BACTERICIDAL ACTION OF HISTONE*

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In the course of investigations on biochemical components of tissue which may exert antimicrobial effect, it has been observed that histone manifests impressive bactericidal activity under certain conditions *in vitro*.

Experimental Techniques

The following procedures were used for culturing microorganisms and for performing antibacterial tests. Clear plastic trays containing rows of cup-like depressions of *ca*. 3 ml. each (disposo-trays, Linbro Chemical Corp., New Haven) were soaked overnight in 95 per cent ethanol, rinsed thoroughly with distilled water, and air-dried. These were placed in containers (plastic refrigerator trays) fitted with hinged lids and were sterilized by exposure to ultraviolet light. Each row of cups served as a test series; 0.9 ml. of medium was placed in the first cup and 0.5 ml. in remaining cups of the series. After addition to the first cup of 0.1 ml of material to be tested, serial twofold dilutions were made by mixing well and transferring 0.5 ml. with a Cornwall automatic syringe, except for the last cup in each row which consisted of medium only and served as a control. Bacteria cultured overnight in penassay broth (Difco) were diluted appropriately in sterile distilled water or in the test medium so that inoculation with 0.02 ml. from a Pasteur pipette yielded 50 to 200 bacteria in the control cup containing no antibacterial agent. After incubation at 38°C. for 2 hours, melted penassay agar at 45–50°C. was added, thus making, in effect, a miniature pour plate of each cup. After drying in the hood for 1 to 2 hours to eliminate excess moisture, the lids were closed and the trays incubated at 38°C. overnight. On inspection the number of colonies was estimated, the end point usually taken being that cup containing the lowest concentration of antibacterial agent producing more than a 50 per cent reduction in number of colonies. Usually this "50 per cent end point" was quite sharp, with complete absence of growth in cups containing 2- or 4-fold higher concentrations of inhibitor. Other techniques, when employed, are discussed in the text.

RESULTS

Preliminary Observations.—The antibacterial activity of histone was encountered as a result of studies on purification of phagocytin, a bactericidal substance extracted from rabbit polymorphonuclear leucocytes (1, 2). Phagocytin preparations were unstable in various salt solutions; one hypothesis related this instability to the presence of lipoproteins. To test this theory, the procedure for extracting phagocytin was changed to include as a first step defatting and

dehydration of leucocytes with cold acetone. Neutral aqueous extracts of these acetone powders were essentially devoid of antibacterial activity, but extracts prepared at an acid reaction (pH 3.5 or lower) showed striking lethal effects on coliform bacteria. Using this technique, bactericidal material was extractable from a wide variety of organs and tissues, in contrast to limitation of phagocytin to the granulocyte when the earlier methods were employed. Further studies on acid extracts of acetone-defatted, dehydrated tissues revealed properties for the antibacterial substance suggesting that it might be a basic protein.

TABLE I

Antibacterial Activity of Various Basic Substances

Substances tested*	Minimal concentration ($\mu g./ml.$) producing >50 per cent reduction in numbers of *E. coli* K-12 in 2 hrs. at 38° C. in	
	0.05 M citric acid–Na$_2$HPO$_4$, 0.01 per cent albumin, pH 5.6	0.05 M citric acid-Na$_2$HPO$_4$, 0.01 per cent albumin, pH 7.0
Lysozyme.............................	>100	>100
Ribonuclease..........................	"	"
Deoxyribonuclease.....................	"	"
Hemoglobin...........................	"	"
Hyaluronidase........................	"	50
Protamine............................	"	6
Histone..............................	0.3	0.6
Spermine.............................	>100	>100
Arginine.............................	"	"

* These basic materials were dissolved at 1 mg. per ml. in 0.01 N HCl and diluted into buffer solutions as indicated at the time of bactericidal assay. Obtained from the following sources: Lysozyme, egg white crystallized, Armour and Co., Chicago; crystallized ribonuclease, kindly provided by Dr. M. Kunitz of The Rockefeller Institute; lyophilized deoxyribonuclease, bovine testicular hyaluronidase, and calf thymus histone, Worthington Biochemical Corp., Freehold, New Jersey; twice crystallized bovine hemoglobin, Nutritional Biochemical Corp., Cleveland; protamine sulfate (salmine), Mann Research Laboratories, Inc., New York; spermine tetrahydrochloride, Hoffmann-LaRoche and Co., Basel.

Antibacterial Activity of Various Basic Proteins.—Studies were therefore made of the antibacterial activity *in vitro* of several basic proteins and protein-like substances. Results are summarized in Table I. Only histone among these materials eliminated coliform microorganisms in a citrate-phosphate buffer at pH 5.6. The activity of histone under these conditions was impressive quantitatively, 0.3 $\mu g.$ per ml. resulting in a significant reduction in number of recoverable *Escherichia coli* K-12. Histone exhibited similar antibacterial activity in a medium at neutral pH. Except for a slight effect of protamine in the system at pH 7, the other basic substances were essentially without antibacterial action under these conditions. Similar results were obtained when these materials were examined for their effect on *Klebsiella pneumoniae*.

