ISOLATION AND CHARACTERIZATION OF PERMEABILITY FACTORS FROM RABBIT NEUTROPHILS*

By N. S. RANADIVE, Ph.D., and C. G. COCHRANE,‡ M.D.

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

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Neutrophils have been found to play an important role in the pathogenesis of several experimental immunologic reactions. Two different groups of substances in rabbit neutrophils have been implicated in the mediation of tissue damage, the catheptic enzymes and certain basic proteins in cytoplasmic granules of neutrophils. Cathepsins D and E have been found capable of degrading vascular basement membranes (1), while the basic proteins have been shown to increase vascular permeability (2–5) without grossly disrupting the basement membrane (1).

Janoff and coworkers (2) investigated one of the neutrophilic cationic proteins of rabbits and elucidated its capacity to degranulate rat mast cells, releasing their content of vasoactive amines. This action apparently brought about the immediate increase in vascular permeability noted upon injection of the protein into the skin of rats. Golub and Spitznagel (5) studied a fraction obtained from rabbit neutrophilic granules that induced vascular permeability. The injury increased over a 2 hr period as opposed to that in the previous study (2) which developed maximally within 30 min. The activity of Golub and Spitznagel's factor was unaffected by prior treatment of the test animal with antihistamine. Studies conducted in this laboratory have demonstrated the immediate, but not the slow-acting, permeability material in extracts of rabbit neutrophilic granules (1). The present studies were undertaken to analyze in greater detail the basic proteins in neutrophilic lysosomal granules. A search was made for permeability factors that possibly act independently of the histamine-releasing mechanism, since several neutrophil-dependent immunologic lesions take place in structures devoid of mast cells. In brief, four distinct cationic proteins have been isolated and characterized from granules of rabbit neutrophils which have the capacity of increasing vascular

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permeability. Only one of these acts via the release of histamine from reservoirs. Various physicochemical properties of the isolated and purified cationic proteins are described. A preliminary report of these findings has appeared (6).

Materials and Methods

Collection of Neutrophils from Rabbits.—Neutrophils were obtained from peritoneal exudates of New Zealand white rabbits and the cytoplasmic granules from these cells were isolated according to the method described by Cohn and Hirsch (7). The granules were washed twice prior to extraction of their contents. While exudates obtained 4 hr after the intraperitoneal injection of glycogen were tested at first, those obtained 16 hr after injection yielded larger numbers of neutrophils. The longer time period was employed when larger quantities were required. No qualitative differences in the proteins isolated at the two times were found. The concentration of neutrophils in the 16 hr exudate was 85% on the average, while greater than 96% neutrophils were found in 4 hr exudates.

Extraction of Cationic Proteins from Neutrophils.—Cationic protein fractions were obtained from neutrophilic granules by extracting the granules with 0.2 N H_2SO_4. The acid extract was then centrifuged at 12,000 rpm for 10 min. The supernatant, which was slightly viscous, was decanted, and the sediment was extracted an additional three times with 0.2 N H_2SO_4. The acid extracts thus obtained were pooled, neutralized by dropwise addition of 1.0 N NaOH, and lyophilized. As an alternative method of preparation, extracts of whole neutrophils were tested. The yield of proteins under study was greater, although qualitatively the proteins obtained were the same as those obtained from isolated granules. The poor yield from granule preparations owed principally to large numbers of intact cells remaining after disruption with 0.34 M sucrose. In order to prepare extracts of whole cells, neutrophils were washed three times, suspended in 5 volumes of distilled water and sonically disrupted for a period of 5 min at 4°C using a Raytheon sonic oscillator. The suspension of disrupted cells was then acidified by dropwise addition of 2 N H_2SO_4 yielding a final H_2SO_4 concentration of 0.2 N. The acidified suspension was then extracted and treated as were the isolated granules.

Separation of the Acid Extract of Neutrophils on Diethylaminoethyl Cellulose (DEAE).—The lyophilized acid extract was dissolved in 15 ml of distilled water. A small insoluble residue was extracted at 0°C once with 10 ml of distilled water and once with 5 ml of acetate buffer (0.01 M), pH 4.0. This extract (final volume 30 ml) was then applied to a pressure-packed column of DEAE cellulose (30.0 cm × 2.5 cm) equilibrated with 0.01 M PO_4 buffer, pH 8.0, at 4°C. Over 50 ml of the equilibrating buffer was passed through the column prior to establishing a gradient of sodium chloride using 0.5 M NaCl in 0.01 M Na_2HPO_4 (pH 8.0) as terminal buffer. The fractions collected were tested for their vascular permeability-inducing activity in the skin of rabbits.

