THE EFFECTS OF MAMMALIAN AND OTHER CATIONIC POLYPEPTIDES ON THE CYTOCHEMICAL CHARACTER OF BACTERIAL CELLS*

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Substances possessing various degrees of antibacterial activity *in vitro* have been isolated from host tissues and body fluids. It has been suspected for many years that these or related tissue substances might participate in the nonspecific resistance of host tissues to microbial infection.

Early contributions in the field were summarized by Metchnikov (1) and Adam[1] (2). Subsequent work, summarized by Skarnes and Watson (4), has with few exceptions been concerned primarily with extraction and characterization of these antibacterial substances. Particular attention has been given to the basic proteins and polypeptides (these will be referred to collectively as cationic polypeptides), which are widely distributed among animal tissues and which possess antimicrobial (6 *a*, 6 *b*) activity *in vitro*. The *in vitro* antibacterial actions of histones (5–8, 10, 11), protamines 5, 6, 9, 12), and certain basic tissue polypeptides (13–17) have been extensively studied. These substances have been found to be toxic to a wide range of bacterial species *in vitro*, under suitable circumstances. They have been isolated from leukocytes, lymphoid tissues, spermatazoa, blood platelets, neoplastic tissues, and other animal sources. They share the property of being highly basic owing to their high arginine and/or lysine content. Lysozyme (18, 34) is a small basic protein possessing highly specific enzymatic activity for the cell wall mucocomplexes of certain bacteria (18). Lysozyme appears to have some antibacterial properties which may not depend on enzymatic activity (19) but may be dependent on the cationic properties of the lysozyme molecule.

Although antimicrobial activities of cationic polypeptides have long been recognized, evidence that the activity is expressed *in vivo* during the course of infection is meager (3). The interactions of cationic polypeptides with microorganisms lack the sharpness of serological specificity; consequently, the techniques of classical immunology have not been applicable to the study of their action *in vivo*. The methods applicable to the study of antibiotics *in vivo* have not been very helpful, for strongly cationic

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polypeptides appear to be so reactive that they are not freely distributed throughout the tissue fluids (20).

The need for direct demonstration of interaction between cationic polypeptides and microorganisms in host tissues has become evident because of considerations which indicate that such interactions may be either spatially or temporally circumscribed or that they may never occur at all. The possibility exists that the substances, or their *in vitro* antibacterial activity may be preparative artifacts (4). In addition it is well known that other materials extractable from tissues, for example organic anions, powerfully antagonize their antimicrobial activities (16). Whatever the validity of objections based on such considerations, it has been evident that the role of these substances in resistance to infection could not be defined without suitable technical means.

The application of cytochemical technique to the problem has been reported by Spitznagel (21–23). It was found that treatment with total histones rendered bacteria stainable with an anionic dye, fast green F.C.F., at pH 8.1. Fast green F.C.F. will be referred to simply as fast green in this paper. The fact that untreated bacteria do not ordinarily become stained under these conditions had been demonstrated earlier (24, 35, 36). Alfert (25) had shown that staining with fast green at pH 8.1 was selective for histones in tissues. It seemed possible that this technique might be used in conjunction with other techniques to demonstrate directly the interaction of bacteria with cationic polypeptides *in vitro* and *in vivo*.

The investigations to be reported here have shown that the cytochemical characteristics of bacterial cells are altered in a predictable fashion through interaction with cationic polypeptides and that the polypeptides appear in highest concentration in the surface layers of the bacterial cells.

Materials and Methods

Bacterial Species.—A strain of *Escherichia coli* and a strain of *Bacillus anthracis* were used in these experiments. Both organisms have been maintained for several years on appropriate solid media in the culture collection of the University of North Carolina. They show typical morphological and biochemical characteristics. The strain of *Bacillus anthracis* used is encapsulated and virulent for mice. The *E. coli* strain is of serotype 026:B6[1] and has a certain degree of pathogenicity for rabbits but it is apparently less virulent for guinea pigs as judged by its ability to multiply *in vivo*.

Media.—Antibiotic assay agar and antibiotic assay broth (Difco Laboratories, Detroit) were used throughout these experiments. Plate counts of *E. coli* were done using nutrient agar.

Polypeptides.—Calf thymus polypeptides were obtained through the kindness of Dr. J. G. Hirsch of the Rockefeller Institute, New York City, and Dr. R. C. Skarnes of the National Institutes of Health, Bethesda, Maryland. These products have been described (15*a*, 16).

[1] Tentative identification made using available antisera. Dr. W. H. Ewing of the United States Public Health Service, Communicable Disease Center, Atlanta, Georgia, has subsequently identified this organism as an antigenically closely related serotype 0117:H27 which has an unidentified *K* antigen. The 0117 antigen is known to cross-react with 026 immune serum.

