PESTICINS

I. Pesticin-bacterium Interrelationships, and Environmental Factors Influencing Activity

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ABSTRACT

Brubaker, Robert R. (U. S. Army Biological Laboratories, Fort Detrick, Frederick, Md.) and Michael J. Surgalla. Pesticins. I. Pesticin-bacterium interrelationships, and environmental factors influencing activity. J. Bacteriol. **82:** 940–949. 1961—A second bacteriocin-like substance produced by all tested strains of *Pasteurella pestis* and *P. pseudotuberculosis* is described. This activity, termed pesticin II, is active against the two avirulent *P. pestis* strains, A12 and Java. These strains do not produce pesticin I, which inhibits the growth of type I strains of *P. pseudotuberculosis*. Pesticin I was also found to be active against certain strains of *Escherichia coli*, strain A12, but not strain Java, and some *P. pestis* isolates which also produce pesticin I. A number of *E. coli* strains produce a substance which also inhibits the growth of strains A12 and Java; the activity of this substance is dependent upon the presence of high concentrations of Ca^{++}.

The activity of both pesticins is inhibited under anaerobic conditions or in the presence of antiserum. The activity of pesticin I, but not pesticin II, is suppressed by Fe^{+++}, hemin, certain hemin-containing proteins, Mg^{++}, and inorganic phosphate. Suppression of pesticin I activity by Fe^{+++} can be reversed by the addition of either Ca^{++} or Sr^{++} or by metal chelating agents. All tested strains of *P. pestis* and *P. pseudotuberculosis* produce a metabolite which suppresses the activity of pesticin I. The activity of this substance, termed pesticin I inhibitor, is enhanced by Fe^{+++}, and to a lesser extent by Mg^{++} or by inorganic phosphate; its activity is suppressed by Mn^{++} and by protamine sulfate.

A differential plating medium containing Mg^{++} and oxalate is selective for avirulent mutants of *Pasteurella pestis* found to occur at a mutation rate of 10^{-4} (Higuchi and Smith, 1961). As reported by Surgalla (1960), this specific mutation to avirulence is characterized, in the cases studied by various workers in this laboratory, by a concomitant loss of sensitivity to glucose (Wessman, Miller, and Surgalla, 1958) and production of VW or virulence antigens (Burrows and Bacon, 1956) in addition to the Ca^{++} requirement reported by Higuchi, Kupferberg, and Smith, (1959). The mechanism whereby the above three bacterial properties plus virulence are simultaneously lost at a high rate may be dissimilar to that of a classical spontaneous mutation involving the change of one property at a low rate. Low mutation frequencies of certain other properties, such as streptomycin-resistance (Garber, Nobel, and Carouso, 1953), are evidence against a general chromosomal instability such as that associated with mutator genes. An interesting possibility is that virulence may be under cytoplasmic or episomic control, as suggested by Ogg et al. (1958).

Episomes are genetic determinants which may exist either as particles integrated on the chromosome, or as autonomous units capable of independent replication in the cytoplasm (Jacob and Wollman, 1958). Temperate phage genomes, bacterial sex factors, and bacteriocinogenic determinants were originally described in the definition of the term episome. The existence of prophage or bacterial sex factors in *P. pestis* has not been verified. Therefore, a study of the bacteriocin-like substance termed pesticin by Ben-Gurion and Hertman (1958) and its determinant was initiated. Pesticin is active against all tested type I (Thal, 1954) strains of *P. pseudotuberculosis* (Burrows and Bacon, 1960) and against certain nonpesticinogenic mutants of *P. pestis* isolated by Hertman and Ben-Gurion (1958).

One purpose of this paper is to describe a

second bacteriocin-like substance (pesticin). Also, in this study which emphasized the role of the pesticins rather than their determinants, we found that certain environmental conditions, which affect the growth or virulence of *P. pestis* and *P. pseudotuberculosis*, also influence the activity of both pesticins.

MATERIALS AND METHODS

Stock cultures. The virulent *P. pestis* strain Poona and its avirulent mutant Ox/P, isolated on the magnesium oxalate agar (MGOX) of Higuchi and Smith (1960), were used in the majority of experiments. The avirulent *P. pestis* strains Java and A1122 were obtained from the Naval Biological Laboratories, Oakland, Calif. *P. pestis* strain A12 is a colony-isolate of strain A1122. The avirulent *P. pestis* strain OX/W was isolated from the virulent strain Washington by selection on MGOX. The virulent type I *P. pseudotuberculosis* strain PB1/+ and its avirulent mutant PB1/− were kindly supplied by T. W. Burrows, Microbiological Research Establishment, Porton, Salisbury, England. Single representative strains of *P. pseudotuberculosis* types I, II, III, and V (Thal, 1954) were obtained from the collection of W. Knapp through the courtesy of S. F. Quan, San Francisco Field Station, Calif. *Escherichia* and *Shigella* species, producing 16 separate colicines, and the universal colicine-indicator *E. coli* ϕ, were generously provided by P. Fredericq, Universite de Liege, Belgium.

