FURTHER STUDIES ON PREPARATION AND PROPERTIES OF PHAGOCYTIN*

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In previous studies (1) phagocytin was prepared by extracting disrupted rabbit granulocytes with a salt solution buffered at pH 5.6. The distinguishing feature of these extracts was a high order of bactericidal activity on several Gram-negative microorganisms under certain conditions *in vitro* (1, 2).

It has now been found that extracts of polymorphonuclear leucocytes prepared at a more acid pH manifest greater bactericidal effects than those described in the original reports on phagocytin. Furthermore, the antibacterial and stability properties of these acid extracts differ in certain regards from those of the preparations made at pH 5.6.

The recent discovery (3) that an arginine-rich histone fraction (histone B), derived from calf thymus, exhibits bactericidal activity similar to that of phagocytin raised the question of a possible relationship between phagocytin and histone B. Results presented in this communication demonstrate that phagocytin and histone B are separate entities.

Methods

Exudates containing polymorphonuclear leucocytes were collected from the rabbit peritoneum by the technique described previously (1) with the following modification:—Three to four hours after injection of glycogen-saline, the exudate was harvested by lavaging the peritoneal cavity with physiological saline rather than with citrated saline. The exudate was collected in a 500 ml. Erlenmeyer flask which contained 10 mg. of heparin. After centrifugation the red cells were lysed in hypotonic saline as previously described. The leucocytes were then washed with saline one additional time on the centrifuge, and the packed cells were stored at −20°C.

Cultures were maintained by passage once monthly on penassay agar (Gram-negative bacilli, staphylococci) or on proteose-blood agar (streptococci). Bactericidal assays were done in plastic cup-trays as described previously (3).

The medium used for bactericidal tests was prepared by mixing 0.1 M citric acid and 0.1 M dibasic sodium phosphate to give a pH of 6.5. Gelatin was dissolved in this buffer at final concentration of 0.02 per cent prior to sterilization in the autoclave. This test medium is called gelatin-citrate-phosphate in the tables which follow.

Results of bactericidal assays are recorded in the tables as the reciprocal of the highest

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dilution of test material producing greater than a 50 per cent kill of microorganisms after incubation for 2 hours at 38°C.

RESULTS

The Demonstration of Certain Differences between Phagocytin and Histone

Various studies were made of acid extracts of rabbit polymorphonuclear leucocytes (presumably containing phagocytin and histone), of similar extracts of rabbit kidney (presumably containing rabbit histone but no phagocytin) and of purified calf thymus histone. These preparations all displayed bactericidal effects on a wide range of enteric bacilli. However, their antimicrobial activities differed strikingly in susceptibility to inactivation by pepsin and in antagonism by high salt concentrations and by spermine.

TABLE I

Demonstration That the Bactericidal Effects of Phagocytin and Histones Differ in Their Susceptibility to Inactivation by Pepsin

Preparation	Bactericidal activity* on *E. coli K-12* in gelatin-citrate-phosphate of preparations at left after exposure for 2 hrs. at 38°C. to	
	0.1 M citric acid	2 γ/ml. crystalline pepsin in 0.1 M citric acid
Rabbit PMN‡ acid extract	320–640	320
Rabbit kidney acid extract	640	10
Calf thymus histone solution	640	<10

* Bactericidal activity in this and in subsequent tables is reported as the reciprocal of the highest twofold dilution producing greater than 50 per cent killing of the test organism in 2 hours at 38°C.

‡ The abbreviation PMN is used for polymorphonuclear leucocyte in the tables.

All steps in preparation of the extract were done at 4°C. Approximately 5 gm. of frozen rabbit polymorphonuclear leucocytes and of rabbit kidneys were disrupted in a Lourdes high-speed mixer (30 seconds) and each was suspended in 40 ml. isotonic NaCl solution. After spinning at 25,000 *G* for 15 minutes, the supernates were decanted and discarded. Extraction with saline and separation in the centrifuge were repeated in similar fashion two additional times. The residues were then well suspended in 40 ml. 0.05 N HCl and allowed to stand for 1 hour. After spinning at 25,000 *G* the clear supernate was decanted and saved for use in tests described below.

Calf thymus histone (Worthington Biochemical Corp., Freehold, New Jersey) was dissolved in 0.05 N HCl at 500 γ/ml.

In early studies it was found that exposure of phagocytin to fairly large amounts of crude pepsin resulted in loss of bactericidal activity. However, certain findings suggested that this might represent antagonism by pepsin,

rather than an enzymatic attack of pepsin on phagocytin. When granulocyte extracts were incubated with small amounts of crystallized pepsin under conditions appropriate for proteolysis, no significant loss of bactericidal activity resulted.