Separation of the Permeability-Inducing Factor by Gel Filtration on a Column of Sephadex G-50.—The permeability-inducing protein fractions from the DEAE column were pooled, lyophilized, and redissolved in 30 ml of water. The aqueous solution after acidification was centrifuged to remove suspended particles. This was then applied to a column of Sephadex G-50 (34 × 4.5 cm). Elution was carried out with 0.02 M acetate buffer pH 4.0 containing 0.1 M NaCl. The fractions were collected in 5 ml volumes and tested for their permeability-inducing activity in rabbit skin.

Preparatory Polyacrylamide Gel Electrophoresis.—Preparatory polyacrylamide gel electrophoresis was performed according to the method described by Maizel (8) with certain modifications. The electrophoresis was carried out with β-alanine buffer, pH 4.5, at 100 v and 30–40 ma of current. 20 ml of small pore gel was layered with 4 ml of concentrating gel

and the sample (1–10 mg) was applied in 4 ml of large pore gel. The bottom chamber was continuously eluted with β-alanine buffer (pH 4.5). The fraction collector was adjusted to collect three tubes per hour containing 3 ml of eluate.

Subfractionation of the third peak obtained from Sephadex with the continuous elution system was not possible. In this case, therefore, the gel block was removed from the chamber after 4 hr of electrophoresis and a thin strip cut from each side for staining with Amidoschwarz, followed by electrophoretic removal of unbound dye. The location of the separated proteins, as shown by the stained strips,[1] was marked on a sheet of paper, the remaining unstained block aligned to this and the fractions containing protein cut out. The protein was then removed from these sections of gel electrophoretically in β-alanine buffer at pH 4.5. The eluted protein fractions were freed from β-alanine either by chromatography on Sephadex G-10 column or by ultrafiltration through Amicon filters (UM-2) with repeated washing with distilled water.

Determination of Increased Vascular Permeability.—Permeability-inducing activity of the test samples was evaluated by injecting intradermally 0.1 or 0.2 ml of the sample into the flank skin of a rabbit which had previously received an intravenous injection of Evan's blue dye solution (1 ml/kg of a 2.5% solution in saline). Wherever necessary, the samples were adjusted to physiological pH and salt concentration. The reactions were normally read after 20 min and when noted, 1, 2, and 4 hr. For comparison of the permeability-inducing activity of various samples, the samples were diluted serially until no reaction was evident. A unit of activity (P. F. unit) was then defined as the amount of injected material required to yield a 4 × 4 mm weak reaction in 0.1 ml.

Analytical Polyacrylamide Gel Electrophoresis.—Analytical polyacrylamide gel electrophoresis was carried out as described by Reisfield et al. (9) using β-alanine buffer (pH 4.5) at 80 v and with 7 to 8 ma of current per tube. Acriflavine was used as a tracking dye, and it was found that within 50 to 60 min the dye could move from top to bottom of the small pore gel, i.e., a distance of 3.5 cm. The gels were stained with 0.2% Amidoschwarz solution in 7.5% acetic acid for 4 hr and the unbound dye removed electrophoretically.

Release of Histamine from Isolated Rat Peritoneal Mast Cells.—Rat peritoneal cells were obtained in Na-citrate-NaCl solution (10). The cells thus obtained were washed three times and suspended in a 2% bovine serum albumin (BSA) solution in medium 199 with a final concentration of approximately 2×10^7 cells/ml. Such suspensions contained 4–6% mast cells.

To determine the histamine-releasing activity of a given sample, 0.1 ml of cell suspension was mixed with 0.5 ml of medium 199 and 0.2 ml of the sample was added to it. In tests comparing the relative capacities of proteins to release histamine, aliquots of mast cells from the same batch were employed. This eliminated errors caused by varied susceptibility of different batches of mast cells to histamine-releasing agents. The reaction mixture was allowed to stand for 30 min at room temperature. The cells were then removed by centrifugation and the quantity of histamine contained in the supernatant and cellular sediment was assayed on atropinized (10^{-7} M of atropine) guinea pig ileum. Preliminary studies indicated that the cationic proteins (50 μg protein) themselves failed to induce contraction of the guinea pig ileum.

