THE ACTIVITY OF PENICILLIN IN RELATION TO BACTERIAL SPORES AND THE PRESERVATION OF MILK

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The activity of penicillin against the spores of *Bacillus subtilis*, *Bacillus megatherium*, *Bacillus cereus*, and *Bacillus stearothermophilus*, was reported recently (Curran and Evans, 1945*a*). As little as 5 Oxford units per ml of the drug effected a marked reduction in the number of viable spores in milk in three of the four species when the samples were suitably incubated for periods of 5 and 27 hours. The plate method of enumeration was employed. Later, Gardner (1945) reported a similar observation based upon the direct microscopical examination of the spores of *Bacillus subtilis* and *Bacillus anthracis* (avirulent) cultivated in the presence of penicillin on nutrient agar blocks.

These findings indicated certain possibilities in the application of penicillin as a preserving agent. Whether, with opportunity for prolonged action, penicillin would be able ultimately to kill all spores of a susceptible species was a question not yet answered, nor was it known whether spores of penicillin-resistant species could be controlled by relatively high concentrations of penicillin. More extended information as to the relative proportion and distribution of susceptible and refractory species was also essential to a critical evaluation of penicillin as a preservative.

In this report an attempt has been made to answer some of these questions. The observations have been limited to the action of penicillin in milk.

METHODS AND MATERIALS

The organisms and their sources were *Bacillus subtilis*, strains 6, 15U, 4149, and LB, recovered from spoiled commercially processed evaporated milk either by the American Can Company or by this laboratory; *Bacillus metiens* and *Bacillus subtilis*, Merck and FDA, from Dr. W. A. Randall of the Food and Drug Administration; *Bacillus albolactis* from the Bureau of Dairy Industry culture collection; the anaerobes 3679, a nontoxigenic proteolytic culture, and *Clostridium botulinum* (62A) from Mr. John Yesair, National Canners Association; and the remaining cultures from the N. R. Smith collection through the courtesy of Dr. Ruth Gordon.

The spores of the aerobic species[1] were produced on standard beef extract tryptone agar medium incubated at the optimum temperature of the organism. They were collected and prepared for use as described by Curran and Evans (1945*b*). Aqueous suspensions of the washed spores were inoculated directly into the test sample. The spores of anaerobes were produced in casein digest

[1] *B. subtilis* FDA was cultivated on glucose agar to prevent its dissociation.

medium, well-mixed suspensions of which served directly as inocula. Pour plates were used for count determinations. For the aerobes the plating medium was that used for the production of spores with 0.5 per cent glucose added. For the anaerobes Brewer plates (Brewer, 1942) and B-B-L anaerobic agar were used. Plates were incubated at the optimum temperature of the organism and counted after 2 to 3 days. Heat treatments were conducted in a thermostatically controlled glycerol bath (±0.5 C).

Some degree of pretreatment heating of milk samples is necessary if the preserving action of penicillin is to be efficiently utilized. This kills many spores, all nonsporulating species which may be resistant to penicillin, and also the vegetative forms of sporing species. Some of the latter are less inhibited by penicillin than are the spores from which they are derived. Mild heating serves also to inactivate preformed microbial penicillinase.

Fresh raw milk and sterile (autoclaved) skim milk, tubed in 10-ml quantities, were the culture mediums. Whenever anaerobic cultures were employed, the dissolved oxygen was driven off by mild heat before inoculation and the samples were sealed by means of sterile agar and mineral oil.

Penicillin[2] sodium was used throughout. The solvent was a buffer mixture consisting of 1 per cent each of K_2HPO_4 and KH_2PO_4, pH 6.0. The penicillinase[3] was the commercial product, dissolved in sterile distilled water.

For testing the presence of botulinus toxin 0.1-ml quantities of each incubated test culture were carefully introduced directly into the stomachs of each of two mice by means of a blunted hypodermic needle. Similar quantities of each incubated or unincubated control were similarly fed to two mice. Animals showing any evidence of bleeding immediately after the operation were discarded and replacements provided. Mice fed from the same culture were placed together in separate cages and observed daily for a period of 5 days. The mice were 3 to 4 months old and weighed about 30 g each.