Table II presents studies of the effect on coliform bacilli of highly purified histones from calf thymus. Histone A (lysine-rich) and histone B (arginine-rich) which had been separated from calf thymus histone by column chromatography (3, 4) were kindly provided by Dr. Charles Crampton, Dr. Stanford Moore, and Dr. William Stein. Histone A manifested no significant antibacterial

TABLE II

Antibacterial Activity of Purified Histone Fractions

Substance tested*	Minimal concentration ($\mu g./ml.$) producing >90 per cent reduction in numbers‡ of	
	E. coli K-12	Klebsiella pneumoniae C
Calf thymus histone, mixed	0.7	1.5
" " " A	>25	>25
" " " B	0.35	0.7

* Calf thymus histone, mixed, was the purified histone obtained from Worthington Biochemical Corp., Freehold, New Jersey. Histone A and histone B separated by column chromatography (3, 4) were kindly donated by Dr. Charles Crampton, Dr. Stanford Moore, and Dr. William Stein of The Rockefeller Institute.

‡ Test performed in 0.1 M citric acid–Na_2HPO_4, 0.01 per cent albumin, pH 5.6.

TABLE III

Failure of Histone A to Antagonize Antibacterial Action of Histone B

Test medium	Minimal concentration ($\mu g./ml.$) of histone B producing >50 per cent reduction in numbers of E. coli K-12
Citrate–phosphate buffer* pH 5.6........................	0.25
10 $\mu g./ml.$ histone A in citrate–phosphate buffer*, pH 5.6...	"
Citrate–phosphate buffer‡ pH 7........................	0.5
10 $\mu g./ml.$ histone A in citrate–phosphate buffer‡ pH 7.....	"

* 0.05 M citric acid–Na_2HPO_4, 0.01 per cent albumin.

‡ 0.1 M citric acid–Na_2HPO_4, 0.2 per cent albumin.

action, while the activity of histone B was approximately twice that of the parent mixed preparation.

Highly purified histones from sources other than calf thymus were not studied, but crude histone preparations from various mammalian organs and tissues all exerted action on *E. coli* K-12 similar to that of calf thymus histone.

The results presented in Table III demonstrate that the presence of histone A did not inhibit detectably the antibacterial activity of histone B. In view of this observation, and of the limited availability of histone B, purified (but mixed) calf thymus histone (Worthington Biochemical Corp., Freehold, New Jersey) was used in the investigations which followed.

Nature of the Effect of Histone on Susceptible Bacteria.—The results presented in Table IV indicate that histone exerted a bactericidal rather than a bacteriostatic action on *E. coli*, *K. pneumoniae*, and a coagulase-negative staphylococcus. In this experiment melted penassay agar was added to mixtures of histone and bacteria after various time intervals. When agar was added promptly, no reduction in number of bacterial colonies was seen in cups

TABLE IV

Evidence That the Action of Histone on Susceptible Microorganisms is Bactericidal Rather than Bacteriostatic

Duration of exposure* (min.) of bacteria to histone *prior* to addition of melted penassay agar	Minimal concentration (μg./ml.) of histone producing >50 per cent reduction in numbers of		
	E. coli K-12	*Klebsiella pneumoniae*	*Staphylococcus albus* (mendita)
0	>25	>25	>25
30	1.6	12.5	1.6
60	0.4	1.6	0.8
120	0.2	0.8	"

* In 0.05 M citric acid–Na_2HPO_4, 0.01 per cent albumin, pH 5.6, 38°C.

TABLE V

Effect of Histone on Bacterial Morphology

Observations after incubation for 2 hrs. at 38°C.	18 hr. old culture of *E. coli* K-12 diluted 1:100 into 0.05 M citric acid–Na_2HPO_4, 0.01 per cent albumin, pH 5.6, containing	
	No histone	5 μg./ml. histone
Gross appearance	No detectable difference in optical density	
Wet mount (phase contrast)	" " " " numbers, no protoplasts, no clumping	
Gram stain	No detectable difference in numbers or appearance	
Surviving bacteria (per ml., by plate count)	2×10^7	2×10^6

containing up to 25 μg. histone per ml.; however, if the mixtures were incubated for an hour prior to addition of penassay agar, even low concentrations of histone were effective in preventing growth. Were the effect bacteriostatic rather than bactericidal, histone should have produced essentially the same suppression of growth irrespective of the time of agar addition.

Table V presents morphological observations on bacteria exposed to histone. Under the conditions of the test 99 per cent of the microorganisms incubated with histone were killed as determined by plate count, yet there was no detectable lysis, clumping, or morphological alteration of these bacterial cells.

Susceptibility of Various Bacteria to Killing by Histone.—Table VI shows the

TABLE VI

Susceptibility of Various Bacteria to Killing by Histone

Microorganism	Minimal concentration (µg./ml.) of histone producing >50 per cent killing in 2 hours at 38°C. in	
	0.05 M citric acid-Na₂HPO₄, 0.01 per cent albumin, pH 5.6	0.05 M citric acid-Na₂HPO₄, 0.1 per cent albumin, pH 7
Escherichia coli K-12	0.3	0.6
" " 28B2	"	"
" " 43B	"	"
" " R	"	0.3
" " Beeson	"	1.2
Klebsiella pneumoniae C	1.2	2.4
Shigella flexneri 9199	0.025	0.4
" sonnei 9290	"	0.1
Salmonella enteritidis	>25	>25
" typhimurium SR-11	0.2	0.4
" " R1A	0.05	0.2
" " 125	"	"
" " 120	0.1	0.4
Pseudomonas aeruginosa 10145	0.025	0.05
Proteus morgani	>25	>25
Serratia marcescens	"	"
Staphylococcus albus Mendita	—	0.8
" " Greaves	—	"
" " McGaffery	—	"
" " Prengle	—	"
" " air	—	0.4
" " JAB	—	"
Staphylococcus aureus MAM	—	>25
" " Stovall	—	"
" " Giorgio	—	25
" " Smith	—	"
" " O'Hara	—	"
" " Stern	—	"
Streptococcus hemolyticus, Group A, D58X/11/1	>25	>25
" " " " D58X	"	"
" " " B, 090 R	"	"
" " " C, C74	"	"
" " " D, D76	"	"
" " " G, D166B	"	"

bactericidal activity of histone on various microorganisms. Tests were performed in citric acid–sodium phosphate buffers at both pH 5.6 and pH 7.