In certain experiments, purified rat mast cells were used. For this purpose, 0.3 ml of rat peritoneal cell suspension in medium 199 was layered on 3 ml of 30% BSA solution (in medium 199) in cellulose nitrate tubes (½ inches diameter by 2 inches). The tubes were then centri-

[1] During staining and destaining procedure, the strips are elongated due to swelling; however, they could be brought back to their original length by suspending them in 70% ethanol.

fuged for 2 to 3 min at 250 rpm. The upper one-third volume of the tube was carefully aspirated and discarded and the bottom half then removed by aspiration in a Pasteur pipette. The cells obtained in this layer were recovered by centrifugation at 2000 rpm and washed twice in medium 199 containing 2% BSA. Greater than 95% of the cells thus obtained were mast cells.

Release of Histamine from Rabbit Platelets.—Washed rabbit platelets (10^8 cells) were incubated for 30 min at 37°C with 20 P. F. units of band 2 protein (see below) (10 μg N) in balanced salt solution either in presence or in absence of 2.5% plasma. The platelets were removed by centrifugation and the supernatant was tested for histamine release using atropinized guinea pig ileum.

Degranulation of Mast Cells in Isolated Rat Mesentery.—Morphologic observation of mast cell degranulation was studied on sections of ileal mesentery of rats (11). The mesentery was incubated in 0.6 ml of testing sample in medium 199 for 30 min. The mesentery was then removed, fixed in 10% formalin, and stained with toludine blue. Degranulated and intact mast cells were counted in over 20 high power fields.

Depletion of Circulating Platelets.—Sheep antiserum to rabbit platelets was prepared, purified, and administered according to methods previously published (12).

Depletion of Neutrophils in Rabbits.—2-kg rabbits were depleted of circulating neutrophils by a single injection of nitrogen mustard (Mustargen HCl, Merck, Sharp & Dohme, West Point, Pa.) (1.75 mg/kg) 3 days prior to testing. The peripheral neutrophil counts were less than 150/mm^3 at that time.

Amino Acid Analysis.—Amino acid analysis was carried out on a Beckman automatic amino acid analyzer. The samples were hydrolyzed with 6 N HCl for 24 hr at 105°C. The analysis was performed as described by Spackman et al. (13).

Ultracentrifugal Analysis.—Molecular weight of isolated band 4 proteins was determined by the Archibald technique of approach to sedimentation equilibrium using a Spinco model E ultracentrifuge. Observations were performed using a schlieren optical system. The sample was dissolved in saline (1.65 mg/ml) and was centrifuged at 29,500 rpm. The photographs were taken at 32 min intervals with schlieren diaphragm of 60°. Molecular weight was calculated using graphical method as described by Schachman (14). The partial specific volume was calculated from amino acid composition (15) and was found to be 0.729 ml/g.

RESULTS

The Isolation and Purification of Basic Proteins of Rabbit Neutrophilic Granules

Separation of Basic Proteins of Neutrophils by Anion Exchange Chromatography and Gel Filtration.—Separation of acid extracted material obtained from the lysosomal granules of neutrophils on a column of DEAE cellulose is shown in Fig. 1. It was found that proteins, passing through the column without binding, were able to induce immediate vascular permeability in rabbit skin. All other fractions were inactive in this regard. Cathepsins (not shown in the figure) were eluted only after NaCl concentration in the elution buffer reached 0.02 M. The fractions containing permeability-inducing activity obtained from the DEAE column were pooled, concentrated by lyophilization and applied to a Sephadex G-50 column. Three major protein fractions were obtained from the Sephadex column and all three fractions showed permeability-inducing activity. The three fractions thus obtained were designated

as G-50 Fr I, G-50 Fr II and G-50 Fr III, according to their order of elution.