The activity of penicillin was tested against a group of 15 aerobic and 2 anaerobic strains representing species which are disseminated widely in nature. The washed spores were uniformly dispersed in sterile (autoclaved) milk in cell concentrations ranging from 50 to 100,000 per ml, heated at 95 C for 15 minutes,[4] and cooled; penicillin was added; and the samples were stored at 30 C. Spoilage was a measure of the capacity of the spores to germinate and to vegetate within the recorded period of observation.

RESULTS

Some measure of inhibition or destruction of the spores was found in all except 4 closely related species. The latter, *Bacillus cereus* (7 strains), *Bacillus mycoides* (2 strains), *Bacillus metiens*, and *Bacillus albolactis*, induced spoilage both with and without penicillin within 1 week. Samples containing penicillin which

[2] Kindly supplied by Chas. Pfizer and Co., Brooklyn, New York.

[3] Obtained through the courtesy of the Schenley Research Institute, Lawrenceburg, Indiana.

[4] Primarily to activate the spores and to kill nonsporulating organisms.

spoiled in 4 weeks were *Bacillus subtilis* Ford, *Bacillus subtilis* var. *aterrimus* 230, *Bacillus subtilis-niger* 6454, and *Bacillus alvei* 680; in 8 weeks, *Bacillus subtilis* Marburg and *Bacillus subtilis* from Merck; in 12 weeks, *Bacillus alvei* 395, 685, and 686; and in 16 weeks, *Bacillus subtilis* 4149, 3679 (anaerobe), and *Clostridium botulinum* 62A. The following members of the genus *Bacillus* produced no change in the presence of penicillin during 16 weeks of observation: *B. subtilis* 6598, 6, 15U, LB, FDA; *B. pumilus* 7061; *B. brevis* 8185, 8186, Penn. S; *B. alvei* 683, 684, 750, 811; *B. firmus* 8247; *B. laterosporus* 8248, 9141; *B. megatherium* 234, 389, 696, 753, 931; *B. circulans* 7049; *B. polymyxa* 8240; *B. sphaericus* 7054, 4525; *B. macerans* 7069. In the absence of penicillin, all except 5 samples spoiled the milk in from 1 to 2 weeks. *B. sphaericus* (2), *B. circulans*, *B. firmus*, and certain strains of *B. alvei* required from 3 to 4 weeks to produce visible spoilage.

These samples, with few exceptions, did not spoil when they were treated with sterile penicillinase at the end of the incubation period and were then incubated, thus indicating the absence of viable organisms. The results with *Bacillus subtilis* differed with the strain, from delayed spoilage to sterilization of the sample. The data on *B. cereus*, *B. megatherium*, and *B. subtilis* are in accord with our previous findings (Curran and Evans, 1945*a*). In the samples containing anaerobes, penicillin delayed but did not prevent spoilage.

Four strains of *B. subtilis* (FDA, 15U, 4149, and LB), *B. brevis* (Penn S), and *B. circulans* (7049) evidenced heat activation (Curran and Evans, 1945*b*). In all of these spoilage was either greatly delayed or prevented.

Although the correlation is not complete, resistance to penicillin among species and among the strains of a species is usually associated with thermolability; pronounced susceptibility to penicillin on the other hand occurs usually among the species of moderate and high thermal resistance. Within a given species, however, the reverse seems to be true; viz., the heat-resistant (surviving 95 C for 15 minutes) spores comprise the largest proportion of penicillin-resistant spores.

Action of penicillin against species relatively resistant to the drug. Some further observations were made upon the action of penicillin against penicillin-resistant species. Four cultures, *Bacillus cereus* 369 and 401, *Bacillus mycoides* 6462, and *Bacillus metiens* were studied. Spores of these cultures were seeded into raw milk in varying concentrations; preliminary heating at 95 C for 15 minutes was combined with incubation of the samples at 30 C for 5 hours, followed by heating at 85 C for 15 minutes. The results (not detailed) revealed that only those samples containing less than 23 viable spores per ml after the initial heating were preserved. These results suggest that all species produce spores which are susceptible to penicillin in some degree—the species differing in the relative proportion of susceptible and resistant cells.