Several strains of *Escherichia coli* were approximately equally susceptible, all being killed by final concentrations of 0.3 to 0.6 µg. histone per ml. Strain

28B2 was known to be resistant to the properdin system. Slightly higher concentrations of histone were required to kill *Klebsiella pneumoniae*. Strains of *Shigellae*, *Salmonella typhimurium*, and *Pseudomonas* were all markedly susceptible to the lethal action of histone. *Salmonella typhimurium* SR-11 and R-1A, respectively mouse virulent and mouse avirulent, were obtained from Dr. Howard Schneider; they did not differ significantly in susceptibility to histone. *Salmonella* strains 120 (smooth) and 125 (rough), kindly provided by Dr. Norton Zinder, were also equally affected. *Salmonella enteritidis*, *Proteus morgani*, and *Serratia marcescens* were not killed by 25 μg. histone per ml.

Staphylococci could not be tested for susceptibility to histone at an acid pH since the controls containing no histone did not regularly survive under these conditions. Observations made in the neutral buffer were, however, of considerable interest. Several strains of *Staphylococcus albus* (all coagulase-negative) were killed by approximately 0.5 μg. histone per ml., while *Staphylococcus aureus* strains (all coagulase-positive) were uniformly much less susceptible. Many of these staphylococcal cultures were furnished by Dr. Stephen Morse and Dr. Zanvil Cohn.

Hemolytic streptococci, obtained through the courtesy of Dr. Rebecca Lancefield, all survived exposure to 25 μg. histone per ml. of neutral or acid buffer medium.

Evidence for Adsorption of Histone to E. coli K-12.—

An 18 hour old culture of *E. coli* K-12 in penassay broth was heated at 85°C. for 45 minutes. The killed bacteria were collected in the centrifuge and suspended in the original volume of 0.05 M citric acid-Na$_2$HPO$_4$, 0.01 per cent albumin, pH 5.6. This cell suspension was divided into two equal parts; one was incubated without further additions (control system), while histone was added to the other to give a final concentration of 10 μg. per ml. (test system). They were held at 38°C. for 2 hours and then centrifuged. Bactericidal assays were done on the supernatants, and on acid extracts (original volume of 0.05 N HCl) of the cell buttons. The acid extracts were dialyzed against a large volume of 0.05 M citrate-phosphate buffer pH 5.6 at 4°C. overnight prior to testing.

As is shown in Table VII, no bactericidal activity was demonstrable in the histone solution after incubation with heat-killed *E. coli* K-12 and removal of the bacterial cells by centrifugation. Histone added to the control supernate (*E. coli* K-12 in buffer, no histone) had the expected activity, showing that histone antagonists were not released into solution from the bacteria during incubation. Adsorption was confirmed by recovery of bactericidal activity in a dilute acid extract of the bacterial cell button which had been incubated with histone. Acid extracts of control bacterial cells were inactive, indicating that the effects observed in extracts of the cell button exposed to histone were not due to "histone" derived from the bacteria *per se*.

Susceptibility to Killing by Histone of Microorganisms Grown and Manipulated under Various Conditions.—The results presented in the upper portion of Table

TABLE VII

Adsorption of Histone to Heat-Killed E. coli K-12 and Elution Therefrom with Acid

Material tested	Reciprocal of highest dilution producing >50 per cent killing of *E. coli* K-12.
Test supernate (killed *E. coli* K-12 + 10 μg./ml. histone in buffer*)	<2
Control supernate (killed *E. coli* K-12 + buffer)	"
10 μg./ml. histone in control supernate	32
Acid extract of control bacterial cell button	<2
" " " test bacterial cell button	32
10 μg./ml. histone in buffer (control untreated solution)	"

* Buffer: 0.05 M citric acid–Na_2HPO_4, 0.01 per cent albumin, pH 5.6.

TABLE VIII

Susceptibility to Histone of Microorganisms Grown and Manipulated in Various Media

E coli K-12			Minimal concentration (μg./ml.) of histone producing >50 per cent killing (all tested in 0.05 M citric acid–Na_2HPO_4, 0.01 per cent albumin, pH 5.6)
Cultured on or in*	Suspended in	Preliminary dilutions in	
Penassay broth (4 hrs.)	—	H_2O	0.3
" " (18 ")	—	"	"
" " (72 ")	—	"	"
Penassay broth	—	Penassay broth	0.6
Beef heart infusion broth	—	H_2O	"
" " " "	—	Beef heart infusion broth	"
Penassay agar	H_2O	H_2O	25
" "	Penassay broth	"	6
" "	H_2O	Penassay broth	0.6
" "	Penassay broth	" "	"
Beef heart infusion agar	H_2O	H_2O	>25
" " " "	Beef heart infusion broth	"	6
" " " "	H_2O	Beef heart infusion broth	2.5
" " " "	Beef heart infusion broth	Beef heart infusion broth	0.6

* 18 hour cultures unless otherwise indicated.

VIII show that cultures varying from 4 to 72 hours in age were equally susceptible to killing by histone.