These three fractions, after recycling on Sephadex G-50 coulmn, were analyzed on analytical polyacrylamide gel electrophoresis and tested for their ability to induce increased permeability of vessels in rabbit skin and to release histamine from rat peritoneal mast cells. The results of these experiments are shown in Fig. 2. It will be seen from the electrophoretic pattern that the G-50 Fr II consisted of one major protein fraction, which is designated band 4, while the G-50 Fr III had three protein fractions, designated as bands 1, 2, and 3. Polyacrylamide gel electrophoresis of G-50 Fr I yielded band 4

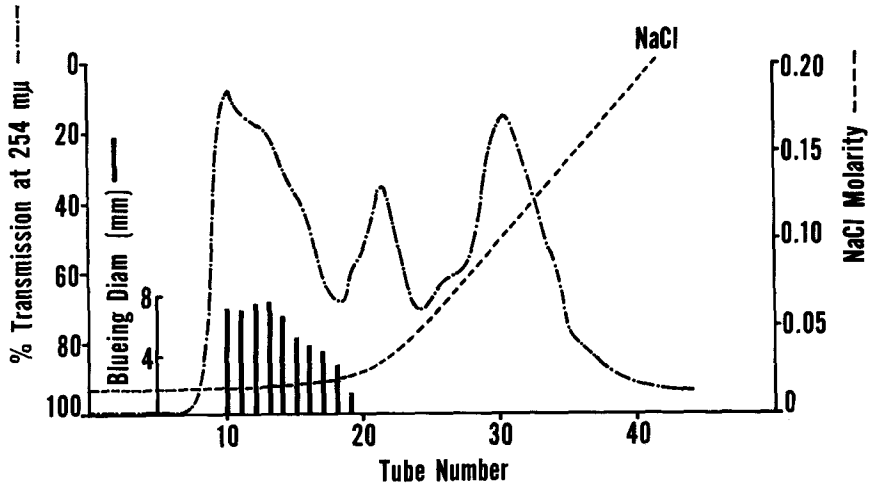

FIG. 1. Fractionation of acid extract of rabbit neutrophilic granules on DEAE-cellulose column (30.0 × 2.5 cm). The elution was carried out with 0.01 M PO_4 buffer, pH 8.0, at 4°C. NaCl gradient was started after 50 ml of the buffer was eluted. Permeability-inducing activity was located in exclusion fraction as shown by solid bars.

protein and other higher molecular weight proteins. G-50 Fr I was also treated with trichloroacetic acid (TCA) at 5% final concentration, followed by removal of the TCA from the supernatant solution by filtration through Sephadex G-10. Most of the permeability-inducing activity in G-50 Fr I was found in the TCA-soluble fraction. Polyacrylamide gel electrophoresis of the material showed proteins electrophoretically identical to those in G-50 Fr III and G-50 Fr II.

Isolation of Four Cationic Proteins of Neutrophilic Granules by Polyacrylamide Gel Electrophoresis.—The biologically active proteins obtained from the Sephadex G-50 columns were finally isolated and purified by preparatory polyacrylamide gel electrophoresis. A typical separation of G-50 Fr II by polyacrylamide gel electrophoresis is shown in Fig. 3. The fractionated material

obtained revealed that the G-50 Fr II band 4 was effectively separated from other proteins by this procedure. Among the proteins separated from band 4 were the three proteins found in G-50 Fr III in small amounts and a large molecular weight protein that migrated more slowly toward the cathode. This