The granulocyte and kidney extracts and the calf thymus histone solution (described above) were diluted 1:2 into 0.2 M citric acid and into a freshly made 0.2 M solution of citric acid containing 4γ/ml. of twice crystallized pepsin (Nutritional Biochemical Corp., Cleveland). After incubation at 38°C. for 2 hours these specimens were examined for bactericidal activity by the standard test system (see Methods).

TABLE II

Resistance of the Bactericidal Action of Phagocytin to Inactivation by Pepsin

Tube contents (incubated at 38°C. for 2 hrs. before use in bactericidal tests)	Bactericidal activity on *E. coli K-12* in gelatin-citrate phosphate	Optical density at 280 λ of trichloracetic acid supernatants diluted 1:3 with H_2O
0.25 ml. rabbit PMN acid extract 0.75 ml. 0.1 M citric acid	320	0.040
0.25 ml. rabbit PMN acid extract 0.65 ml. 0.1 M citric acid 0.1 ml. 20 γ/ml. pepsin	320	0.043
0.4 ml. 0.1 M citric acid 0.1 ml. 20 γ/ml. pepsin 0.5 ml. albumin solution	<10	0.100
0.25 ml. rabbit PMN acid extract 0.1 ml. 20 γ/ml. pepsin 0.25 ml. 0.1 M citric acid 0.5 ml. albumin solution	320	0.113
0.5 ml. 0.1 M citric acid 0.5 ml. albumin solution	<10	0.040
0.25 ml. rabbit PMN acid extract 0.25 ml. 0.1 M citric acid 0.5 ml. albumin solution	320	0.044

As is shown in Table I, the antibacterial activity of an acid extract of rabbit leucocytes was essentially unaltered by a 2 hour exposure to peptic digestion, while that of rabbit kidney extract and of calf thymus histone was destroyed. This result did not establish that the bactericidal material in the acid extract of granulocytes differed from histone; the white cell extracts might contain histone and also a pepsin inhibitor not present in the other extracts. This possibility was excluded by the following experiment which demonstrated

that pepsin exerted proteolytic action in the presence of the acid extract of rabbit granulocytes.

Bovine plasma fraction V (Armour and Co., Chicago) was dissolved at 1 mg./ml. in 0.1 M citric acid. The rabbit PMN acid extract, the albumin solution, 0.1 M citric acid, and a fresh solution of 20 γ/ml. of crystalline pepsin in 0.1 M citric acid were added to tubes as indicated in Table II. After incubation for 2 hours at 38°C., 0.1 ml. was removed from each tube for bactericidal assay. An equal volume of 10 per cent trichloracetic acid was added to the remainder of each test solution. After standing at 4°C. for 30 minutes, the tubes were centrifuged and the clear supernatant solutions were diluted with water and their optical density measured at 280 λ in a 1 cm. cuvette in the Beckman spectrophotometer.

The results in Table II show again that the bactericidal activity of rabbit leucocyte acid extracts was not lowered by exposure to pepsin. That conditions were suitable for peptic digestion, even in the presence of this preparation, is demonstrated by the release of trichloracetic acid–soluble aromatic material from the indicator albumin solution included in the test.

TABLE III

Demonstration That the Bactericidal Activities of Phagocytin and Histones Differ in Susceptibility to Antagonism by Spermine

Preparation	Bactericidal activity on *E. coli K-12* in gelatin-citrate-phosphate when the following were added to the test medium (final concentration):		
	No addition	1 γ/ml. spermine tetrahydrochloride	10 γ/ml. spermine tetrahydrochloride
Rabbit PMN acid extract	640	640	320
Rabbit kidney acid extract	640	80	20
Calf thymus histone solution	640	160	20

In the course of comparing the granulocyte acid extract to the histone preparations, observations were made on effects of various antagonists of their bactericidal activity. Heparin proved to be approximately equally effective as an antagonist for all these preparations, but, as is shown in Table III, the activity of the acid extract of white cells was not significantly influenced by spermine, while the rabbit kidney extract and calf thymus histone solution showed markedly diminished bactericidal effects in the presence of this amine.

In previous studies (3) the bactericidal activity of histone was noted to diminish or disappear as the ionic strength of the test medium exceeded that of 0.2 M NaCl, while the lethal effects of phagocytin on coliforms were apparently not antagonized by levels of salt concentration up to at least 0.4 M NaCl (2). Table IV presents results of studies on the comparative susceptibility of polymorphonuclear leucocyte extracts and of histones to antagonism by increasing ionic strength of the test medium.