Both polypeptides are reported to be cationic and are rich in lysine. Hirsch's preparation also contains a large proportion of arginine. Total histone preparations were obtained from commercial sources[2] and through the kindness of Dr. Claude McClure of the Department of Biochemistry of the University of North Carolina.

Fraction V bovine serum albumin, bovine gamma globulin, *E. coli* 026:B6 immune serum, protamine, polymyxin B, hen's egg lysozyme, and pancreatic ribonuclease were also obtained from commercial sources.

Organic Anions.—Hyaluronic acid and deoxyribonucleic acid were obtained from various commercial sources. Hog gastric mucin was obtained from the Wilson Company, Chicago.

The Cytochemistry of Bacteria.—The staining reactions of bacteria were studied in wet mounts and in air-dried and fixed preparations. Wet mounts were found more useful in studying bacterial morphology because the bacteria were better able to retain their shapes if they were not dried. Wet mounts were not suitable, however, for all of the experiments to be described.

A modification of Alfert's (25) method for staining histones with fast green at pH 8.1 was devised to detect the presence of cationic polypeptides in or on the bacterial bodies. A 0.1 per cent aqueous solution of fast green was titrated electrometrically to pH 8.1 using N/20 sodium hydroxide just prior to use in staining. For use in wet mounts one loop of bacterial suspension was mixed with one loop of fast green and sealed with petroleum jelly under a 22 mm^2 No. 1 coverslip. The preparation was allowed to sit at room temperature for 1 hour prior to study. Although the dye solution is blue at pH 8.1, stainable structures in bacteria or tissues acquire a definite green color.

Air-dried smears were immersed for 1 hour in fresh fast green at pH 8.1, washed briefly in distilled water, and then stained 30 seconds in 0.25 per cent aqueous azure A, washed 1 minute, and dried. Bacteria treated with strongly cationic polypeptides such as histone stain green and untreated bacteria take the azure stain. Bacteria treated with strongly cationic polypeptide do not take the azure stain even when the fast green stain is omitted.

Staining for protein-bound arginine was done on air-dried bacterial smears using the Sakaguchi oxime technique (26 *a*). It was found that celloidin coating prevented the bacteria from being washed off the glass. Structures containing large amounts of arginine stained an orange color.

Protein-bound amino groups were looked for using the azomethine technique (26 *b*). Structures high in protein-bound amino groups such as the epsilon amino group of lysine stained a pink or plum color.

The fluorescence of bacteria in ultraviolet light was studied using a Leitz fluorescence microscope. Bacteria were generally viewed suspended in 0.05 molar phosphate-citrate buffer at pH 5.6 in wet mounts. Fluorescence was observed best using a 2 mm UGI filter in the light source of the ultraviolet fluorescence microscope.

RESULTS

Sorption of Cationic Polypeptides to Bacterial Cells In Vitro.—The interactions of mammalian cationic polypeptides and bacterial cells were studied by treating a Gram-negative and a Gram-positive species with total histone *in vitro*. *Escherichia coli* and *Bacillus anthracis* were chosen for this study. They have been found to be sensitive to the toxic effects of cationic polypeptides and this sensitivity has been extensively investigated by others as noted above. *B.*

[2] Worthington Biochemical Corp., Freehold, New Jersey; Mann Research Laboratories, New York City.

anthracis is highly virulent for certain animal species but not so virulent for others. *E. coli*, on the other hand, is generally not considered particularly virulent yet it possesses a considerable degree of pathogenicity under a variety of circumstances which appear to be associated in a rather ill defined fashion with disturbances in host resistance. Total histones were chosen as a type of cationic polypeptide because their antibacterial properties (10) and their chemistry (30) have been extensively studied.

Cultures of *E. coli* and *B. anthracis* were prepared by cultivation of large inocula in antibiotic assay broth for 18 hours at 37°C. The bacteria were removed from the medium by centrifugation and resuspended to original volume in 0.05 M phosphate-citrate buffer at pH 5.6 and pH 6.8 (10). 0.2 ml of the resuspended bacteria were then inoculated into tubes containing total histone in 0, 12 μg/ml, 25 μg/ml, 50 μg/ml, and 100 μg/ml concentrations in a volume of 1.8 ml per tube. These tubes were incubated in a water bath at 37°C for 1 hour. The bacteria were then resuspended with vigorous shaking and a loopful of each suspension was mixed on a coverslip with aqueous fast green at pH 8.1 and sealed to a glass slide with petroleum jelly for microscopic examination.