Media. All cultures were grown for 24 hr at 26 C on slants of Difco Blood Agar Base (Difco Laboratories, Detroit, Mich.) plus 0.1% glucose and 0.04% Na_2SO_3 (BAB). BAB supplemented with 0.01 M $CaCl_2$ was employed in preliminary studies concerning the activity of pesticins. Quantitative determinations of the reagent concentration necessary for suppression or enhancement of the activity of pesticins were performed on a medium (NZAA) composed of 2% N-Z-Amine, type A (Sheffield Products, Inc., Norwich, N. Y.), 0.85% Oxoid Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.), and 0.04% Na_2SO_3. Hemin, $FeCl_3$, K_2HPO_4, and sodium citrate, when added to NZAA, were autoclaved in the medium. Concentrated $CaCl_2$ and Na_2SO_3 solutions were autoclaved separately and added aseptically. The media were adjusted to pH 6.7 with 1 N NaOH or HCl before sterilization.

TABLE 1. *Effect of various reagents on the activity of PI, PII, and PIi as determined qualitatively by the modified cross-streak method*

Reagent	Concentration tested	Effect on activity[a]		
		PI	PII	PIi
NH_4Cl, NaCl, KCl	1.0 M	0	0	0
$MgCl_2$[b, d]	1.0 M	S	0	E
$CaCl_2$[c]	1.0 M	EE	EE	0
$SrCl_2$	1.0 M	EE	EE	S
$ZnCl_2$	0.01 M	0	0	0
$MnCl_2$[d]	0.01 M	0	0	SS
$FeCl_3$[e]	0.01 M	SS	0	EE
Hemin[e]	0.01 M	SS	0	0
Hemoglobin, Myoglobin	5.0%	S	NT	0
Ferritin	1.0%	0	NT	S
K_2HPO_4	0.2 M	S	0	E
Antiserum (PB1/+)	Undiluted	SS	SS	NT
Normal serum	Undiluted	0	0	NT
Citrate, Tartrate	1.0 M	E	0	S
α/α'-dipyridyl	Saturated	E	NT	SS
O-phenanthroline	Saturated	E	NT	SS
Ca-EDTA	1.0%	E	NT	SS
Deoxyribonuclease, ribonuclease	0.1%	0	0	0
Lysozyme	1.0%	0	0	0
Protamine sulfate	1.0%	0	0	SS
Catalase	0.1%	0	0	NT
Trypsin	1.0%	SS	SS	NT

[a] (S) suppresses activity; (SS) strongly suppresses activity; (E) enhances activity; (EE) strongly enhances activity.

[b] Suppresses growth of virulent cells at 37 C in the absence of Ca^{++} (Higuchi et al., 1959).

[c] Necessary for growth of virulent cells at 37 C; may be replaced by Sr^{++} or Zn^{++} (Higuchi *et al.*, 1959).

[d] Reverse glucose toxicity of virulent cells (Wessman, *et al.*, 1958).

[e] Enhances virulence of certain non-pigmented strains of *P. pestis* in mice (Jackson and Burrows, 1956).

Activity of pesticins. The inhibitory or enhancing effect of various reagents on the activity of pesticins was qualitatively determined by use of a modification of the cross-streak method of Fredericq (1948). A 2.0 × 0.5 inch strip of type A glass fiber paper (Gelman Instrument Co., Chelsea, Mich.) was soaked in reagent solution and placed across a streak of growth previously

developed on BAB, BAB plus 0.01 M $CaCl_2$, or NZAA for 24 hr at 26 C. The plate was then sterilized with chloroform vapor, over-layered with 5 ml of seeded-agar medium, and, after incubation for 48 hr at 37 C, the effect of cross-diffusion of reagent and pesticin on the growth of the indicator strain was noted.

Quantitative determinations of the effects of $CaCl_2$, $FeCl_3$, and hemin on pesticin activity were performed on NZAA by use of the double agar layer technique. A drop of a suspension containing 10^8 OX/P cells per ml of 0.033 M phosphate buffer, pH 7.0, was placed on plates containing 25 ml hardened NZAA, or NZAA containing either 0.1 M sodium citrate, or 0.025 M PO_4^{---}, or both. After incubation for 48 hr at 26 C, the plates were sterilized with chloroform vapor, and the developed colony was overlayered with 5 ml of seeded NZAA containing various dilutions of $CaCl_2$ and $FeCl_3$, or $CaCl_2$ and hemin. After incubation of the indicator strain for 48 hr at 37 C, the ratio of reagents which permitted partial or complete inhibition of growth adjacent to the producer colony was recorded. It should be noted that in order to differentiate between the effects of $CaCl_2$, $FeCl_3$, and hemin on pesticin production and pesticin activity these reagents were added only to the upper agar layer. Sodium citrate and K_2HPO_4 were added to the basal agar layer since these reagents do not affect pesticin production. Diffusion into the basal layer yields a reagent concentration $\geqq$ 1:6 of that originally added, however, all figures relate to the original $CaCl_2$, $FeCl_3$, and hemin concentrations employed.

Reagents. Reagent grade chemicals were used in all experiments. Rabbit antisera were supplied by W. Lawton. Vitamins were obtained from Nutrition Biochemicals Corporation, Cleveland, Ohio. Hemoglobin (bovine), myoglobin (equine), and ferritin (equine) were received from Pentex Incorporated, Kankakee, Ill.