In confirmation of results presented in the earlier separate studies, it is seen that the antimicrobial effects of the granulocyte extract were but little altered by addition of salt to the test medium, while the rabbit kidney extract and calf thymus histone lost their capacity to kill *Escherichia coli* K-12 as the ionic strength of the medium was raised.

Thus, from these experiments it appeared that the rabbit polymorphonuclear leucocytes contained a bactericidal material (phagocytin) for coliform organisms, which was different from the histones of calf thymus or of rabbit kidney.

TABLE IV

Demonstration That the Bactericidal Action of Phagocytin Is More Resistant Than That of Histones to Antagonism by Increasing Ionic Strength of the Medium

Preparation	Bactericidal activity on *E. coli K-12* in 0.1 M citric acid-Na citrate pH 5.5 containing final concentrations of:			
	No addition	0.05 M NaCl	0.1 M NaCl	0.2 M NaCl
Rabbit PMN acid extract	1280	1280	640	640
Rabbit kidney acid extract	1280	160	80	20
Calf thymus histone solution	1280	320	40	<10

Separation of the Bactericidal Proteins of Rabbit Granulocytes by Fractional Extraction

Another line of experimentation attempted to distinguish between phagocytin and histone by demonstrating a different location for these materials within the phagocytic cell. The Mirsky-Pollister technique (4) for preparation of nuclei from calf thymus cells by extraction with dilute citric acid was slightly modified to permit separation of disrupted rabbit polymorphonuclear leucocytes into three gross fractions: (*a*) cytoplasmic constituents soluble in isotonic sucrose, (*b*) cytoplasmic and a small portion of nuclear components insoluble in isotonic sucrose but soluble in dilute citric acid, and (*c*) nuclear fraction, largely insoluble in dilute citric acid. The fractionation procedure employed was as follows:—

All operations were carried out at 4°C. unless otherwise noted. Approximately 0.5 gm. of packed rabbit polymorphonuclear leucocytes was well suspended in 5 ml. of 0.25 M sucrose–0.002 M $CaCl_2$. This suspension was frozen at −12°C. in a salt–alcohol–ice water bath and homogenized while still frozen in a Lourdes high speed mixer for 1 minute. A smear showed debris and broken cells; all nuclei stained readily with trypan blue. Sucrose-$CaCl_2$ solution was added to a total of 25 ml. The mixture was then centrifuged at 4,600 *G* for 10 minutes and the supernate decanted and saved. A second extraction with 25 ml. sucrose-$CaCl_2$ solution was done in the same manner. Next the residue was suspended in 25 ml. 0.01 M citric acid and allowed to stand with occasional mixing for 30 minutes. A smear showed the remaining formed elements to be mainly nuclei, to some of which cytoplasmic tags were attached.

After centrifugation at 4,600 *G* for 10 minutes citric acid extraction of the residue was then repeated. The residue was next suspended in 25 ml. 2 M NaCl and allowed to stand for 2 hours. Most of the solids appeared to pass into solution, and the small amount of residue was removed by centrifugation. One-half of this 2 M NaCl-soluble fraction was diluted with 10 volumes of distilled water. The fibrous precipitate (presumably nucleoprotein) which formed was collected in the centrifuge and extracted in 12 ml. of 0.2 N HCl to dissolve the histones.

Susceptibility of these fractions to peptic digestion was tested by diluting each 1:5 into 0.1 M citric acid and adding freshly dissolved crystalline pepsin to a final concentration of 1 γ/ml. After 2 hours of digestion at 38°C. standard bactericidal tests were done. Activities of pepsin-digested samples given in the table have been corrected for the fivefold dilution made during incubation with pepsin. Lysozyme activity was measured by making serial

TABLE V

Separation of Phagocytin from Lysozyme and Histones by a Series of Extractions on Rabbit Polymorphonuclear Leucocytes

Preparations derived from sequential operations on a single sample of disrupted rabbit granulocytes	Bactericidal activity on *E. coli K-12* in gelatin-citrate phosphate	Bactericidal activity on *E. coli K-12* of pepsin digested specimens	Lysozyme content γ/*ml.*	Protein content (optical density by Folin test)
First 0.25 M sucrose extract	320	160	100	1.2
Second " " " "	10	—	<10	0.18
First 0.01 M citric acid extract	5120	5120	<10*	1.5
Second " " " " "	80	—	<10*	0.14
2 M NaCl extract	5120	640	<10	0.66
0.2 N HCl extract of nucleoprotein precipitate formed on diluting above 2 M NaCl extract with water	2560–5120	<10	<10	0.60