Preliminary observations indicated that coliform bacilli grown on penassay agar slants were much less susceptible to histone than cultures in penassay

broth.[1] It should be emphasized that the tests were performed in the *same* medium; the differences in susceptibility under discussion here were presumably based on variations in the microorganisms related to cultural and suspending media. The resistance to histone of bacteria grown on agar media might well have been explained by the presence in suspensions of these organisms of acid polysaccharides derived from the agar, since, as is demonstrated in a section below, such substances exerted powerful antagonistic action on the bactericidal effect of histone. However, attempts to remove antagonists derived from the agar by repeated washings of the *E. coli* K-12 with water failed to change their resistance to histone. Furthermore, as is demonstrated by the results in the lower portion of Table VIII, *E. coli* grown on agar slants became more susceptible to histone when suspended and diluted in liquid culture medium. Agar slant cultures suspended in broth and then diluted in water remained relatively

TABLE IX

Recovery of Susceptibility to Histone of Agar Grown E. coli K-12 on Incubation in Broth

Inoculum (*E. coli* K-12) cultured on penassay agar slant	Minimal concentration ($\mu g./ml.$) histone producing >50 per cent killing in 0.05 M citric acid–Na$_2$HPO$_4$, 0.01 per cent albumin, pH 5.6
Washed off agar and suspended in ice cold penassay broth; promptly diluted in ice cold water and added to test system.	6
Above penassay broth suspension incubated at 38°C. 30 min., then diluted in water and added.	0.4
Penassay broth suspension incubated at 38°C. 120 min., then diluted in water and added.	0.2

resistant to histone killing, while those suspended in water or broth and diluted in broth became susceptible. This finding suggested that recovery of susceptibility of coliforms cultured on solid media might be related to the duration of exposure to broth prior to addition to the test system. Table IX presents results of an experiment directed toward this point. *E. coli* washed off an agar slant with cold broth and promptly diluted in water and tested were relatively resistant to killing by histone; the same suspension of organisms, however, increased remarkably in susceptibility after incubation at 38°C. The precise factors related to histone susceptibility of the bacteria as influenced by these manipulations are unknown.

Stability of the Bactericidal Activity of Histone.—Solutions of histone at 250 $\mu g./ml.$ in 0.01 N HCl manifested unchanged bactericidal activity on storage at 10°C. for several weeks. These solutions could be heated in a boiling water

[1] Similar observations on differences in susceptibility to phagocytin of agar and broth grown cultures have been made by Michael and Braun at the Institute for Microbiology, Rutgers. (personal communication).

bath for 15 minutes with but little diminution in potency. In neutral mixed phosphate buffer, however, bactericidal activity was quite unstable, over a tenfold reduction occurring in 1 hour at 38°C. The antimicrobial effect of histone solutions in 0.01 N HCl was rapidly destroyed on incubation with pepsin. These observations were in keeping with recognized properties of histones; namely, stability in acid, marked susceptibility to proteolytic enzymes, and instability at neutral reactions thought to be due to aggregation, attack by contaminating proteases, or adsorption (4–6).

Bactericidal Activity of Histone in Various Media.—Table X summarizes experiments inquiring into the influence of ionic strength, osmolarity, and salt composition of the medium on bactericidal activity of histone. Salt concentration influenced the results strikingly; histone at a concentration of 1 μg. per ml. or less exerted a lethal action in various buffers of low molarity, while the microbes survived exposure to 25 μg. histone per ml. when the molarity was raised. Although the effect of molarity on bactericidal activity varied somewhat depending on the salts used, ionic strength higher than 0.15 to 0.2 molar NaCl in general led to increased survival of *E. coli* K-12. Histone was equally active in citrate, citrate-phosphate, or phosphate buffers of low molarity; the bactericidal activity was considerably less in acetate or lactate buffers. Inclusion in the medium of high molar concentrations of sucrose or glucose resulted in only slight reduction in killing. The microorganisms were essentially unaffected by histone in simple NaCl solution, but addition of low concentrations of versene to this medium resulted in restoration of bactericidal activity.

The mechanism by which high concentrations of NaCl antagonized the bactericidal action of histone was investigated in one experiment not shown in Table X.

Histone dissolved in 0.02 N HCl was diluted 1:10 into 0.05 M citrate-phosphate buffer, 0.01 per cent albumin, pH 5.6, and also into this same buffer containing 0.4 M NaCl. After incubation at 38°C. for 2 hours, these solutions were diluted 1:10 into 0.05 M citrate-phosphate buffer containing no added salt, and tested for bactericidal activity in standard manner. Both manifested the same capacity to kill *E. coli* K-12.

This experiment demonstrated that incubation of histone in buffer containing 0.4 M NaCl did not result in loss of the capacity to kill *E. coli* K-12. Thus, the antagonistic action of high ionic concentration appeared to operate by affecting the bacteria or the combination of bacteria with histone, rather than by denaturing or otherwise inactivating histone.

The experiment presented in Table XI shows the relationship between pH and bactericidal activity of histone in a medium of relatively constant ionic strength. Activity at pH 5.6 was approximately twofold greater than that at pH 7. Observations could not be made at reactions more acid than pH 5 because of death under these conditions of *E. coli* K-12 in control specimens containing no histone.