RESULTS

In preliminary studies, ill-defined and only partially cleared zones of inhibition were observed surrounding pesticin-producing colonies on BAB. However, we found that by adding 0.01 M $CaCl_2$ to BAB, clear and sharply-defined zones of inhibition could be obtained. Employing this medium, 80 virulent and avirulent strains and substrains of *P. pestis* were screened for pesticin-

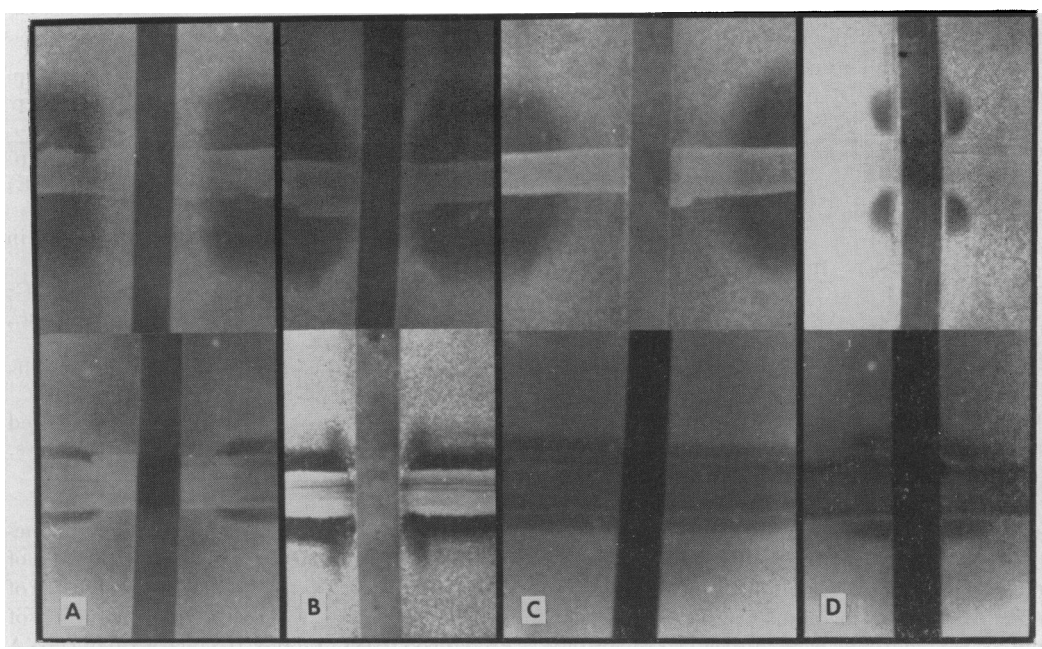

FIG. 1. *The effect of various reagents on the activity of PI (top row) and PII (bottom row) tested by the modified cross-streak method.* A, *Trypsin (1.0%);* B, *Antiserum (PB1/+);* C, *$FeCl_3$ (0.01* M*) all assayed on BAB plus 0.01* M *Ca^{++};* D, *$CaCl_2$ (1.0* M*) assayed on BAB containing 0.001* M *Fe^{+++} in the top agar layer.*

production. In addition to strain TRU, previously reported to be non-pesticinogenic (Ben-Gurion and Hertman, 1958), we found that strains A12 and Java also fail to produce pesticin.

Pesticin II. Unlike strain TRU, strains A12 and Java are sensitive to a second bacteriocin-like substance produced by all tested strains of *P. pseudotuberculosis* and *P. pestis* including strain TRU and the A12 and Java strains themselves. This new activity has been termed pesticin II (PII) as opposed to the activity described by Ben-Gurion and Hertman which will be referred to as pesticin I (PI). Strain A12, but not strain Java, is also sensitive to PI. Certain properties of PII are similar to those previously described for PI (Ben-Gurion and Hertman, 1958). For example: both pesticins are destroyed by trypsin; activity appears in culture supernatants following the induction of lysis by ultraviolet light; and their activities are temperature-dependent and not transferable by sub-culture.

Activity of PI and PII. The effect of Ca^{++} and other reagents on the activity of both pesticins was qualitatively assayed on BAB and on BAB plus 0.01 M $CaCl_2$ by use of the modified cross-streak test. PI activity was determined by employing *P. pestis* OX/P as the producer strain and *P. pseudotuberculosis*, strain PB1/+, as the indicator, whereas PB1/+ was used as the producer strain with strain Java as indicator in studies of PII activity. The concentration and effect of the reagents tested on the activity of PI, PII, and PIi, an inhibitor of PI described below, are illustrated in Table 1. In addition, a summary is given in Table 1 of the effects of some reagents previously shown to affect the growth and virulence of *P. pestis*. It was found that Ca^{++} and Sr^{++} strongly enhanced the activity of both pesticins. Fe^{+++}, hemin, and to a lesser extent Mg^{++} or inorganic phosphate suppressed the activity of PI but not PII, even in the presence of 0.01 M Ca^{++}. Again, both the hemin-containing proteins, hemoglobin and myoglobin, suppressed the activity of PI but not PII. Ferritin had no apparent effect at the concentration tested. PI and PII activity was not inhibited by catalase.