Action of penicillin against naturally produced spores. It is generally recognized that organisms developing in natural environments are often endowed with greater resistance than those produced in the laboratory. To test the action of penicillin (5 u per ml) against naturally produced spores, raw milk was treated

TABLE 1

The preserving action of penicillin (5 u per ml) in milk not artificially inoculated

DATE	TOTAL COUNT (AEROBIC)	COUNT AFTER HEATING 95 C—15 MIN (AEROBIC)	PRELIMINARY INCUBATION	AFTER 3 MONTHS' STORAGE AT 30 C	
				Aerobic	Anaerobic
	per ml	*per ml*			
21/3	16,000	2.6	None	U	
23/3	1,500	1.3	None	U	
28/3	10,400	2.3	None	U	
4/4	68,000	134 (147)*	None	S	
11/4	44,600	39	None	S	
18/4	77,000	15	None	S	
25/4	17,700	1	None	U	
3/5	2,600	4	None	U	
9/5	2,100	2	5 hr at 30 C plus 85 C for 15 min	U	
16/5		3	5 hr at 30 C plus 85 C for 15 min	U	
25/6	4,300	7 (8)	5 hr at 30 C plus 85 C for 15 min	U	
10/7	28,000	5 (11)	5 hr at 30 C plus 85 C for 15 min	U	
14/8	27,000	12 (58)	5 hr at 30 C plus 85 C for 15 min	U	
21/8	116,000	10 (17)	5 hr at 30 C plus 85 C for 15 min	U	
23/8	16,000	6 (13)	5 hr at 30 C plus 85 C for 15 min	U	U
28/8	51,000	6 (19)	5 hr at 30 C plus 85 C for 15 min	U	
5/9	24,000	19 (35)	5 hr at 30 C plus 85 C for 15 min	S	S
7/9	133,000	2 (35)	5 hr at 30 C plus 85 C for 15 min	U	S
12/9	169,000	3	5 hr at 30 C plus 85 C for 15 min	S	
20/9	190,000	16 (70)	5 hr at 30 C plus 85 C for 15 min	S	S
21/9	14,000	1 (27)	5 hr at 30 C plus 85 C for 15 min	U	U
25/9	54,000	46	5 hr at 30 C plus 85 C for 15 min	S	S
26/9	98,000	24	5 hr at 30 C plus 85 C for 15 min	U	U
28/9	6,000	1	5 hr at 30 C plus 85 C for 15 min	U	S
6/11	25,000	4 (13)	5 hr at 30 C plus 85 C for 15 min	U	U

Cereus-mycoides types predominated in the organisms which survived preliminary heating.

U = Unchanged.

S = Spoiled.

* Figures in parentheses = after heating at 85 C for 15 min.

after being heated at 95 C for 15 minutes, with and without a short incubation period. Anaerobic, as well as aerobic, cultivation was employed in part of these tests. The milk was collected over a period of 8 months to provide seasonal variations in the spore flora.

The results (table 1) show that the count of the aerobic thermostable spores

TABLE 2

The sterilizing levels of penicillin in milk containing both naturally occurring and artificially introduced penicillin-resistant spores (Bacillus cereus 720)