TABLE X

Effect of Salt Concentration and of Buffer System on Bactericidal Action of Histone

Molarity and buffer*	Minimal concentration ($\mu g./ml.$) of histone producing >50 per cent killing of *E. coli* K-12 in 2 hrs. at 38°C.
0.01 M citric acid–Na$_2$HPO$_4$, pH 5.6	0.2
0.03 M " " " " "	"
0.1 M " " " " "	0.4
0.5 M " " " " "	>25
0.01 M citric acid–sodium citrate, pH 5.6	0.2
0.03 M " " " " " "	"
0.1 M " " " " " "	6
0.5 M " " " " " "	>25
0.01 M Na$_2$HPO$_4$–KH$_2$PO$_4$, pH 6.7	0.8
0.03 M " " " "	"
0.1 M " " " "	6
0.5 M " " " "	>25
0.01 M citric acid–sodium citrate, pH 5.6, 0.05 M NaCl	0.1
" M " " " " " " 0.1 M "	0.2
" M " " " " " " 0.15 M "	1.2
" M " " " " " " 0.2 M "	25
0.05 M citric acid–Na$_2$HPO$_4$, pH 5.6, 0.05 M NaCl	0.4
" M " " " " " 0.1 M "	"
" M " " " " " 0.2 M "	0.8
" M " " " " " 0.4 M "	>25
0.05 M citric acid–Na$_2$HPO$_4$, pH 5.6, 0.05 M KCl	0.1
" M " " " " " 0.1 M "	0.4
" M " " " " " 0.2 M "	6‡
" M " " " " " 0.4 M "	>25‡
0.05 M citric acid–Na$_2$HPO$_4$, pH 5.6, 0.025 M Na$_2$SO$_4$	0.2
" M " " " " " 0.05 M "	0.4
" M " " " " " 0.1 M "	1.6
" M " " " " " 0.2 M "	>25
0.05 M citric acid–Na$_2$HPO$_4$, pH 5.6, 0.1 M sucrose	0.8
" M " " " " 0.5 M "	1.6
" M " " " " 0.1 M glucose	0.8
" M " " " " 0.5 M "	1.6
0.03 M acetic acid–sodium acetate, pH 5.6	2.5
0.1 M " " " " " "	>25

TABLE X (Concluded)

Molarity and buffer*	Minima ‡concentration (μg./ml.) of histone producing > 50 per cent killing of E. coli K-12 in 2 hrs. at 38°C.
0.03 м lactic acid–NaOH, pH 5.6	2.5
0.1 м " " " " "	25
0.05 м NaCl	25
0.1 м "	"
0.15 м "	"
0.05 м NaCl, 10 μg./ml. versene Na₂	0.4
0.1 м " , " /" " "	"
0.15 м " , " / " " "	25

* Final concentration of 0.01 per cent albumin included in all buffer systems.
‡ Partial killing of the bacterial inoculum in control cups (containing no histone).

TABLE XI

The Effect of pH on the Bactericidal Action of Histone

Medium	Minimal concentration (μg./ml.) of histone producing >50 per cent killing of E. coli K-12 in 2 hrs. at 38°C.
0.1 м NaCl, 0.01 м citric acid–Na₂HPO₄, pH 5.0	0.2
" " " " " " " " " 5.6	"
" " " " " " " " " 6.0	0.4
" " " " " " " " " 6.5	"
" " " " " " " " " 7.0	"

The effects of rabbit serum and of bovine plasma albumin on the susceptibility of a coliform microorganism to histone are shown in Table XII. In citrate-phosphate buffer at pH 5.6 relatively high concentrations of rabbit serum or of bovine albumin did not significantly antagonize the lethal effect of histone. In fact, under these conditions low concentrations of rabbit serum potentiated the bactericidal action. In contrast, in the medium at pH 7 rabbit serum (at concentrations higher than 3 per cent) and bovine albumin (at concentrations above 0.3 per cent) reduced the capacity of histone to kill the coliform microbes.

Antagonism of the Bactericidal Action of Histone.—The effect on antibacterial activity of histone of adding various basic substances to the test medium is presented in Table XIII. Under these conditions protamine and spermine were markedly antagonistic; for example addition to the pH 5.6 medium of 1 μg. of protamine per ml. blocked the bactericidal action on *E. coli* K-12 of 1 μg. per

TABLE XII

The Effect of Rabbit Serum and of Bovine Plasma Albumin on the Bactericidal Activity of Histone

Rabbit serum* added to the test medium, final concentration	Minimal concentration ($\mu g./ml.$) of histone producing >50 per cent killing of *E. coli* K-12 in 2 hrs. at 38°C. in	
	0.05M citric acid-Na_2HPO_4, pH 5.6	0.05 M citric acid-Na_2HPO_4, pH 7
per cent		
30	0.8	12
10	0.2	3
3	0.05	0.8
1	0.025	0.2
0.3	0.1	"
None	0.2	"
Bovine plasma fraction V added to the test medium, final concentration		
per cent		
1	0.4	12
0.3	0.2	1.5
0.1	"	0.8
0.03	"	0.4
0.01	"	0.2
None	"	"

* Fresh rabbit serum was dialyzed overnight at 4°C. against 100 X volumes of respective citrate-phosphate buffer and clarified by centrifugation prior to addition to the test medium.

TABLE XIII

Antagonism of the Bactericidal Action of Histone by Various Basic Substances

Antagonist* tested	Minimal concentration ($\mu g./ml.$)of antagonist producing reversal of the lethal effect on *E. coli* K-12 of	
	1 $\mu g./ml.$ histone in citrate-phosphate buffer,§ pH 5.6	4 $\mu g./ml.$ histone in citrate-phosphate buffer,§ pH 7
Protamine sulfate	1	—‡
Spermine hydrochloride	0.05	1.5
Lysozyme "	>100	>100
Hemoglobin	"	"
Arginine hydrochloride	"	"

* Some of these materials were obtained from the following sources: Protamine sulfate (salmine), Mann Research Laboratories, Inc., New York; spermine tetrahydrochloride, Hoffmann-LaRoche and Co., Basel; crystalline lysozyme hydrochloride, Armour and Co. Chicago; twice crystallized bovine hemoglobin, Nutritional Biochemical Corp., Cleveland.