TABLE I

Fast Green Staining of Bacteria Exposed to Various Concentrations of Histone at Two Hydrogen Ion Concentrations

pH	*E. coli*					*B. anthracis*			
	Histone μg/ml					Histone μg/ml			
	0	12	25	50	100	0	12	25	50
5.6	0*	11	22	53	80	0	28	70	86
	0	9	20	58	75	0	20	70	86
6.8‡	0	8	35	76	73	0	20	51	95
	0	11	41	65	64	0	11	64	95

* The numbers represent the percentages of bacteria stainable with fast green after 1 hour at 37°C in histone as indicated.

‡ The percentage figures for pH 6.8 preparations tend to be too low because of the severe structural damage which was produced in the bacteria. The damage to *B. anthracis* was particularly severe and left only stained fragments thought to be crumpled cell walls.

The bacteria in the wet mounts were examined immediately after they had been prepared and no staining was noted. After 30 minutes, however, (see Table I) a varying number of bacteria from the histone-containing tubes, of both *E. coli* and *B. anthracis*, had taken the green stain. Bacteria from control tubes without histone did not stain.

Fig. 1 shows the appearance of *E. coli* stained with fast green after exposure to total histone 100 μg/ml. The stain affected areas similar to those stained by the periodic acid-Schiff technique in *E. coli* and was most intense at the pe-

riphery of the organisms especially about one end or a side. Occasionally a granule which appeared to be near the surface and in the center of the bacterial cells became stained. The histone-treated *E. coli* were no longer motile although the control suspensions were so, even in fast green.

Figs. 2 and 3 show the appearance of *B. anthracis* stained with fast green after exposure to total histone 100 and 50 μg/ml respectively. Staining tended to occur in discrete areas at the periphery and under the capsule (not visible). With the higher histone concentrations a very densely stained area appeared surrounding the middle fourth of the anthrax cells. With the highest histone concentrations the cells of both species tended to stain uniformly and intensely. The cells of both species were agglutinated.

It seemed likely that the basic histones were becoming attached to particularly acid portions of the bacterial cells. To test this notion *E. coli* cells which had not been treated with histones were stained with aqueous azure A or with crystal violet 0.1 per cent. These cationic dyes were adjusted to various pH values electrometrically using N/20 NaOH or N/20 HCl. It was found that staining occurred with these dyes in patterns similar to those which histone treatment induced *vis-a-vis* fast green. The staining of these areas near the cell surface ceased at or below pH 3. It appeared, therefore, that these areas were quite acidic. This supported the concept that the basic proteins combine with acidic sites on the bacterial cells.

The Effects of Concentration and pH on the Interactions of Cationic Protein with Bacterial cells.—Only a variable proportion of bacteria exposed to histones became stainable with fast green at pH 8.1. It appeared likely that this might be the result of variables which could be defined and controlled. To investigate this possibility a constant, known concentration of bacterial cells was allowed to interact with several different concentrations of total histones at two pH levels in 0.05 M buffer solution. Table I shows that the percentage of green-staining cells was directly related to the histone concentration. Although it could not be quantitated with techniques available to us, it was evident that the degree of fast green staining of individual bacterial cells became more intense with the increase in histone concentration.

The influence of the pH levels chosen on the interaction of total histone with *E. coli* and *B. anthracis* was found to be rather equivocal in terms of the degree or percentage of fast green staining of the organisms. It was evident, however, that both species had undergone considerable cellular damage in histone at pH 6.8. *B. anthracis* cells were particularly damaged (see Fig. 4) with the result that only stained material associated with the crumpled cell walls remained recognizable. The fate of the protoplast was not determined. The extent of this cellular damage is demonstrated by Table II. These data were obtained by studying, with phase microscopy, the total number and morphology of bacteria from the several *B. anthracis* cultures of Table I. The

data show that the proportion of normally shaped bacteria decreased markedly at pH 6.8 and that increasing histone concentration accentuated this.

The Effects of Varying the Mass of Bacterial Cells on Their Interaction with Total Histone.—When different concentrations of *E. coli* and *B. anthracis* were exposed to a constant histone concentration at pH's 5.6 and 6.8 (Table III) the percentage of bacteria stainable with fast green increased as the bacterial mass decreased. As in the experiments shown in Table II severe cellular damage was encountered in the suspensions of *B. anthracis* exposed to histone at pH 6.8. This was most marked in the tubes with the lowest bacterial concentrations.

TABLE II

Loss of Normal Cellular Architecture in Suspensions of B. anthracis Exposed to Histone Related to Hydrogen Ion Concentrations

pH	Histone	Total countable bacteria*/ml suspension	Total bacteria with normal shape
	μg/ml		
5.6	0	2.2×10^7	2.2×10^7
	12	1.2×10^7	3.4×10^6
	25	1.1×10^7	7.4×10^5
	50	7.0×10^6	8.7×10^5
6.8	0	2.1×10^7	2.1×10^7
	12	8.8×10^6	2.5×10^6
	25	1.1×10^7	4.1×10^5
	50	1.5×10^7	0

* Counted in 10 per cent formalin with phase microscope and Petroff-Hauser counting chamber.