Thiamin, riboflavin, pyridoxal chloride, nicotinic acid, pantothenic acid, biotin, and folic acid had no effect (in saturated or 0.01% solutions) on the activity of pesticin. The activity of both pesticins was not significantly affected by 19 amino acids or by 16 additional metallic cations. Neither PI nor PII was active under any test condition when the indicator strains were incubated anaerobically in a Brewer jar.

Antisera prepared against *P. pestis* strains Alexander, M41, M23, EV76, TS, TRU and *P. pseudotuberculosis* strain PB1/+ neutralized the activity of both pesticins. Normal serum did not exhibit a neutralizing effect. Since strains TRU and PB1/+ do not produce PI, it is probable that neutralization is not due to an antibody. Instead, antibody may be directed against the PI adsorption site or substrate, a situation similar to that described by Bordet (1948) for certain colicine-sensitive strains. Figure 1 illustrates the effects of trypsin, antiserum, Fe^{+++}, and Ca^{++} on the activity of PI and PII.

The Ca^{++} concentration necessary for the enhancement of PII activity was determined by using Poona, Java, and PB1/+ as producer strains, *E. coli* ϕ as a control, and Java as the indicator strain. After sterilization of the developed colonies, grown on BAB, the plates were overlayered with seeded BAB containing 2-fold dilutions of $CaCl_2$. Activity was recorded by measuring the distance from the edge of the producer colony to the edge of the zone of inhibition. Unexpectedly, we found that the non-colicinogenic strain *E. coli* ϕ also produced an activity which slightly inhibited the indicator strain at a Ca^{++} concentration of 0.037 M. At

TABLE 2. *The effect of Ca^{++} on the inhibition of growth at 37 C of Pasteurella pestis strain Java by various bacterial species*

Added $CaCl_2$[a]	Producer strain, zone size[b]			
	P. pestis Poona	*P. pestis* Java	*P. pseudotuberculosis* PB1/+	*E. coli* −
M	mm	mm	mm	mm
0.600	N.G.[c]	N.G.	N.G.	N.G.
0.350	12	12	12	12
0.150	10	10	10	8
0.075	8	8	7	4
0.037	6	6	5	1
0.018	5	4	4	0
0.009	4	2	2	0
0.004	4	2	2	0
0.002	4	2	2	0
0.001	4	2	2	0

[a] Contained in the top layer only.

[b] Measured from edge of producer colony to edge of zone.

[c] (N.G.) No growth of indicator strain Java.

Ca^{++} concentrations of 0.3 M, the zone size surrounding the *E. coli* ϕ colony was equal in size to the zones surrounding the colonies of strains Poona, Java, and PB1/+. The results of this titration are given in Table 2.

Subsequent investigation of 22 additional strains of *E. coli* indicated that 17 inhibit the growth of the PII indicator strains A12 and Java in the presence of 0.2 M but not 0.01 M Ca^{++}. No inhibition was observed when strain A1122 was tested under identical conditions. At 0.2 M Ca^{++}, a single tested strain of *Shigella boydii* also inhibited the growth of strain A12 but not A1122; however, tests of a limited num-