‡ Protamine sulfate was itself bactericidal under these conditions and therefore could not be examined for antagonistic effect.

§ 0.05 M citric acid-Na_2HPO_4, 0.01 per cent albumin.

ml. of histone. Lysozyme, hemoglobin, and arginine exerted no antagonistic action up to concentrations of 100 μg. per ml.

Table XIV shows similar tests for antagonism of antibacterial action of histone by various polysaccharides. Heparin was the most potent blocking agent among the substances tested. Nucleic acids, chondroitin sulfate, and bacterial lipopolysaccharides also interfered with the bactericidal effects of histone. Hyaluronic acid was only weakly antagonistic and dextran showed no neutralizing effect.

TABLE XIV

Antagonism of the Bactericidal Action of Histone by Various Polysaccharides

Antagonist* tested	Minimal concentration ($\mu g./ml.$) of antagonist producing reversal of the lethal effect on *E.coli* K-12 of	
	2 μg./ml. histone in 0.05 M citric acid-Na$_2$HPO$_4$, 0.01 per cent albumin, pH 5.6	4 μg./ml. histone in 0.05 M citric acid-Na$_2$HPO$_4$, 0.01 per cent albumin, pH 7
Heparin	0.25	0.5
Ribonucleic acid	1	2.5
Deoxyribonucleic acid	0.4	1
Yeast nucleic acid	4	8
Chondroitin sulfate	"	4
E. coli lipopolysaccharide	8	8
S. marcescens lipopolysaccharide	4	"
Hyaluronic acid	50	16
Dextran	>100	>100

* Some of these materials were obtained from the following sources: heparin sodium, Connaught Medical Research Laboratories, Toronto; calf thymus ribonucleic acid, Mann Research Laboratories, Inc., New York; calf thymus deoxyribonucleic acid and hyaluronic acid, Worthington Biochemical Corp., Freehold, New Jersey; *E. coli* and *S. marcescens* lipopolysaccharides, Difco Laboratories, Detroit; dextran, high molecular weight, Commercial Solvents Corp., New York.

It seemed likely that the acid polysaccharides antagonized by combining with histone rather than by interacting with the bacterial cells. An experiment to establish this point is presented in Table XV. The concentration of heparin required to block the bactericidal effect varied directly with the concentration of histone in the test system, and was independent of the concentration of bacteria.

Kinetic Features of the Bactericidal Action of Histone.—Kinetic features of the lethal effect of histone on *E. coli* K-12 were studied by the following technique.

The test medium employed was 0.05 M citric acid-Na$_2$HPO$_4$, 0.01 per cent albumin, pH 5.6. Appropriate volumes of this medium, and of solutions of histone (diluted in 0.01 N HCl), of 0.01 N HCl, and of *E. coli* K-12 diluted in the above citrate-phosphate buffer were allowed to stand in the water bath for 30 minutes to reach the desired temperature (38°C.

TABLE XV

Mechanism of Heparin Antagonism on the Bactericidal Effect of Histone; Relationship to Size of Bacterial Inoculum and to Histone Concentration

Test medium	Minimal concentration (µg./ml.) of heparin required to antagonize the bactericidal action of histone on	
	50 E. coli K-12 per ml.	1000 E. coli K-12 per ml.
1 µg./ml. histone in citrate–phosphate buffer, * pH 5.6	0.125	0.125
10 " " " " " _ " " * " "	2	2
1 µg./ml. histone in citrate–phosphate buffer, * pH 7	0.125	0.125
10 " " " " " _ " " * " "	2	2

* 0.05 M citric acid–Na_2HPO_4, 0.01 per cent albumin.
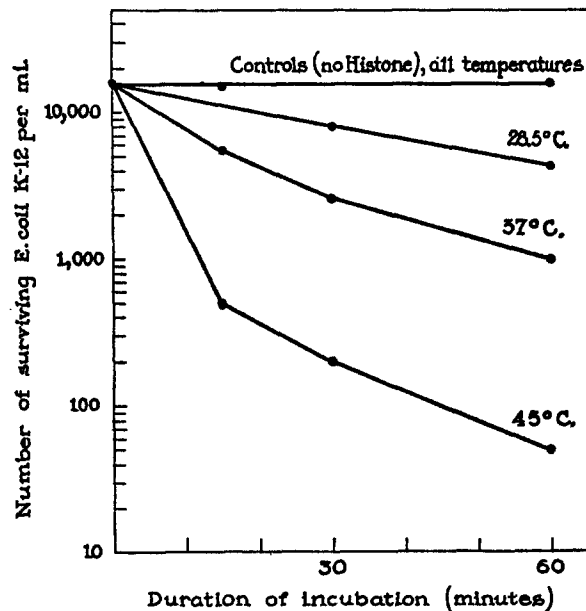
FIG. 1. Effect of temperature on kinetic features of the bactericidal action of histone.