* Clumping but no lysis of *M. lysodeikticus* occurred with this extract.

twofold dilutions of each fraction and of crystalline egg white lysozyme solutions in 0.05 M mixed phosphate buffer pH 6.5, after which a drop of a heavy suspension of *Micrococcus lysodeikticus* was added to each sample. After incubation at 38°C. for 1 hour, lytic end points were recorded and lysozyme content calculated by comparison to activity of the lysozyme standard solutions. For chemical measurement of protein content each sample was diluted 1:2 with water and subjected to a modified Folin test (5). Optical densities at 650 λ were measured in a Coleman Jr. spectrophotometer.

The following may be concluded from the results presented in Table V. Isotonic sucrose extracts of disrupted rabbit granulocytes contained phagocytin as evidence by their lethal effects on coliform bacilli and the preservation of this activity following pepsin digestion. These extracts also contained large amounts of lysozyme; the lysozyme demonstrable in this dilute extract indicates that 3 to 4 mg. of lysozyme is contained in 1 ml. of packed rabbit polymorpho-

nuclear leucocytes. A second extraction with isotonic sucrose contained but trace amounts of lysozyme and phagocytin. However, extraction of the remaining insoluble material with 0.01 M citric acid yielded a solution with bactericidal activity approximately 10 times higher than that in the first isotonic sucrose extract. Resistance of the citric acid-soluble material to inactivation by pepsin indicated that it was phagocytin and not histone. Since nuclei remained largely intact following extraction with citric acid, phagocytin would appear to be a partially bound cytoplasmic substance with limited solubility in physiological pH ranges. The second extract of residual solids with citric acid gave a solution with only a low level of bactericidal activity. Neither citric acid extract had demonstrable lysozyme activity. The residue remaining after repeated citric acid extraction was largely dissolved by 2 M NaCl. Formation of fibrous precipitate on dilution of this solution with water was in keeping with the recognized behavior of nucleoprotein. Acid extracts of the nucleoprotein precipitate demonstrated potent bactericidal activity which was completely lost following peptic digestion, suggesting that this activity was due to histone, rather than to phagocytin.

These findings gave further support to the notion that lysozyme, phagocytin, and histone were separate entities. Lysozyme in rabbit polymorphonuclear leucocytes apparently was largely free in the cytoplasm. Phagocytin on the other hand was released into neutral solvents only to a limited extent (approximately 10 per cent), while the remainder, whether by reason of solubility *per se* or binding to cell particulates, was dissolved only on exposure to acid. Nuclear histones, as would be expected, were not appreciably extracted in neutral aqueous solvents or in dilute citric acid, and required stronger measures, such as strong salt solution or relatively strong mineral acids, to separate them from their attachments in nuclei.

Further studies on extraction of phagocytin showed that rabbit polymorphonuclear leucocytes, whether fresh, stored in the frozen state, lyophilized, or dehydrated and defatted with cold acetone, all gave results similar to those presented in Table V; that is, extraction of any of these granulocyte preparation with neutral solvents (sucrose, physiological salt solutions, phosphate buffers) yielded about one-tenth of the total phagocytin, the remainder being extracted in dilute acids. Initial extraction with neutral aqueous solutions containing ethyl alcohol, butanol, or glycerol did not increase the proportion of phagocytin taken into solution.

Range of Antimicrobial Action of Phagocytin Prepared by Extraction of Polymorphonuclear Leucocytes with Dilute Organic Acid

The range of bactericidal activity of phagocytin prepared by extraction with dilute citric acid was studied to determine whether it differed significantly from that of the less potent aqueous extracts previously reported.

Approximately 1 gm. of frozen packed rabbit polymorphonuclear leucocytes was homogenized and suspended in 30 ml. 0.25 M sucrose–0.002 M $CaCl_2$. The soluble fraction, separated by centrifugation at 25,000 *G* for 15 minutes, was discarded. Extraction of the residue with sucrose solution was repeated twice in the same fashion. The residue was then suspended in 0.01 M citric acid and allowed to stand at 4°C. overnight. The clear supernatant solution after spinning at 25,000 *G* for 15 minutes was used for the tests described below.