VIABLE SPORES AFTER INOCULATION	PRELIMINARY TREATMENT	PENICILLIN	CONDITION OF MILK AFTER 3 MONTHS' STORAGE AT 30 C		
			B. cereus	Penicillin	*B. mycoides*
per ml		*u/ml*		*u/ml*	
220,000	95 C-15 min; no incub.	100	U	100	S 1 wk
220,000	95 C-15 min; no incub.	500	U	500	U
220,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	S 1 wk	100	S 1 wk
220,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	100	U	500	U
22,000	95 C-15 min; no incub.	50	S 8 wk	50	S 1 wk
22,000	95 C-15 min; no incub.	100	S 8 wk	100	U
22,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	10	S 2 wk	10	S 1 wk
22,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	S 8 wk	50	U
2,200	95 C-15 min; no incub.	50	S 2 wk	50	U
2,200	95 C-15 min; no incub.	100	S 8 wk	100	U
2,200	95 C-15 min; 30 C-5 hr; 85 C-15 min	10	S 8 wk	10	S 4 wk
2,200	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	U	50	U
200	95 C-15 min; no incub.	50	S 2 wk	50	U
200	95 C-15 min; no incub.	100	S 8 wk	100	U
200	95 C-15 min; 30 C-5 hr; 85 C-15 min	10	S 1 wk	10	U
200	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	U	50	U
No inoc.	No heat; no incub.	500 aerobic	S 1 wk		
		500 anaerobic	S 1 wk		
No inoc.	No heat; no incub.	1,000 aerobic	S 1 wk		
		1,000 anaerobic	S 2 wk		
No inoc.	95 C-15 min; no incub.	50 aerobic	S 2 wk		
		50 anaerobic	S		
No inoc.	95 C-15 min; no incub.	100 aerobic	S 8 wk		
		100 anaerobic	S 8 wk		
No inoc.	95 C-15 min; 30 C-5 hr; 85 C-15 min	10 aerobic	S 1 wk		
		10 anaerobic	S 1 wk		
No inoc.	95 C-15 min; 30 C-5 hr; 85 C-15 min	50 aerobic	S 4 wk		
		50 anaerobic	S 1 wk		

Raw milk, unheated, 48,000 per ml; raw milk after 95 C for 15 min, 48 per ml.
Aerobic and anaerobic refer to storage conditions.

was low throughout. Qualitatively, *B. cereus* and *B. mycoides* types greatly predominated. In only 4 of the 25 samples did the count of thermostable (aerobic) spores exceed 20 per ml. There is little correlation between the total count and the number of spores which survived heating at 95 C for 15 minutes; also there is little correlation between the total count (vegetative and spores)

and spoilage, but a rather close correlation between the thermostable spore count and spoilage. With one exception, in all the samples that spoiled the spore count was 15 to 134 per ml, whereas, with one exception, all the preserved samples initially contained 10 or less thermostable spores per ml. As would be expected, the counts after heating at 85 C for 15 minutes were substantially higher than those after heating at 95 C for 15 minutes; however, the average ratio of difference was in this instance much smaller than that found in previous experiments in which artificially produced spores were used. This indicates that

TABLE 3

*The effect of penicillin upon the preservation of autoclaved milk seeded with the spores of a penicillin-sensitive species**

NO. OF SPORES AFTER INOCULATION	PRELIMINARY TREATMENT	LEVEL OF PENICILLIN	CONDITION OF MILK AFTER STORAGE AT 30 C FOR			
			10 days	1 month	2 months	3 months
per ml		*u/ml*				
119,000	95 C-15 min	5	10-U	3-S 7-U	8-S 2-U	10-S
119,000	95 C-15 min	50	2-U	2-U	2-U	1-S 1-U
11,900	95 C-15 min	5	10-U	10-U	10-U	10-U
11,900	95 C-15 min	50	2-U	2-U	2-U	2-U
1,190	95 C-15 min	5	10-U	10-U	10-U	10-U
1,190	95 C-15 min	50	2-U	2-U	2-U	2-U
710	No heat	5	7-S 3-U	9-S 1-U	9-S 1-U	9-S 1-U
710	No heat	20	2-U	2-U	2-U	1-S 1-U
710	95 C-15 min	5	10-U	10-U	10-U	10-U
710	95 C-15 min	20	2-U	2-U	2-U	2-U
65	No heat	5	1-S 9-U	1-S 9-U	1-S 9-U	1-S 9-U
65	No heat	20	2-U	2-U	2-U	2-U
65	95 C-15 min	5	10-U	10-U	10-U	10-U
65	95 C-15 min	20	2-U	2-U	2-U	2-U
65	95 C-15 min	No penicillin	2-S			

Numbers in storage period columns refer to the number of tubes.