unless otherwise indicated). Histone solution or 0.01 N HCl was then added to the buffer tubes. The bacterial inoculum was added at timed intervals so as to permit sampling of each tube after a measured period of incubation. At each sampling an aliquot was promptly diluted into a solution of 10 µg.ml. of heparin in distilled water, heparin being used in the diluent to prevent further bactericidal action (see section above on antagonists). Serial tenfold dilutions were made in heparin-water and 0.1 ml. aliquots were plated on penassay agar. After overnight incubation at 38°C. colonies were counted and the number of surviving E. coli K-12 per ml. calculated.
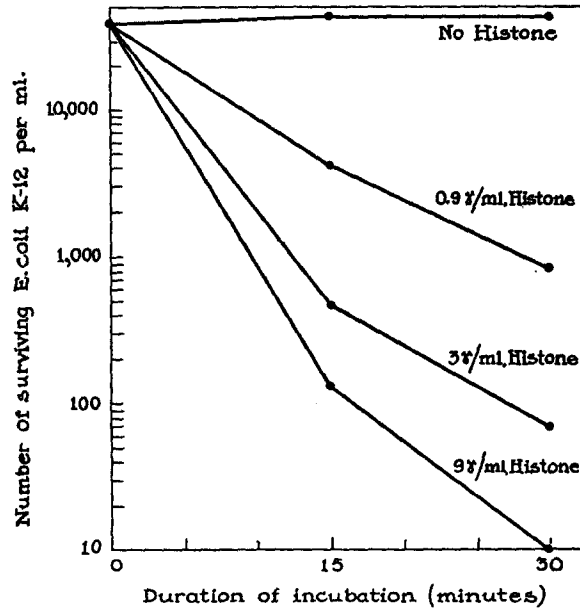
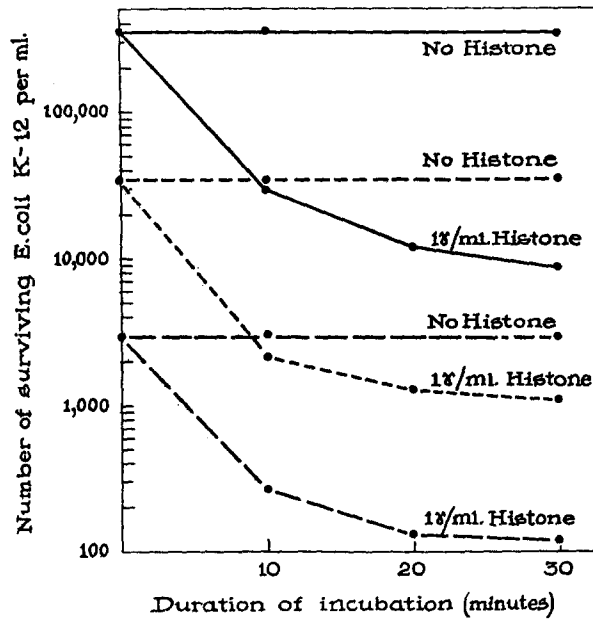FIG. 2. Kinetic features of bactericidal action of various concentrations of histone.

FIG. 3. Kinetic features of bactericidal activity of histone in relation to concentration of microorganisms.

Fig. 1 shows the relationship between temperature and rate of killing of
E. coli K-12 by histone. Killing took place more rapidly the higher the tempera-
ture. The technique was not sufficiently accurate to permit calculation of
reliable rates in the early course of the reaction; thus quantitative relationships
between rate and temperature were not established.

Fig. 2 reveals that the rate of the bactericidal effect was also a function of
the concentration of histone in the system. Killing of *E. coli* K-12 was more
rapid at higher concentrations of histone.

The relationship between concentration of bacteria and the rate of their
killing by histone is presented in Fig. 3. It is seen that the per cent of micro-
organisms killed in a given time period by the same concentration of histone
was approximately the same, regardless of the concentration of bacteria (within
the limits studied). Stated in another way, these data show that the rate of
the reaction (number of bacteria killed per minute in the first 10 minutes) was
directly proportional to the concentration of microorganisms in the system.

DISCUSSION

Although several previous studies have dealt with toxic effects of protamines
and histones on microorganisms and mammalian cells (7–14), in no instance did
the authors report specific and potent *bactericidal* activity for histone. There is,
however, no real conflict in results; the previous and present investigations
differ in several regards, including the histone preparations, the bacteria, and
the conditions under which antimicrobial effects were observed.

The bactericidal activity reported here seems to be specific in the sense that
very low concentrations of the arginine-rich histone fraction (histone B of
Crampton, Moore, and Stein) kill susceptible organisms, while many other
similar basic protein-like substances, including the lysine-rich histone A, are
inactive even when added to the medium in fairly large amounts. The lethal
action is not likely due to arginine content or basic nature *per se*, since pro-
tamine, which has a higher isoelectric point and contains more arginine than
histone B, exhibits little or no bactericidal action under certain of the test
conditions. The high degree of purity of histone B separated by column chro-
matography, considered along with the fact that concentrations of this prepara-
tion lower than 1 μg. per ml. kill bacteria, make it quite unlikely that the ob-
served activity might be due to a contaminating material; however, since
histone B is recognized to be inhomogeneous, it is at present not possible to
ascribe the bactericidal effect to a pure substance.

The survey of susceptibility to histone of various types of microorganisms
has thus far been limited to enterobacteria and micrococcci as reported above.
The behavior of other bacteria, and of viral and fungal agents on exposure to
histone under various conditions, has yet to be fully investigated.

The mechanism by which histone fractions kill certain microorganisms is
unknown. In view of the size and charge of histone, it seems reasonabe to assume

that it may act as do the bactericidal cationic detergents; *i.e.*, by adsorption to the cell surface with consequent damage to the osmotic barrier (reviewed in reference 15). Fischer and Wagner have in fact demonstrated recently that protamines and histones are absorbed to mammalian cells and microorganisms with resulting alteration of osmotic integrity, impairment of respiration, and death (14).