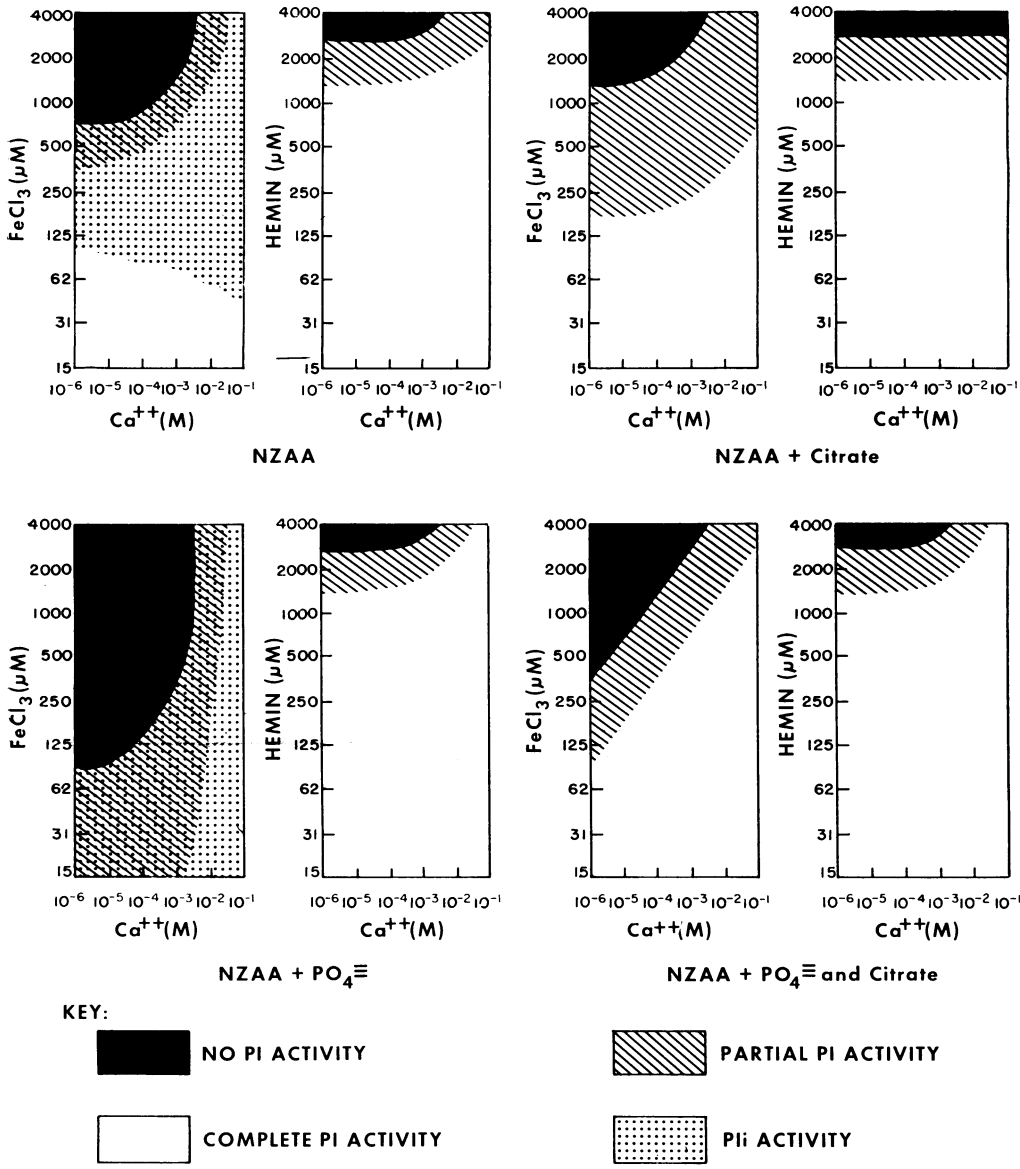

FIG. 2. *The influence of Ca^{++} plus Fe^{+++}, and Ca^{++} plus hemin (contained in the top agar layer), and 0.1 M sodium citrate, 0.1 M sodium citrate plus 0.025 M inorganic phosphate (contained in the basal agar layer), on the activity of PI at 37 C. Pasteurella pestis OX/P was employed as the producer strain and P. pseudotuberculosis strain PB1/+ was used as the indicator.*

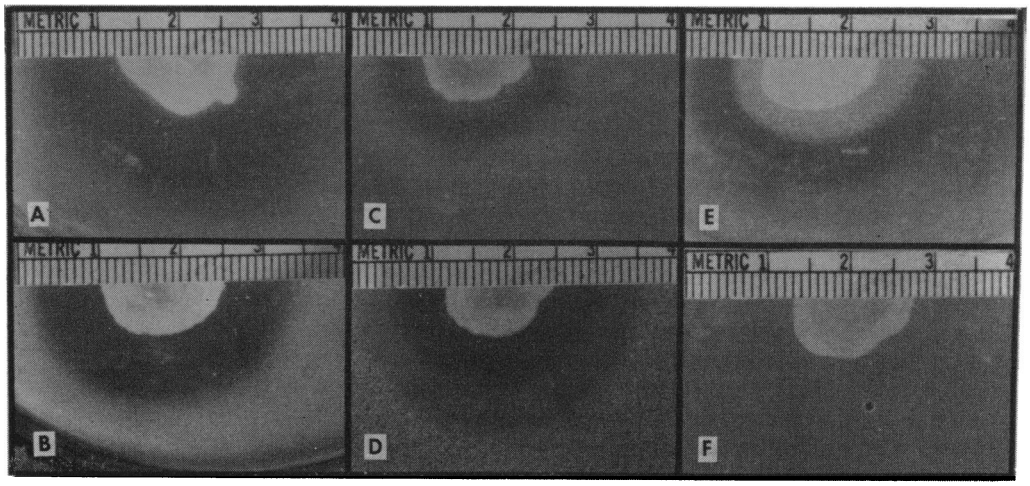

FIG. 3. *The effect of various reagents on the activity of PI and PIi tested on NZAA.* A, *No addition; B, 0.1* M Ca^{++} *(top layer) plus 750* μM Fe^{+++} *(top layer) and 0.6* M *citrate (top layer); C, 0.025* M $PO_4^{\equiv}$ *(basal layer);* D, *0.025* M $PO_4^{\equiv}$ *(basal layer) and 0.6* M *citrate (top layer);* E, *0.1* M Ca^{++} *(top layer) and 750* μM Fe^{+++} *(top layer);* F, *750* μM Fe^{+++} *(top layer).*

ber of *Salmonella* species and other *Shigella* were negative. The activity exhibited by *E. coli* strains is also sensitive to trypsin. Tests concerning induction by ultra-violet light were not performed on this species.

It seemed desirable to determine the quantity of iron and hemin which is required to suppress the activity of PI in the presence of both high and low concentrations of Ca^{++}. NZAA was used for this purpose since this medium requires no added Ca^{++} to support activity of PI. The results of preliminary titrations indicated that the activity of PI was suppressed in the presence of inorganic phosphate and enhanced in the presence of sodium citrate; Fe^{++} had little effect suggesting that this metal may be active only after oxidation to Fe^{+++}. Therefore, the final titrations compared the effects of Fe^{+++} and Ca^{++}, or hemin and Ca^{++}, in the presence and absence of inorganic phosphate and sodium citrate. The results of these experiments are illustrated in Fig. 2. With the exception of the plates containing hemin and citrate, partial or complete activity of PI was always observed in the presence of 10^{-1} M but not 10^{-3} M Ca^{++} when the medium contained 4,000 μM Fe^{+++} or hemin. At a Ca^{++} concentration of 10^{-5} M, 2,000 μM Fe^{+++} was required to suppress completely the activity of PI in the presence of sodium citrate, but only 125 μM Fe^{+++} suppressed activity in the presence of inorganic phosphate. Sodium citrate and inorganic phosphate showed no significant effect on the plates containing hemin. Some typical plates exhibiting complete and partial activity of PI are illustrated in Fig. 3.