The Effects of Organic Anions on the Interaction of Histones with Bacteria.—Anionic molecules have been reported (6, 10, 16, 17, 27–29) to inhibit the antibacterial effects of cationic polypeptides. If the interaction of total histones with the bacterial cells was required in order that they become fast green–positive, it was expected that organic anions would block the interaction and thus prevent staining. To test this, aqueous solutions of several organic anions were mixed with the bacterial suspensions prior to adding the histones. Oleic acid, gastric mucin, deoxyribonucleic acid (DNA), hyaluronic acid, and fraction V bovine albumin were tried in various concentrations. Inhibition of the interaction between bacteria and histone was evaluated by staining with fast green at pH 8.1. In Table IV it may be seen that albumin did not appear to inhibit, DNA and mucin seemed most inhibitory weight for weight, and hyaluronic acid was almost as effective as DNA and mucin. Addition of these substances subsequent to the addition of histone had little effect on the inter-

action of bacteria with histones as tested by fast green staining. These substances did not appear to block the staining with fast green when they were added to suspensions of histone-treated bacteria.

Because magnesium (15 *b*, 17) and sulfate (15 *b*) ions had been reported to inhibit the antibacterial effects of basic calf thymus polypeptide on *Mycobacterium* the effect of $MgCl_2$ and K_2SO_4 on the interaction of *E. coli* and histone was evaluated by the same method used for acidic molecules (see Table V). It was found that 40 mM/liter of these salts appeared to inhibit interaction completely according to the criterion of fast green staining. 4 mM/liter of $MgCl_2$ was partially inhibitory. These results supported the hypothesis that fast green at pH 8.1 stained only cells which had interacted with the total histones.

TABLE III

The Effects of Bacterial Concentration on the Interaction between Bacteria and Histone 25 μg/ml Measured by Fast Green Staining

Organism	pH	Bacterial concentration mg dry wt/ml			
		0.08	0.04	0.02	0.01
E. coli	5.6	55*	62	72	81
	6.8‡	50	58	70	90
B. anthracis	5.6	64	94	100	100
	6.8‡	61	Unreadable	Unreadable	Unreadable

* Per cent bacterial cells stained by fast green.

‡ Counts uncertain owing to severe alterations in bacillary structure, especially in the *B. anthracis* suspensions.

The Selectivity of Fast Green Staining at pH 8.1 as an Indicator of Interaction between Bacterial Cells and Cationic Polypeptides.—In order to gain information concerning the specificity of the effect of histone on the fast green staining of *E. coli* and *B. anthracis*, albumin, polymyxin B, egg lysozyme, ribonuclease, γ-globulin, protamine, and total histone were allowed to react with *E. coli* and *B. anthracis* under similar conditions. The bacteria were then stained with fast green. Table VI shows that only protamine and total histone induced the bacteria to stain with fast green at pH 8.1. Polymyxin B had a slight effect on *E. coli* at pH 6.8. Ribonuclease had a slight effect on *B. anthracis* at pH 5.6. The basic calf thymus polypeptides (not shown in table) furnished by Hirsch and Skarnes both induced the bacteria to stain with fast green in a fashion comparable to that observed with total histone. *E. coli* agglutinated by specific antiserum did not stain with fast green at pH 8.1 but did stain with azure A.

Other Cytochemical Evidence for the Adsorption of Histones to Bacterial Cells.—Staining of materials by anionic dyes such as fast green appears to be due to

TABLE IV

Effects of Anionic Organic Molecules on the Interaction between Histone 50 μg/ml and E. coli 0.08 mg/ml as Measured by Fast Green Staining*

Organic anion	Total histone	Concentration of organic anions μg/ml										
		0	0.25	1.0	2.5	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Oleic acid	+	−‡	59§	−	80	−	−	0	−	−	−	−
	None	−	−	−	−	−	−	−	−	−	−	0
Hog gastric mucin	+	−	−	57	−	−	26	−	−	12	−	−
	None	−	−	−	−	−	−	−	−	−	−	0
Deoxyribonucleic acid	+	−	−	46	−	−	46	−	−	0	−	−
	None	−	−	−	−	−	−	−	−	−	−	0
Hyaluronic acid	+	−	−	−	58	−	−	28	−	−	16	−
	None	−	−	−	−	−	−	−	−	−	−	0
Albumin	+	−	−	−	−	67	−	−	64	−	−	69
	None	−	−	−	−	−	−	−	−	−	−	0
None	+	63	−	−	−	−	−	−	−	−	−	−

* Dry weight bacteria/ml.