* *Bacillus subtilis* var. *aterrimus*.

the naturally occurring *B. cereus* and *B. mycoides* spores are relatively more resistant to heat than are the artificially produced spores of these species.

The ineffectiveness of penicillin against spores capable of growing anaerobically is apparent from these data. All samples that spoiled under anaerobic cultivation included all those that spoiled aerobically and some that did not.

Levels of penicillin required for sterilization. In table 2 are shown the sterilizing levels of penicillin in milk containing both naturally occurring and artificially introduced penicillin-resistant spores. From 100 to 500 u per ml of penicillin were required to preserve the inoculated cultures depending upon the initial

concentration of spores and the prestorage treatment. The preliminary incubation reduced the amount of penicillin required for preservation in some samples; in others no differences are apparent. The effect of concentration of spores upon the limiting concentration of penicillin varied greatly with the two species. The data on the uninoculated sample furnish further evidence of the presence in milk of spores resistant to penicillin and of the uncertainty of penicillin as an aid to preservation. As is evident in table 2, in the absence of pretreatment heating the preservation of milk by penicillin is practically unattainable. As

TABLE 4

The influence of penicillin upon the development of the spores of Clostridium botulinum in milk and upon the formation of toxin

VIABLE SPORES AFTER INOCULATION	TREATMENT	CONDITION OF CULTURE AFTER 7 WEEKS, INCUBATION	CULTURE AFTER 13 WEEKS' INCUBATION	
			Condition	Toxicity* (Mice)
per ml				
80	No penicillin	GP—3 weeks	GP	1 dead <24 hr 1 very sick
80	Penicillin 5 u/ml	U	U	2 normal
800	No penicillin	GP—3 weeks	GP	1 dead <24 hr 1 dead <48 hr
800	Penicillin 5 u/ml	U	U	2 normal
8,000	No penicillin	GP—3 weeks	GP	1 dead <24 hr 1 very sick
8,000	Penicillin 5 u/ml	U	U	2 normal
80,000	No penicillin	GP—3 weeks	GP	2 dead <48 hr
80,000	Penicillin 5 u/ml	GP—(6 weeks)	GP	2 dead <48 hr
80,000	Penicillin 50 u/ml	U	U	
800,000	No penicillin	GP—10 days	GP	2 dead <36 hr
800,000	Penicillin 50 u/ml	GP—3 weeks	GP	1 dead 48 hr 1 very sick
800,000	Penicillin 100 u/ml	GP—3 weeks	GP	
No inoc.	Penicillin 5 u/ml	U	U	2 normal
No inoc.	No penicillin	U	U	2 normal
318,000	Initial inoculum	U	U	2 normal

GP = Gas, peptonization.

U = Unchanged.

*0.1 ml of the incubated test culture was forcibly fed to each mouse. All the sick animals evidenced the typical symptoms of botulinus intoxication.

much as 1,000 u per ml in this instance was ineffective in preventing rapid spoilage.

Action of penicillin against a species relatively sensitive to the drug. In the next experiment, spores of a penicillin-sensitive species, *Bacillus subtilis* var. *aterrimus*, served as inocula in sterile milk. This strain, together with *Bacillus niger* and the Ford strain of *B. subtilis*, is more resistant to penicillin than other members of the subtilis group. The data (table 3) show that the number of spores has an important influence upon the treatment necessary to ensure ster-

ilization. When the number of spores was relatively high, 5 u per ml of penicillin was not a consistently effective preservative even when combined with preliminary heating. When the spores numbered 10,000 per ml or less, 5 u per ml was effective when used in conjunction with prior heating. Without the latter, sterility was not attained with as few as 65 spores per ml, although 20 u per ml did preserve these samples.