The influence of salt content of the medium on survival of bacteria exposed to histone seems to be related to ionic strength rather than to osmolarity. Ionic strength might play a role in this system primarily by affecting adsorption of histone onto bacterial receptor sites. Recent evidence suggests that reactive acid groups at the surface of coliform bacilli are of the polycarboxylic type (16); if this be so, then the interaction between histone and these organisms may be analogous to that between basic proteins and polycarboxylic acid resins such as IRC-50, in which cation concentration of the medium is recognized to be critical in determining adsorption.

Kinetic features of the bactericidal action of histone have thus far been only partially determined, but the general effects of temperature, histone concentration, and concentration of microorganisms on rate of the reaction suggest that it is a complicated one, perhaps at least in part enzymatic in nature.

Antagonism of the bactericidal effect of histone by various acid polysaccharides probably operates *via* formation of a relatively undissociated acid-base salt, thus impeding or preventing histone from reaching the presumed bacterial receptor sites. Heparin at a concentration of 1 μg. per ml. blocks the lethal effect on *E. coli* K-12 of 5–10 μg. histone per ml.; these quantitative relationships agree well with findings of Kent *et al.*, who observed that 1 μg. sodium heparinate combined with 4.5 μg. of histone in experiments on displacement fractionation of nucleoprotein (17).

Among the basic substances tested for antagonism of the bactericidal action of histone on coliform organisms, only protamine and spermine were effective. Since these were the only materials investigated which were more strongly basic than arginine-rich histone B, it seems likely that they blocked by successfully competing for acid receptor sites on the bacilli, thus protecting these sites from lethal combination with histone.

As pointed out in a recent review (18), much remains to be learned about tissue factors which exert antimicrobial action. The information available at present does not permit more than speculation as to whether histone may exert bactericidal activity under certain conditions *in vivo*. Although present in all cells as a major chemical component of the nucleus, significant concentrations of histone might be present in tissues and body fluids only in pathological states such as necrosis. Furthermore, it remains to be determined whether or not conditions (ionic strength, presence of antagonists, etc.) *in vivo* would be favorable for lethal action of histone on invading microorganisms.

Several features of the bactericidal effect of histone, phagocytin (1, 2),

hemoglobin (19), and leukins (18) are similar. That histone and hemoglobin are different is apparent; their lethal action on microbes *in vitro* also differs in media of various ionic strength and pH.

It also seems likely that histone and phagocytin are separate entities. For example, phagocytin is found in physiological saline extracts of certain disrupted polymorphonuclear leucocytes; such extracts, according to experience with other tissues, should be essentially free of nuclear basic proteins. It is worth mentioning, however, that the arginine-rich histone fraction is readily extractible from nucleoprotein by dilute organic acids (20); since granulocyte cytoplasm develops at times quite a low pH (reviewed in reference 21), perhaps some histone might be free in these cells under certain conditions. Histone and phagocytin also differ significantly in their capacity to kill certain bacteria and in their activity in relation to ionic strength, pH, and protein composition of the medium. Although these differences seem convincing, there is a remote possibility that constituents other than basic proteins in the crude extract called phagocytin might alter features of the bactericidal activity.

The relationship between histone and so-called leukins is difficult to define, since the term leukin has been applied to a variety of white cell extracts manifesting inhibitory activity on Gram positive microbes. Perhaps, as postulated by some workers (18), leukins are in fact histones and protamines, and might thus exert bactericidal action on Gram-negative as well as Gram-positive microorganisms under appropriate test conditions. The fact that susceptibility of many bacteria to killing by tissue basic proteins is markedly influenced by composition of the test medium, may well account for some of the confusion and conflicting results in the literature dealing with antimicrobial action of cell extracts and tissue fluids.

SUMMARY

The arginine-rich fraction of calf thymus histone (histone B) exerts bactericidal activity on various coliform bacilli and micrococci under certain conditions *in vitro*. Final concentrations of less than 1 μg. histone per ml. kill susceptible microbes without detectable morphological alteration or lysis. Among the microorganisms highly susceptible to histone are *Escherichia*, *Salmonella*, *Shigella*, *Pseudomonas*, *Klebsiella*, and *Micrococcus pyogenes* var. *albus*. Less susceptible or completely resistant are *Proteus*, *Serratia*, *Micrococcus pyogenes* var. *aureus*, and various types of hemolytic streptococci.

Coliforms grown on solid media are much more resistant to the lethal effect of histone than are those cultured in liquid media. This difference is apparently related to the physiological state of the bacteria; agar grown microorganisms washed with water remain resistant to histone, whereas incubation in broth rapidly renders them more susceptible.

Histone is adsorbed onto heat-killed *E. coli* K-12 under conditions suitable for lethal action on this organism.

The bactericidal activity of histone is but little affected by pH of the test system, but ionic strength of the medium exerts a marked influence, the lethal action being reduced or blocked as the salt concentration reaches levels higher than that of 0.15–0.2 M NaCl. Relatively high concentrations of rabbit serum or of bovine plasma albumin reduce the bactericidal activity of histone in a medium at pH 7; these serum preparations are, however, essentially without effect in the test system at pH 5.6. The bactericidal effect of histone is antagonized by addition to the medium of small amounts of certain basic substances (protamine, spermine), or of various acid polysaccharides (heparin, nucleic acid, bacterial lipopolysaccharides).

The rate of killing of E. coli K-12 by histone increases as the temperature and the concentration of histone are raised. Within the limits studied, this rate also appears to be directly proportional to the concentration of bacteria in the system.

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