Pesticin I inhibitor. A peculiar protective phenomenon was observed in the above titrations when media lacking citrate were employed. As illustrated in fig. 3E, there was growth of the indicator strain over the producer colony which was surrounded by a ring of inhibition. Indicator cells subcultured from colonies growing within the central area of growth remained sensitive to PI. We assumed that the lack of inhibition of growth in the immediate vicinity of the producer colony was due to the action of a metabolite which suppressed the activity of PI. As shown in Fig. 4, the activity of this substance, termed Pesticin I inhibitor (PIi), is dependent upon small concentrations of Fe^{+++} or the presence of inorganic phosphate and the absence of sodium citrate. A corresponding inhibitory activity directed against PII was not observed.

PI has an isoelectric point of 7.5 as determined by use of the Beckman continuous-flow curtain electrophoresis apparatus (Beckman Instruments Co., South Pasadena, Calif.). When cell extracts, prepared by the French pressure cell, are adjusted to pH 4.7, PI is precipitated, presumably as the nucleate, and PIi activity remains in solution. If the acid-insoluble matter is removed by centrifugation, and dissolved in a

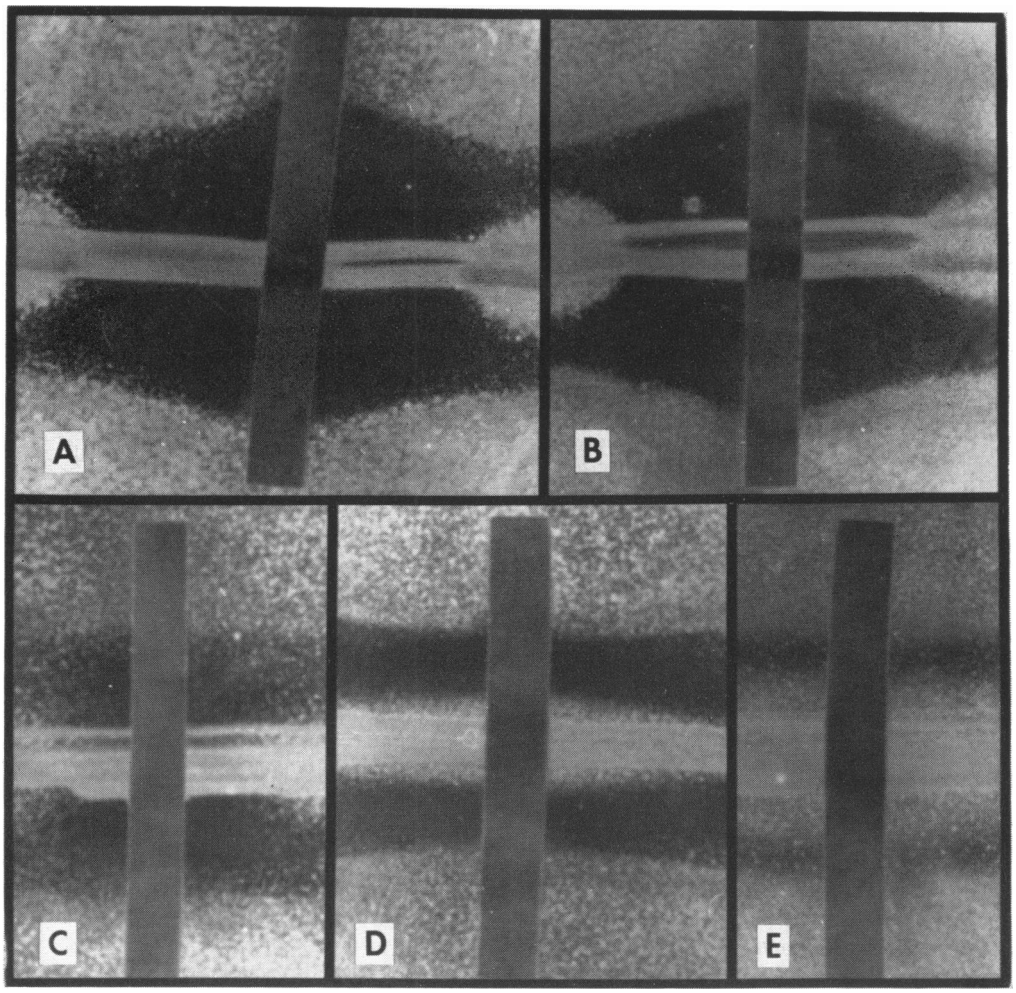

FIG. 4. *The effect of various reagents on the activity of PIi tested by the modified cross-streak method.* A, *$MnCl_2$ (0.01* M*);* B, *Ca-EDTA (1.0%);* C, *sodium citrate (1.0* M*);* D, *$MgCl_2$ (1.0* M*);* E, *Saline (0.85%) control.*

neutral buffer, a dramatic increase of PI titer is observed upon subsequent testing with the method described by Ben-Gurion and Hertman (1958). If the acid-soluble fraction is neutralized and added back to the acid-insoluble fraction, the PI titer returns to that originally observed. These observations will be dealt with more fully in a subsequent communication. The purpose of describing this experiment here is to demonstrate that PIi is an acid-soluble substance which reversibly inhibits the activity of PI.

