT WHEN IN CONTACT WITH FOOD PRODUCTS.

SANITARY—PURE

ODORLESS—TASTELESS

NON-TOXIC

This Fine Mist-like **HAYNES-SPRAY** *should be used to lubricate:*

SANITARY VALVES
HOMOGENIZER PISTONS — RINGS
SANITARY SEALS & PARTS
CAPPER SLIDES & PARTS
POSITIVE PUMP PARTS
GLASS & PAPER FILLING MACHINE PARTS
and for ALL OTHER SANITARY MACHINE PARTS which are cleaned daily.

The Modern HAYNES-SPRAY Method of Lubrication Conforms with the Milk Ordinance and Code Recommended by the U. S. Public Health Service

The Haynes-Spray eliminates the danger of contamination which is possible by old fashioned lubricating methods. Spreading lubricants by the use of the finger method may entirely destroy previous bactericidal treatment of equipment.

PACKED 6-12 oz. CANS PER CARTON SHIPPING WEIGHT—7 LBS.

THE HAYNES MANUFACTURING COMPANY
4180 LORAIN AVENUE • CLEVELAND, OHIO 44113

HAYNES SNAP-TITE GASKETS

LOW COST...RE-USABLE

LEAK-PREVENTING

NEOPRENE GASKET for Sanitary Fittings

Check these SNAP-TITE *Advantages*

- Tight joints, no leaks, no shrinkage
- Sanitary, unaffected by heat or fats
- Non-porous, no seams or crevices
- Odorless, polished surfaces, easily cleaned
- Withstand sterilization
- Time-saving, easy to assemble
- Self-centering
- No sticking to fittings
- Eliminate line blocks
- Help overcome line vibrations
- Long life, use over and over

Available for 1", 1½", 2", 2½" and 3" fittings.
Packed 100 to the box. Order through your dairy supply house.

THE HAYNES MANUFACTURING CO.
4180 Lorain Avenue • Cleveland 13, Ohio

HAYNES SELF-CENTERING SNAP-TITE® *Gaskets*

SIZES 1" - 1½"
2" - 2½" - 3"

*MADE FROM **TEFLON®**

"The Sophisticated Gasket"

THE IDEAL UNION SEAL FOR BOTH VACUUM AND PRESSURE LINES

Gasket Color . . . slightly off-white

SNAP-TITE self-centering gaskets of TEFLON are designed for all standard bevel seat sanitary fittings. They SNAP into place providing self-alignment and ease of assembly and disassembly.
HAYNES SNAP-TITES of TEFLON are unaffected by cleaning solutions, steam and solvents. They will not embrittle at temperatures as low as minus 200° F. and are impervious to heat up to 500° F.

FOR A FITTING GASKET THAT WILL OUT-PERFORM ALL OTHERS...

Specify . . . HAYNES SNAP-TITES of TEFLON

- *TEFLON ACCEPTED SAFE FOR USE ON FOOD & PROCESSING EQUIPMENT BY U. S. FOOD AND DRUG ADMINISTRATION*

*Gaskets made of DuPont TEFLON® TFE-FLUOROCARBON RESINS

THE HAYNES MANUFACTURING COMPANY
4180 LORAIN AVENUE • CLEVELAND, OHIO 44113

A HEAVY DUTY SANITARY LUBRICANT

Available in both

SPRAY AND TUBE

All Lubri-Film ingredients are approved additives and can be safely utilized as a lubricant for food processing equipment when used in compliance with existing food additive regulations.