TABLE VI

Range of Antibacterial Action of Phagocytin Prepared by the Citric Acid Extraction Technique

Test microorganism	Bactericidal activity of phagocytin (gelatin-citrate-phosphate used as test medium)
E. coli K-12	1280
" " Beeson	640
Salmonella typhimurium RIA	640
" " SR11	320
Salmonella enteritidis	<10
Klebsiella pneumoniae	160
Shigella sonnei	1280
Pseudomonas aeruginosa	2560
Proteus morganii	<10
Serratia marcescens	<10
Group A streptococcus D58X*	640
" B " 090R	20
" C " C-74	640
" D " D-76	<10
" G " D166B	40
Staphylococcus albus mendita	640
" *aureus* Smith	320

* Streptococcus cultures were kindly provided by Dr. Rebecca Lancefield. Surviving streptococci after exposure to phagocytin solutions were cultured by adding proteose blood agar to the test cups.

Table VI demonstrates that the bactericidal activity on several Gram-negative bacilli of phagocytin prepared by the newer technique was similar to that previously found with crude white cell extracts. In the particular experiment reported there was no lethal action on *Salmonella enteritidis*, *Proteus morganii*, and *Serratia marcescens*; however, in other instances moderate bactericidal action has been observed on these microbes by similar phagocytin preparations. This variability in results has not yet been explained.

In contrast to experience initially reported with phagocytin, the citric acid extracts of granulocytes also killed several Gram-positive bacteria. As is shown in the table, some hemolytic streptococci, including Group A strains, were

susceptible to phagocytin. Both a coagulase-negative and a coagulase-positive staphylococcus were killed on exposure to phagocytin under these conditions. Bactericidal tests were also done on streptococci in Todd-Hewitt broth medium and on staphylococci in penassay broth. Little killing occurred in whole broth, but marked bactericidal action was exerted on these Gram-positive cocci in broth diluted with three or more volumes of water.

Distribution of Phagocytin in Various Tissues

In the initial studies phagocytin was found in polymorphonuclear leucocytes but not in other phagocytic cells or in tissues. The new information on more efficient techniques for extracting phagocytin made it necessary to reinvestigate tissue distribution of this material.

TABLE VII

Bactericidal Activities of Extracts of Various Rabbit Tissues

Rabbit tissue or cell	Bactericidal activity of the following extracts on *E. coli K-12* in gelatin-citrate-phosphate				
	First 0.25 M sucrose	Second 0.25 M sucrose	First 0.01 M citric acid	Second 0.01 M citric acid	0.2 N HCl
PMN	80	<10	1280	40	1280
Monocyte	<10	<10	10	<10	640
Kidney	<10	<10	<10	<10	1280
Liver	<10	<10	<10	<10	640
Lung	<10	<10	10	<10	1280

A normal rabbit was killed by exsanguination and small portions of various organs were stored at −20°C. A packed cell button of monocytes, prepared by collection of a rabbit peritoneal exudate 4 days following injection of mineral oil, was kindly provided by Dr. George Mackaness and was stored under the same conditions. Weighed portions of these frozen tissues and of a similarly frozen rabbit polymorphonuclear leucocyte button were repeatedly frozen and thawed, homogenized in a high speed blendor, and then extracted sequentially in the cold twice with sucrose–$CaCl_2$, twice with 0.01 M citric acid, and finally with 0.2 N HCl. In all operations 30 ml. of extracting solution was used per gram, wet weight, of starting material. For each extraction residue was suspended in fresh solution with the high speed blendor and the suspension was allowed to stand for 30 minutes before separation by centrifugation at 25,000 *G* for 15 minutes.

As is seen in Table VII, bactericidal activity was present in the isotonic sucrose and especially in the citric acid extracts of rabbit polymorphonuclear leucocytes, but not in a similarly prepared extract of rabbit monocytes, kidney, liver, or lung. In contrast, the 0.2 N HCl extract of all these tissues and cells contained a bactericidal material. The citric acid preparation from granulocytes retained its antimicrobial effect when spermine or NaCl was added to the test medium, and its activity was unaltered by peptic digestion. These properties

were thus typical of phagocytin. The bactericidal activity of the 0.2 N HCl extracts of monocytes, liver, kidney, and lung was antagonized by spermine and by high ionic strength medium, and was completely lost following pepsin digestion, indicating that the active material in these extracts was histone, and not phagocytin. The activity of the 0.2 N HCl extract of polymorphonuclear white cells was reduced slightly by spermine or salt added to the medium, and was lowered approximately fourfold by pepsin digestion. These results were thus in keeping with the possibility that the strong acid extract of rabbit granulocytes contained both phagocytin and histone; if this were so then it follows that extraction with 0.01 M citric acid did not completely liberate phagocytin from disrupted granulocytes. In any event, the results in Table