By use of the modified cross-streak method of Fredericq we found that all tested strains of *P. pestis* and *P. pseudotuberculosis* produce PIi activity when grown at 26 C. At 37 C, *P. pseudotuberculosis* strains of four serotypes produce PIi including the pesticin-sensitive strains PB1/+, PB1/−, and the Type I strain of Thal. *P. pestis* grows poorly on NZAA at 37 C, therefore, PIi production by this species was not determined at elevated temperatures.

The effect of a number of reagents on the activity of PIi was determined by use of the modified cross-streak method. A basal layer of NZAA and a top layer of NZAA containing 0.1 M Ca^{++} and 750 μM Fe^{+++} were used in all tests. The results of these experiments are shown in Table 1. In addition to citrate, it was found that tartrate, calcium-ethylenediaminetetraacetic acid (Ca-EDTA), Sr^{++}, Mn^{++}, and protamine sulfate

inhibited the activity of PIi. Fe^{+++} and to a lesser degree Mg^{++} and inorganic phosphate enhanced the activity of PIi. Crystalline deoxyribonuclease, ribonuclease, catalase, and egg white lysozyme had no observable effect. The chelating agents α, α'dipyridyl, O-phenanthroline, and Ca-EDTA enhanced the activity of PI when tested on BAB. Presumably, this effect is due to the removal of iron, thus inhibiting the activity of PIi. The effects of Mn^{++}, Ca-EDTA, sodium citrate, and Mg^{++} on the activity of PIi are illustrated in Fig. 4.

TABLE 3. *Pesticin-bacterium interrelationships*

Strain	Production		Sensitivity	
	PI	PII	PI	PII
P. pestis (wild type)	+	+	0	0
P. pestis, PW12, PW18, PW39	+	+	+	0
P. pestis A12	0	+	+	+
P. pestis Java	0	+	0	+
P. pestis TRU	0	+	0	0
P. pseudotuberculosis (Type I)	0	+	+	0
P. pseudotuberculosis (Type II)	0	+	0	0
E. coli ϕ	0	?	+	0

Pesticin schema. Since strains Java and A12 both produce PII and are sensitive to this substance, it was of interest to determine if a similar phenomenon occurs in the case of PI. Therefore, 50 colonies of the avirulent *P. pestis* strain OX/W were picked from BAB plates previously incubated for 48 hr at 26 C. Upon subsequent testing by the double agar-layer technique, employing BAB plus 0.01 M Ca^{++}, we found that all 50 produced PI; however, three of these, strains PW12, PW18, and PW39, were also sensitive to PI. All produce, but are not sensitive to, PII. Limited attempts to obtain mutants, which both produce and are sensitive to PI, from strains other than OX/W have not been successful.

Burrows and Bacon (1960) demonstrated that type II strains of *P. pseudotuberculosis* were not inhibited by PI. In this study, we found that single representative types II, III, and V strains of *P. pseudotuberculosis* were not sensitive to PI; however, a number of *E. coli* strains, including *E. coli* ϕ, were inhibited by this activity. Sensitivity to colicines B, D, I, and S1 was lost by four of nine *E. coli* mutants selected for resistance to PI. The remaining five mutants retained sensitivity to all 16 tested colicines. Sensitivity of *E. coli* strains to PII was not observed, and those *P*