‡ A negative sign indicates no test was done under these conditions.

§ Percentage of bacteria which stained with fast green at pH 8.1 after 1 hour incubation at 37°C with the organic anions and histone in the concentrations stated.

TABLE V

Effects of Divalent Ions on the Interaction between Histone 50 μg/ml and E. coli at pH 5.8 in 0.05 M McIlvaine Buffer

	Salt Concentration in mM/liter*			
	0	0.4	4.0	40
$MgCl_2$	63‡	51	14	0
K_2SO_4	−	59	52	0

* These figures do not include the buffer salt present.

‡ The figures in the boxes represent percentage of bacteria which stained with fast green at pH 8.1.

the presence of free, dissociated cationic groupings such as the guanidinium of arginine and the epsilon amino of lysine (25). The anionic dye apparently forms an electrovalent linkage with the cationic groupings on the material stained (25, 26 *c*). At high pH values only proteins of high isoelectric point such as histones and protamines have sufficient dissociated anionic groups to

combine with the dye in amounts sufficient to produce visible staining. It seemed likely that cationic polypeptides cause bacteria to be stainable with anionic dyes by providing the necessary cationic groupings for combination with the dye. An alternative mechanism considered was that the cationic polypeptides might combine with the anionic groupings which ordinarily determine bacterial surface charge. The cationic groupings of the bacterial cell might be freed in this way to combine with the anionic dye. That this is possible is easily demonstrated by esterification (26 *d*) of bacterial cells. Cells treated thus stain brilliantly with fast green at pH 8.1. It seemed possible too that the fast green staining might have been caused by increased cellular permeability

TABLE VI

Interaction of Various Proteins and Polypeptides with Bacteria as Reflected by the Affinity of the Bacteria for Fast Green

Organism	pH	Albumin*	Polymyxin B*	Lysozyme*	RNAase*	Globulin*	Protamine*	Histone*
E. coli 0.07 mg	5.6	0‡	0	0	0	0	54	82
dry wt/ml	6.8	0	15	0	0	0	45	90
B. anthracis 0.02 mg dry wt/ml	5.6	0	0 cells fragmented	0	17	3	92	76
	6.8	0	0§	0§	0	0	85	—

* 50 μg of each substance were added to the *E. coli* suspensions and 25 μg of each substance were added to the *B. anthracis* suspensions.

‡ Percentage of organisms stained with fast green after 1 hour at 37°C with the agent indicated.

§ Suspension cleared grossly.

brought about by the death of the cells or the detergent action of cationic polypeptides and was not primarily related to the physicochemical characteristics of the bacterial cell.

It was found that treatment of *E. coli* and other bacterial species by autoclaving or heat fixation did not render them stainable by fast green at pH 8.1. Polymyxin (see Table VI) and penicillin had little or no effect on such staining. Altered permeability of the cell did not seem to be an important cause for staining.

E. coli were deaminated with nitrous acid (26 *e*). The procedure was effective as the bacteria were no longer stained by acid dyes at low pH. Histone treatment rendered the deaminated bacteria stainable with fast green. Thus the dye-binding sites appeared to have been provided by the histone.

Trypsinization of histone-treated *E. coli* rapidly abolished their reactivity with fast green. Since histones are quite sensitive to tryptic digestion (31) this

supported the concept that the polypeptide must be present on the bacterial cell in order that the cell be stainable with fast green at pH 8.1.

During attempts at studying the histone sorption by bacteria with fluorescent antibodies it was noted that *E. coli* and *B. anthracis* treated with histones became fluorescent in ultraviolet light. In Fig. 5 it may be seen that *E. coli* treated with 50 μg/ml of total histones in 0.05 molar pH 5.6 phosphate citrate buffer fluoresce in ultraviolet light. Comparison of Fig. 5 with Fig. 1 shows that the fluorescence is most intense in areas similar to those which stain most intensely with fast green. The mechanism by which this fluorescence is produced is not clear. However, we have found that excitation at 2900 A causes total histones to fluoresce at 3300 A and 6200 A. The fluorescence in the visible wavelengths is considerably weaker than that in the ultraviolet. Fluorescence and phosphorescence of proteins and polypeptides have been more extensively studied by others (31).

The presence of guanidinium (arginine) and free amino groups (epsilon amino of lysine) on the histone-treated bacteria was studied by means of the Sakaguchi oxime staining technique and the azomethine technique. Cells of *E. coli* treated with total histones *in vitro* were compared with cells from the same cultures treated only in the phosphate-citrate buffer. Smears of human blood were used as positive controls. It was found that histone-treated *E. coli* gave a positive reaction with both techniques while the control *E. coli* cells did not stain by either method. This was interpreted to mean that the positive staining was due to the presence of histone on the bacterial cells.