ESPECIALLY DEVELOPED FOR LUBRICATION OF FOOD PROCESSING AND PACKAGING EQUIPMENT

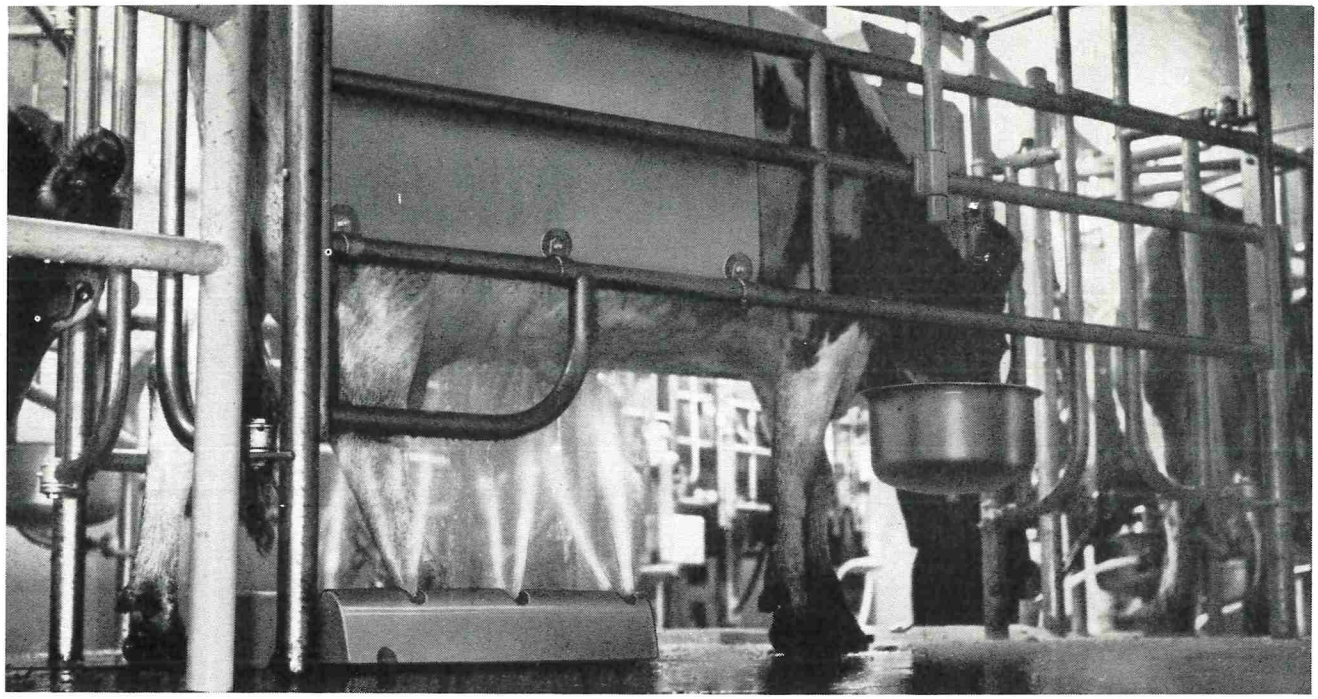
For Use in Dairies — Ice Cream Plants — Breweries — Beverage Plants — Bakeries — Canneries — Packing Plants

SANITARY • NON TOXIC • ODORLESS • TASTELESS

SPRAY — PACKED 6 — 16 OZ. CANS PER CARTON
TUBES — PACKED 12 — 4 OZ. TUBES PER CARTON

THE HAYNES MANUFACTURING CO.
CLEVELAND, OHIO 44113

It's like having a third hand



Automated
SURGE PREP-STALL

The first mass produced Automated Prep-Stall was recently introduced by Babson Bros. Co. Dairymen say it's like having a third hand. Depending upon the present system, it can increase milking efficiency in individual stall parlors by as much as 30 per cent.

The Automated Surge Prep-Stall is not a cow washer. While it does clean and sanitize, its prime purpose is to stimulate the udder so the cow is ready to be milked when she enters the milking stall.

Automatic prepping contributes to greater milking efficiency in several ways:

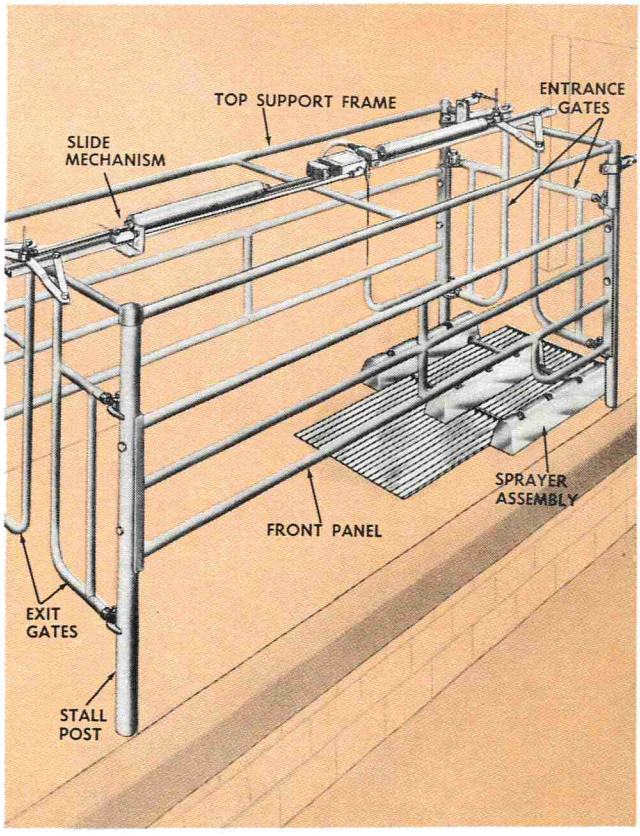
First, it automatically performs the job of cow prepping in a uniform and approved manner.

Second, it cleans and sanitizes the udder.

Third, it shortens the time each cow spends in the milking stall and increases parlor efficiency.

Fourth, it frees the operator to devote more time to better cow milking.

For more specific information on the Automated Surge Prep-Stall and other dairy equipment innovations write BABSON BROS. CO.



SURGE...the accent is on YOU

SURGE

BABSON BROS. CO., OAK BROOK, ILLINOIS
BABSON BROS. CO., (Canada) LTD., PORT CREDIT, ONTARIO