TABLE VIII

Effect on Bactericidal Action of Phagocytin of Soluble Cytoplasmic Constituents from Various Tissues

Material tested	Bactericidal activity on *E. coli K-12* in gelatin-citrate-phosphate containing 10 per cent final concentration of the initial isotonic sucrose extracts of					
	PMN	Monocytes	Kidney	Liver	Lung	No addition
First 0.01 M citric acid extract of polymorphonuclear leucocytes (phagocytin)	5120	5120	2560	2560	2560	1280

VII show that phagocytin was demonstrable only in extracts of polymorphonuclear leucocytes, while, as expected, histones could be prepared from various rabbit tissues.

It was of interest to determine whether the initial isotonic sucrose extracts of the various tissues contained antagonists for the bactericidal effects of phagocytin. The results presented in Table VIII show that the cytoplasmic material soluble in isotonic sucrose from the various rabbit cells and tissues, including that from granulocytes, did not antagonize the bactericidal effects of phagocytin; in fact, some degree of potentiation of this effect appeared to occur with these extracts.

The Presence of Phagocytin in Polymorphonuclear Leucocytes from Various Animal Species

In the original report on phagocytin contained in extracts at pH 5.6 of polymorphonuclear leucocytes, considerable activity was found in preparations from rabbit cells while human and guinea pig granulocytes yielded only low levels of activity and rat and mouse cell extracts were inactive. Reinvestigation seemed indicated of phagocytin content of polymorphonuclear white cells from various species, employing the newer technique of citric acid extraction.

Guinea pig granulocytes were obtained from peritoneal exudates by a technique similar to that used for the rabbit. Four young adult white male guinea pigs were each injected intraperitoneally with 50 ml. isotonic saline containing 1 mg./ml. of glycogen. Four hours later the animals were sacrificed and exudate cells were collected by lavaging the peritoneal cavity with heparinized saline. Red cell lysis and washing of the white cells with saline were done as described previously for rabbit exudate cells. The yield was 3×10^8 white cells, 95 per cent of which were polymorphonuclear ones. The washed cell button was stored at −20°C. until used.

Human and horse leucocytes were obtained from blood of these animals. Fifty ml. of blood was collected in a syringe moistened with heparin solution, the final concentration of heparin being 0.1 mg./ml. To this was added 2 ml. of a 12 per cent solution of dextran in water (Abbott Laboratories, Chicago). The samples were mixed and then allowed to sediment at room temperature. After 30 minutes (horse blood) and 2 hours (human blood) the white

TABLE IX

Extraction of Phagocytin from Leucocytes of Various Animals

Polymorphonuclear leucocyte extract from	Bactericidal activity on *E. coli K-12* in gelatin-citrate-phosphate		
	Isotonic sucrose solubles	0.01 M citric acid solubles	0.2 N HCl solubles
Rabbit	160	1280	2560
Human	<10	1280	2560
Horse	<10	640	1280
Guinea pig	<10	320	640

cell rich plasma layer was drawn off. Spinning at 500 R.P.M. for 10 minutes in the size 2 International centrifuge brought down most of the leucocytes while leaving platelets largely in suspension. Red cells which contaminated the leucocyte button were removed by hypotonic lysis as described previously for rabbit leucocyte preparations. The washed human white cell button contained 2×10^8 leucocytes, 75 per cent of which were neutrophiles; the horse cell button contained 2×10^8 leucocytes, 80 per cent of which were polymorphonuclear ones. These white cell buttons were stored at −20°C. until used.

Extraction of rabbit, guinea pig, human, and horse packed white cells was performed by the same technique as described for rabbit tissue extracts in the preceding section. Five ml. of extracting fluid per 10^8 cells was used at each step. Initial extracts in 0.01 M citric acid were allowed to stand at 4°C. overnight before separation.

The results in Table IX show that bactericidal activity was present in acid extracts of polymorphonuclear leucocytes from rabbit, man, horse, and guinea pig. A similar pattern of activity was obtained when tests were done on *Salmonella typhimurium* and on *Shigella sonnei*. The bactericidal effect of the citric acid extract from all four animal species was unaffected by peptic digestion, while in each case the activity of the 0.2 N HCl extract was reduced fourfold or more. Thus, extraction with dilute citric acid yielded phagocytin from the polymorphonuclear leucocytes of all four animal species thus far tested. Of special interest is the fact that phagocytin was demonstrated in

horse and human cells obtained from blood rather than from exudates, suggesting that this material was present in normal circulating white cells and not formed only in response to inflammation and injury accompanying the peritoneal exudate.