DISCUSSION

These studies have shown that cationic polypeptides, sorbed to bacterial cell walls, impart to the cells some of the physicochemical properties of a cationic particle. This has made possible cytochemical differentiation of bacterial cells which have sorbed sufficient cationic polypeptide. It has long been known that cations of different sorts are bound with varying affinities to bacterial cells. Bacterial interaction with colored compounds such as the triphenylmethane dyes can be studied microscopically, a circumstance of great importance in microbiology.

The cationic polypeptides are colorless in visible light. Consequently it has not been possible to observe directly their interaction with bacteria. The interaction of bacterial cells with polymyxin, which is a cationic polypeptide has been observed directly using a fluorescent derivative of the polymyxin (32 *a*). Prior to this the removal of polymyxin by bacterial cells from the suspending medium had been demonstrated (33). We have shown that total histone sorbed to bacterial cells causes them to fluoresce. This is a relatively weak fluorescence, however, and requires the application of relatively high concentrations of histone.

Evidence of a less direct nature than that cited for polymyxin has been submitted to show that cationic polypeptides from tissue combine with bacterial cells. Fleming (34) concluded from direct microscopic observation that the cell wall became swollen during the action of lysozyme and this observation has been amply confirmed by several workers. Protamines and synthetic lysine polypeptides have been shown to cause bacterial cells to agglutinate (12, 27, 29), presumably by combining with and altering the character of the bacterial surface. We have observed this agglutination repeatedly and have found, as others have, that the cellular aggregates are easily dispersed by shaking. Tomcsik (9) found that certain proteins, including protamines, produced changes in the refractive properties of the surface layers of bacterial cells which were visible with the use of phase microscopy. This work was of particular interest because it was directed toward a study of the interactions between bacteria and substances produced in inflammatory reactions. Unfortunately, as Tomcsik pointed out, the method had a low degree of specificity. Elution of a substance possessing antibacterial properties from heat-killed, histone-treated *E. coli* has been demonstrated (10). Amano (14) observed that plakin-treated cells of *Bacillus* species were easily stained by neutral red although untreated cells did not combine with the dye. Metchnikov (1) observed that *E. coli* organisms in phagocytic vacuoles became stainable with neutral red. From the observations with neutral red one may infer that a change in cell surface has occurred but the nature of the change is not clear.

We wished to have supporting evidence for the notion that the sorbed cationic polypeptides were cytochemically demonstrable on the bacterial cell. It was especially desirable that the methods employed operate to demonstrate this through independent mechanisms. The Sakaguchi cytochemical technique which has been recognized as being quite specific (26 *f*) for arginine depends on the formation of a colored compound as oxime reacts with the guanidinium of arginine in the presence of hypochlorite. Methylation and deamination, which depend on the blocking or the destruction of anionic and cationic groupings of the bacterial cell, were found to produce the anticipated cellular changes. The deaminated cells stained with fast green only after histone treatment. Since the amino groups of the bacterial cell were no longer free to bind the dye, it appeared that the epsilon amino and/or guanidinium groups of the histone were binding the dye. The fluorescence which appeared in histone-treated bacteria was similar in color to the fluorescence seen in uncombined histone. The sites which fluoresced were apparently identical with those which took the fast green at pH 8.1. These results, as well as those obtained with tryptic digestion of histone-treated cells, appear to indicate that the cationic polypeptides combine with and are physically present on the bacterial cells. The polypeptides appear to impart to the cell certain of their properties.

In order that a method be useful for discriminating between bacteria which

have sorbed cationic host proteins *in vivo* and ones which have not, it must be reasonably selective. The use of fast green at pH 8.1 was adapted from a technique developed by Alfert (25) for the study of histones in cell nuclei. He found that only histones and protamine bound the dye in high concentration. We have confirmed this. In addition we found that the calf thymus polypeptides of Hirsch, and of Skarnes, also bind the dye. These experiments were carried out on filter paper models and with bacteria. A wide variety of substances from animal tissues, including lysozyme and ribonuclease and serologically specific antibody failed to give bacterial cells the capacity to bind fast green. Subsequent experiments have added globin and spermine to this list. It was found very important to avoid acid fixatives such as Carnoy's or fixatives with an appreciable tendency to mask carboxyl groups. Such fixatives render acidic proteins stainable with fast green at pH 8.1. Air drying or formalin fixation followed by thorough washing appeared to be best. Except for situations where these precautions were ignored, fast green at pH 8.1 was selective for highly cationic polypeptides and successfully discriminated between bacteria treated with these and other biological compounds.