The action of penicillin against the spores of Clostridium botulinum. Varying numbers of spores of *Clostridium botulinum* were incubated anaerobically in milk with different concentrations of penicillin. At the recorded intervals, gross changes in the appearance of the samples were noted and animal feeding tests[5] performed. The data presented in table 4 show that penicillin in low concentration (5 u per ml) greatly delayed growth and the formation of toxin when the concentration of spores was substantially below 800,000 per ml. At the latter level, the effect of penicillin up to 100 u per ml was almost negligible; however, in the presence of relatively few spores ($<$ 8,000 per ml) the growth- and toxin-delaying action of 5 u per ml of penicillin was pronounced, the data giving no indication as to the limits of the sporistatic period. No explanation is offered for the lack of complete uniformity in some of the feeding tests. The importance of the number of spores in determining the length of the inactive period suggests that a very small proportion of the spores in the initial population was insusceptible to the inhibitory action of penicillin, rapid spoilage in a particular instance depending upon the presence in the sample of 1 or more resistant cells. Bigger (1944) has applied the name "persisters" to the very small minority of *Staphylococcus pyogenes* cells found to be resistant to the activity of penicillin. The resistant cells rarely exceeded 1 per million of the cocci originally present. A closely analogous situation seems to exist for spores.

DISCUSSION

It is evident from the foregoing report that penicillin in low concentration (5 u per ml) is remarkably sporistatic and sporicidal for a wide range of organisms. Certain deficiencies of the drug as a spore-controlling agent are also apparent; a small but widely disseminated group of sporing aerobes is strongly resistant to penicillin. When such spores are present, even in small numbers, penicillin is not an effective preserving agent except in concentrations which would be impracticable. With certain other aerobes, penicillin in low concentration delays but does not consistently prevent the germination of their spores and subsequent growth. Data obtained with anaerobic sporeformers show that the spores of some species can develop rather quickly in the presence of about 5 u per ml of penicillin. Likewise, this level of penicillin will not consistently prevent the growth and the formation of toxin originating from the spores of *Clostridium bolulinum*, though these may be greatly delayed.

Delayed spoilage indicates an inhibiting action upon the germination of the spores. This continues presumably until the penicillin falls below a sporistatic

[5] Our thanks are due to Dr. Samuel R. Hall of this Bureau for his assistance in the feeding and care of the animals.

level; in some strains of *B. subtilis* the threshold concentration is less than 0.015 u per ml. In view of the rather slow deterioration of penicillin even at incubator temperatures (Rammelkamp and Helm, 1943; Kirby, 1944; Benedict *et al.*, 1945), 5 u per ml of penicillin might be expected to exert a prolonged sporistatic action against susceptible species.

The possible utility of penicillin in the preservation of nonfood materials would depend upon the reaction of such materials and upon the kind and number of spores encountered. In combination with mild heating penicillin might find application in special situations, either as a preservative or spoilage-delaying agent.

SUMMARY

A study was made of the preserving action of penicillin in milk containing viable bacterial spores. Fifteen aerobic and two anaerobic species were examined.

In their reaction to penicillin, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus albolactis*, and *Bacillus metiens* were relatively resistant. When they were present penicillin was not an effective preserving agent except in concentrations that would be impracticable. The remaining 13 species were relatively susceptible to penicillin as was manifested by strong sporicidal or prolonged sporistatic activity in a drug concentration of 5 u per ml. This concentration sterilized many of these cultures.

Five units per ml of penicillin greatly delayed but did not prevent spoilage by *Clostridium botulinum* and an unidentified anaerobic species (3679). In the former, toxin formation accompanied spoilage, both in the control and penicillin samples. The sporistatic period varied with the concentration of spores and the level of penicillin. The data indicate that an extremely small proportion of the spores of *Clostridium botulinum* are highly resistant to the sporistatic action of penicillin.

The evidence suggests that all spore cultures contain spores susceptible to penicillin, the species differing in the relative proportion of resistant and sensitive cells.

On the basis of this study, it is concluded that penicillin has no application in the preservation of food. In combination with mild heating, it might have utility as a preservative or spoilage-delaying agent in certain nonfood materials.

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