Relationship between pH of the Medium and Bactericidal Activity of Phagocytin

Originally it was reported (2) that pH of the test medium exerted a striking influence on the antimicrobial effects of phagocytin, this material being the more active the more acid the test conditions. Subsequent studies on histone and on phagocytin demonstrated the antagonistic role of high ionic strength on bactericidal activity (3). Since the original observations on relations between

TABLE X

Influence of pH and of Ionic Strength of the Medium on the Bactericidal Effects of Phagocytin

Medium	Bactericidal action of phagocytin on		Medium	Bactericidal action of phagocytin on	
	E. coli K-12	*Salmonella typhimurium*		*E. coli* K-12	*Salmonella typhimurium*
ICS pH 5.0	10240	320	ICS diluted 1:5 with H_2O pH 5.0	5120	1280
" " 5.5	5120	320	" " " " " " 5.5	2560	1280
" " 6.0	2560	320	" " " " " " 6.0	2560	640
" " 6.5	1280–2560	320	" " " " " " 6.5	1280–2560	640
" " 7.0	1280	320	" " " " " " 7.0	1280–2560	640

pH and phagocytin action were made in a medium of fairly high ionic concentration containing citrate, it seemed possible that the influence of pH may have operated *via* alterations in ionic strength, rather than by hydrogen ion concentration *per se.*

The medium used in these tests was that described in full and termed ICS in the earlier publication (1): it contained KH_2PO_4, KCl, $MgSO_4$, and sodium citrate, with a final pH of 5.6. This medium was adjusted to the various pH values listed in Table X by addition of 1 N NaOH or 1 N HCl under constant mixing and observation with a pH meter. Portions of these media were diluted 1:5 with water to reduce ionic strength. A citric acid extract of rabbit granulocytes as described in the section above was used in these tests.

As is demonstrated by the results in Table X, pH of the test medium had an influence on the bactericidal activity of phagocytin on *E. coli* K-12, confirming the findings presented in the original paper (2). A somewhat less striking but still significant pH effect was noted when this microorganism was studied in medium diluted with water in order to minimize the influence on bactericidal activity of variation of ionic strength. In contrast, studies with *Salmonella typhimurium* showed that this organism was approximately equally susceptible

to killing by phagocytin at all pH levels tested, while reduction of ionic strength of the medium by dilution with water significantly increased its susceptibility. Thus, it appears that both factors, pH and ionic strength, were of importance in reactions between phagocytin and bacteria, and that their relative importance varied depending on the particular species of microorganism.

Stability and General Chemical Properties of Phagocytin

The original report on phagocytin prepared by extraction of granulocytes with salt solutions at or near neutrality pointed out that activity in such extracts was unstable even when stored in the cold. This instability made difficult any attempt to purify and isolate the active component. Phagocytin extracted from rabbit polymorphonuclear leucocytes by the citric acid technique described above manifested quite different stability properties. Extracts in citric acid or dilutions of such extracts in water, 0.1 M mixed phosphate buffer pH 6.5, 0.1 M tris-HCl buffer pH 8.1, 0.1 N HCl, or 2 M urea all exhibited constant bactericidal effects after storage at 8°C. for periods as long as 30 days. Furthermore, the bactericidal activity of citric acid extracts was remarkably stable to heat; treatment at 56°C. for 30 minutes brought about no change, and only a twofold diminution in activity resulted from heating for 30 minutes at 100°C.

On dialysis of phagocytin solutions against large volumes of dilute mineral or organic acid, all bactericidal activity remained within the cellophane sac. Dialyzed preparations gave positive chemical tests for proteins, but contained only trace amounts of lipid and anthrone-reactive carbohydrates.

Precipitation of citric acid extracts with trichloracetic acid at a final concentration of 5 per cent removed all bactericidal activity from the solution. One-half to one-fourth of the original activity was recovered in aqueous solutions of the precipitate.

In view of the remarkable resistance of phagocytin solutions to inactivation by pepsin, observations were made on the effects of other proteolytic enzymes.

Enzymes employed were the following: pepsin, twice crystallized (Nutritional Biochemicals Corp., Cleveland); trypsin, twice crystallized (Worthington Biochemical Corp., Freehold, New Jersey); chymotrypsin, thrice crystallized, and papain, twice crystallized (Mann Research Laboratories, New York). Buffer systems used are listed in Table XI. Portions of each enzyme solution in its buffer were inactivated by heating at 100°C. for 15 minutes. A dialyzed phagocytin solution, prepared from rabbit granulocytes, was diluted 1:5 into the various enzyme solutions and into appropriate controls. After incubation at 38°C. for 2 hours, 0.1 ml. was removed from each tube for bactericidal assay. The remainder of each specimen was mixed with an equal volume of 10 per cent trichloracetic acid and gross estimates were made of the quantity of precipitate formed.