We have found that various anionic substances, including nucleic acids and gastric mucin, prevent cationic polypeptides from being sorbed to bacterial cells. Inhibition of sorption by divalent anions and cations was likewise demonstrated. The antagonisms were readily recognized by the absence of cationic properties among cells treated with cationic polypeptides in systems containing the antagonists. Fast green failed to stain such cells. This was not due to the anions interfering with interactions between the anionic dye and a polypeptide-bacterial cell complex, for the staining of cell-polypeptide complexes formed prior to the addition of the anions was not blocked. Several workers have found anionic substances antagonistic to the antibacterial effects of cationic polypeptides (6 *a*, 6 *b*, 15 *b*, 16, 17, 27–29). The inhibition of cationic-polypeptide activity by divalent ions and by certain cations has been observed (15 *b*, 32 *b*).

Staining with fast green showed that the greatest concentration of cationic polypeptide appeared near the cell surfaces of *E. coli* and *B. anthracis*. This has been generally true in similar experiments with various other genera and species (unpublished data). The use of fluorescence induced in ultraviolet light confirmed this finding. Agglutination of bacterial cells and loss of motility in *E. coli* were also noted. The locus of action appears to be in the cell wall or cell membrane or both in the case of *E. coli*. In *B. anthracis* a similar situation is present but in addition areas of high polypeptide concentration were observed extending out into the capsule. The latter patterns resembled changes observed by Tomcsik (9) in *B. anthracis* treated with cationic polypeptides. The action in cell wall and/or cell membranes is of interest in the light of Newton's (32 *a*) demonstration that these are the loci of action of a fluorescent polymyxin-B derivative. Evidence that cell walls and/or cell membranes are important loci

of action of cationic polypeptides has been adduced by others (14, 18, 33, 34). Our observations have shown that cellular integrity is disrupted by non-lysozyme cationic polypeptides. This action appeared to resemble that of polymyxin-B.

The action of cationic polypeptides on microbial cells has been attributed to: combination of polypeptide with acidic components of the cell surfaces (12, 16, 28, 32 *a*, 32 *b*); combination of cationic-polypeptide with anionic sites such as the phosphoric groups of nucleic acids not necessarily in the surface layers (17, 27, 29, 32 *b*); detergent action (5, 32–33); chelation of sulfate (15 *b*); interference with cations (17, 32 *b*); enzymatic activity on mucocomplexes of cell surface components (18). Our evidence indicates that the cationic polypeptides do combine with what are probably anionic sites in the cell surface layers over a pH range from 5.6 to 7.0. At pH 6.8 and higher, cellular disruption takes place which might be interpreted to indicate that a detergent action had occurred. It could not be ascertained whether or not combination with nucleic acids occurred. The antagonistic action of nucleic acids against cationic polypeptides may simply be due to their anionic character and have nothing to do with their being nucleic acids. The antagonistic action of divalent ions is probably related to interactions with the ion exchange activities of the baterial cell itself. We made no specific effort to detect enzymatic activity on the cell mucocomplexes themselves. Morphological evidence indicated that lytic action of the lysozyme variety did not occur.

It appeared most likely that electrovalent binding accounted at least in part for the interaction between cationic polypeptides and anionic bacteria. Interactions at cell surface are generally thought to be quite complex. Consequently, the binding forces are likely to be more complex than those of electrovalent nature.

SUMMARY

Cationic polypeptides interact with bacterial cells of *E. coli* and *B. anthracis*. They confer upon the cells some of the characteristics of cationic particles. Since bacterial cells usually behave as anions, acidic dyes at high pH levels differentiate between cells which have and those which have not interacted with cationic polypeptides. Under the conditions of these experiments it appeared that cationic polypeptides tend to be sorbed in highest concentration in the surface layers of the cells. Electrovalent binding to anionic cell components and detergent action are probably among the mechanisms involved in the interaction between the polypeptides such as histones and bacterial cells.

The differential staining of bacterial cells which have interacted with cationic polypeptides is feasible and reasonably selective. It should be useful in determining whether bacterial cells interact with host cationic polypeptides *in vivo*.