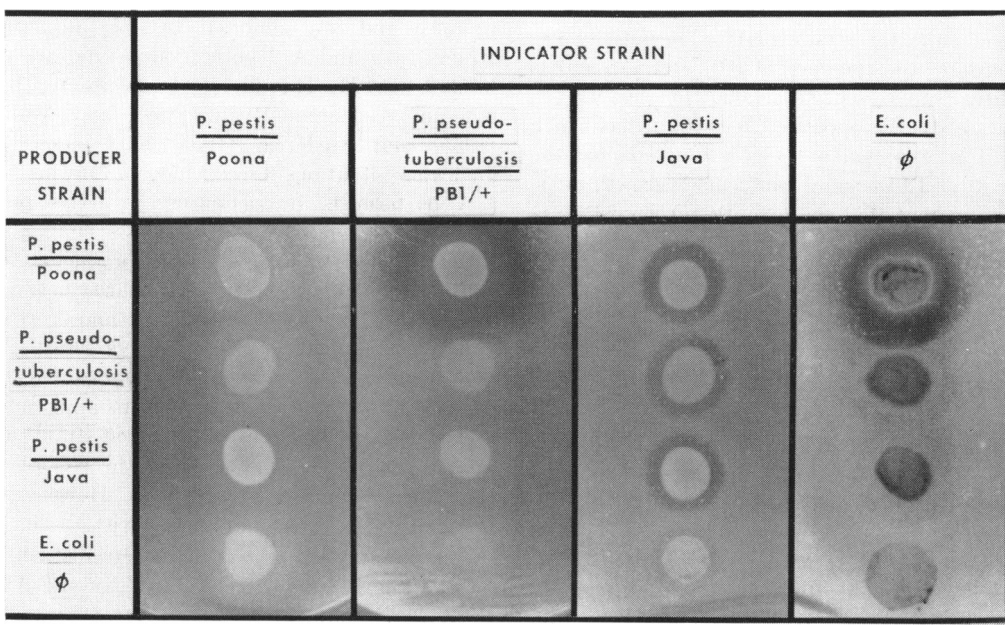

FIG. 5. *Production and activity of PI and PII assayed on BAB plus 0.01 M Ca^{++}.*

pseudotuberculosis strains which were inhibited by PI were resistant to all 16 tested colicines.

The pesticin-bacterium interrelationships described in this paper are outlined in Table 3. Fig. 5 illustrates inhibition of indicator strains by PI and PII.

DISCUSSION

In any test concerning the assay of an antibacterial substance by agar diffusion, a number of factors may affect the size of the zone of inhibition. For instance, Linton (1958) showed that if a sufficient concentration of streptomycin is not built up soon enough by diffusion, permitting a population of indicator cells to exceed a critical threshold of 1.58×10^7 viable cells per ml, no zone of inhibition, or reduction of zone size is observed upon further incubation. An analogous situation might result upon the addition of various reagents to the pesticin-assay media. In this case, an induction of a lag in initial multiplication of indicator cells by Ca^{++} might permit the accumulation of sufficient PI or PII to inhibit the formation of subsequent visible growth. Conversely, in the case of PI, the addition of Fe^{+++} might stimulate rapid initial multiplication of the indicator cells resulting in the eventual appearance of confluent growth about the producer colony. In the case of PI, this explanation must be discarded since growth of the indicator strain PB1/+ is initially enhanced by the presence of 0.01 M Ca^{++} and is slightly suppressed by 4,000 μmoles Fe^{+++}. Since this is the opposite effect from that which would be expected if these cations affected the activity of PI by merely influencing growth rates, it is apparent that more subtle mechanisms are involved. However, growth of the PII indicator strains A12 and Java is markedly suppressed at 37 C in the presence of 0.01 M Ca^{++}. This fact suggests that the mechanism described above accounts for the increased zone sizes that were observed at high concentrations of Ca^{++}.

In contrast to PII, the substance produced by most of the tested *E. coli* strains is not active at low concentrations of Ca^{++}. The zones that are observed when a high (0.3 M) concentration of Ca^{++} is employed are identical in size, regardless of the origin of the antibacterial activity. Therefore, it is probable that a chemical difference exists between the inhibitory substance produced by the *E. coli* strains and that produced by the two tested *Pasteurella* species. It was not possible to verify this hypothesis by employing PII resistant mutants since mutations of this type appear to be unstable.

At least four factors influence the activity of PI. These are: O_2; temperature; the ratio of Ca^{++} to Fe^{+++}; and the presence of cationic substances such as protamine. PI, which is a basic protein, may exist as neutral pH as a PI-PIi complex, or as a salt. The addition of cationic substances such as Mn^{++} or protamine would then result in a competition between these reagents and PI for PIi. Thus PI might be liberated from PIi resulting in an increase of antibacterial activity. The influence of Fe^{+++}, Mg^{++}, and PO_4^{---} on the activity of PIi, and the reversal of the Fe^{+++} effect by Ca^{++} is not understood. Speculation as to the specific roles of these and other reagents on the activity of PI must include the possibility that the four factors listed above act synergystically. In addition, with the method of assay employed, it is not possible to distinguish between effects due to interference with the activity of the PI molecule as opposed to effects which may influence the potential sensitivity of the indicator cells. We hope that further studies employing cell-free systems may clarify some of the complex-interrelationships controlling the activity of PI.

P. pestis strains PW12, PW18, and PW39 both produce and are inhibited by PI. Similarly, strains Java and A12 both produce and are inhibited by PII. This finding is unexpected but not unprecedented. Ryan, Fried, and Mukai (1955) and Fredericq (1956) have described analogous situations for colicinogenic strains.

A bacteriocin, by definition, is active only against cells of a species which is similar or closely related to the bacterium producing the colicine (Fredericq, 1958). The similarity between *P. pestis*, *P. pseudotuberculosis*, and certain enteric bacilli with regard to phage sensitivity and common antigens has been reviewed by Meyer (1958). Since these organisms apparently are related, the fact that some *E. coli* strains are sensitive to PI does not preclude the possibility that this substance is a bacteriocin. However, it is highly improbable that the activity produced by the majority of tested *E. coli* strains, which inhibits the PII indicator strains Java and A12, is an almost ubiquitous and heretofore undiscovered bacteriocin. If the PII molecule produced

by *P. pestis* and *P. pseudotuberculosis* should be identical to the substance produced by strains of *E. coli*, then it would be inappropriate to consider PII a bacteriocin-like substance, or pesticin. Further study of the production, chemistry, and genetics of PI, PII, and PIi will be necessary to determine what role, if any, these substances play in the pathogenic process.

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