The results recorded in Table XI show that bactericidal activity remained in phagocytin solutions after exposure to pepsin, trypsin, chymotrypsin, and papain. Reduction in amount of material precipitable by trichloracetic acid

confirmed that conditions in the test system were appropriate for enzymatic activity. Exposure to active proteolytic enzymes resulted in a slight but reproducible two- to fourfold reduction in bactericidal activity, whereas heated enzyme solutions were essentially without effect. These findings were incon-

TABLE XI

Susceptibility of Phagocytin to Inactivation by Various Proteases

Enzyme, final concentration	Buffer	Phagocytin diluted 1:5 into enzyme solutions at left and incubated 2 hrs. at 38°C.	
		Bactericidal activity on *Salmonella typhi-murium* in gelatin-citrate-phosphate	Precipitation with trichloracetic acid
None	0.1 M citric acid	80	+++
Pepsin, 2 γ/ml	" " " "	40–80	+
" , 20 γ/ml	" " " "	20	0
Heat-inactivated pepsin, 2 γ/ml	" " " "	80	+++
" " " , 20 γ/ml	" " " "	80	+++
None	0.1 M phosphate pH 7	80	+++
Trypsin, 10 γ/ml	" " " " "	40–80	++
" , 100 γ/ml	" " " " "	20	++
Heat-inactivated trypsin, 10 γ/ml	" " " " "	80	+++
" " " , 100 γ/ml	" " " " "	80	+++
None	0.1 M phosphate pH 7	80	+++
Chymotrypsin, 2 γ/ml	" " " " "	40	++
" , 20 γ/ml	" " " " "	20–40	+
Heat-inactivated chymotrypsin, 2 γ/ml	" " " " "	80	+++
" " " , 20 γ/ml	" " " " "	40	+++
None	0.1 M phosphate pH 6.5–0.01 M cysteine	80	+++
Papain, 2γ/ml	" "	20–40	+
" , 20 γ/ml	" "	10–20	0
Heat-inactivated papain, 2 γ/ml	" "	80	+++
" " " , 20 γ/ml	" "	40	+++

clusive and subject to several interpretations: (*a*) phagocytin is a protein which is only slowly attacked by proteases; in view of the marked destruction of protein in the extracts as demonstrated by trichloracetic acid precipitation following proteolysis, this hypothesis, if correct, would indicate that the extracts contain large amounts of proteins other than phagocytin; (*b*) phagocytin is not a protein and is resistant to attack by various proteases, the slight

reduction in activity being due to antagonism by the enzymes or by split products of digested contaminating proteins; (*c*) phagocytin is susceptible to proteolytic digestion, but the liberated fragments retain antimicrobial properties.

SUMMARY

Phagocytin and histone differ significantly in the following regards: (*a*) the bactericidal action of histone is rapidly lost on peptic digestion, while that of phagocytin is but little affected; (*b*) the lethal effect of phagocytin on coliform bacteria is much more resistant than that of histone to antagonism by spermine or by increasing ionic strength of the medium; (*c*) phagocytin can be extracted from disrupted granulocytes with dilute citric acid whereas effective extraction of histone requires stronger mineral acid or strong salt solution; (*d*) phagocytin is limited in distribution to polymorphonuclear leucocytes while histone is demonstrable in many tissues.

A new technique has been devised which permits extraction of phagocytin essentially free of lysozyme and histones. Phagocytin thus prepared kills certain Gram-positive bacteria as well as Gram-negative bacilli under appropriate *in vitro* test conditions. Among susceptible Gram-positive microbes are Group A streptococci and staphylococci.

Phagocytin is demonstrable in citric acid extracts of granulocytes obtained from rabbit, man, horse, and guinea pig, the only species thus far investigated. Circulating blood leucocytes as well as exudate cells contain this bactericidal substance.

The lethal effects of phagocytin on bacteria may be influenced, depending on the particular microorganism, by either pH or ionic strength of the medium.

The bactericidal action of phagocytin is only slightly reduced following digestion with trypsin, chymotrypsin or papain. The active ingredient is, however, non-dialyzable and apparently precipitated by trichloracetic acid. Data available at present are insufficient to define the chemical nature of phagocytin.

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