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EXPLANATION OF PLATE 104

PLATE 104

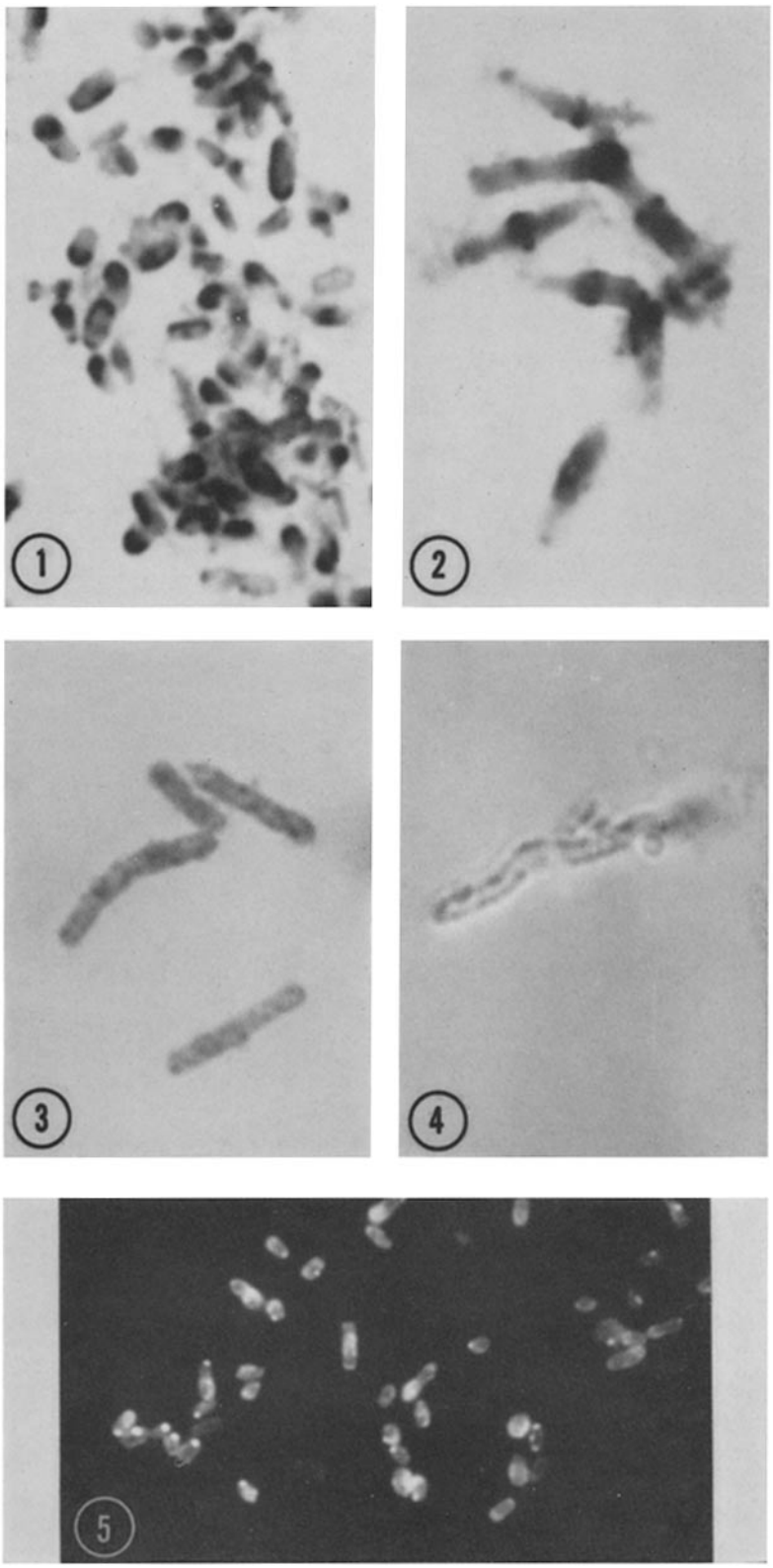
FIG. 1. 18 hour culture of *E. coli* incubated 1 hour with total histone 100 μg/ml at pH 5.6 and stained in a wet mount with aqueous 0.1 per cent fast green at pH 8.1. Magnification × 3000. The dark areas were green.

FIG. 2. *B. anthracis* cells from a 24 hour culture treated at pH 5.6 with total histone 100 μg/ml for 1 hour. Stained with aqueous 0.1 per cent fast green at pH 8.1 in wet mount. Total magnification × 3000. The dark areas were green.

FIG. 3. *B. anthracis* cells from a 24 hour culture, treated with 25 μg/ml total histone at pH 5.6 for 1 hour and stained with aqueous 0.1 per cent fast green at pH 8.1 in wet mount. Total magnification × 3000. The dark areas were green.

FIG. 4. *B. anthracis* cells from culture used for Fig. 3 treated with 25 μg/ml of total histone at pH 6.8 for 1 hour and stained at pH 8.1 with aqueous 0.1 per cent fast green. Total magnification × 3000. The dark areas were green-stained cell wall and possibly cell membrane.

FIG. 5. Fluorescence of *E. coli* in ultraviolet light. This 18 hour culture was treated with total histone 50 μg/ml for 1 hour at pH 5.6. Wet mount in pH 5.6 phosphate-citrate buffer. Total magnification × 2700.



(Spitznagel: Effects of polypeptides on bacterial cells)