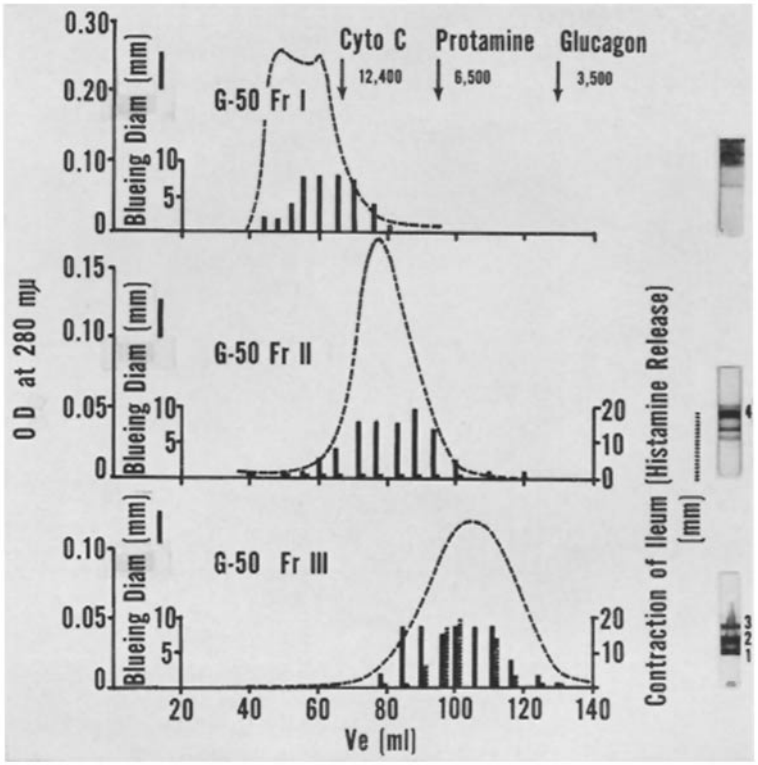

FIG. 2. Gel filtration chromatography of three vasoactive fractions on a column containing Sephadex G-50. These fractions (G-50 Fr I, G-50 Fr II, and G-50 Fr III) were obtained from a previous separation on Sephadex G-50 of the exclusion peak from DEAE-cellulose column (shown in Fig. 1). The eluted fractions were tested for permeability activity (solid bars) and histamine-releasing activity (broken bars). The analytical acrylamide gel electrophoretic patterns (β-alanine buffer, pH 4.5) of each fraction are shown on the right hand side.

latter protein was found not to induce increased vascular permeability in the skin of rabbits.

G-50 Fr III, which contained band 1, 2, and 3 proteins, was subjected to preparatory electrophoretic separation on polyacrylamide gel as shown in Fig. 4. Each of the three protein preparations yielded a single band when tested in analytical polyacrylamide gel electrophoresis. When tested in the skin of rabbits, each induced an increase in vascular permeability.

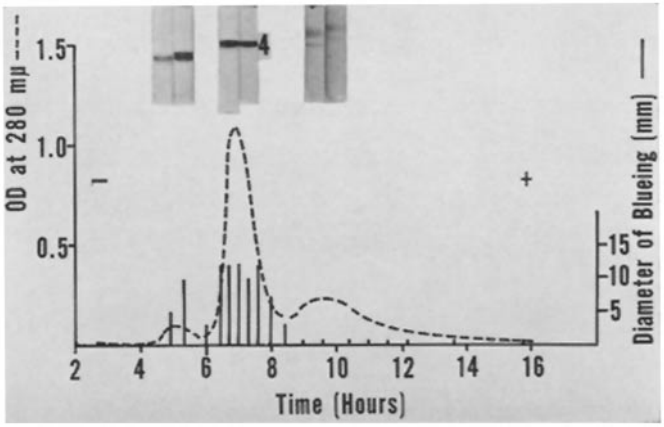

FIG. 3. Preparatory polyacrylamide gel electrophoresis of G-50 Fr II with continuous elution. Electrophoresis was carried out in β-alanine buffer, pH 4.5, at 100 v and 50 ma current at 4°C. The proteins were eluted according to their electrostatic charges, the most basic protein being eluted first (left hand side of figure) and the least basic being eluted last (right hand side). The permeability activity of eluted fractions is shown by solid bars. The representative analytical acrylamide gel electrophoretic patterns of the fractions are shown on the top of each fraction.

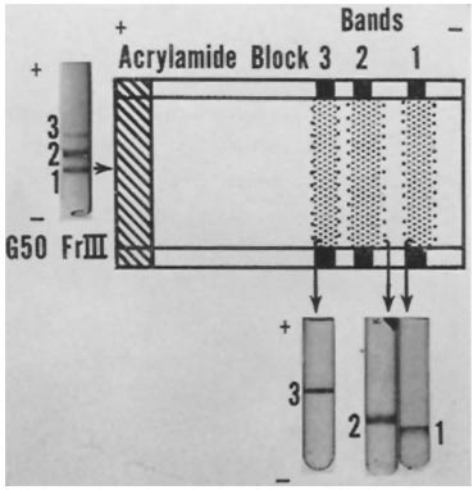

FIG. 4. Separation of band 1, 2, and 3 proteins from G-50 Fr III by preparatory polyacrylamide gel electrophoresis. The electrophoresis was carried out in β-alanine buffer, pH 4.5, at 4°C for 4 hr at 100 v and 50 ma current. The strips from either side of the block were cut and stained. The block was then cut as shown in the figure. The proteins in cut fractions were eluted electrophoretically. The analytical electrophoretic pattern of each eluted protein is shown below the respective cut portion of the block.

The vasoactive basic proteins of neutrophils were thus separated into four different protein fractions. These protein fractions are referred to according to their electrophoretic characteristics in polyacrylamide gels, i.e. protein bands 1, 2, 3, and 4, named from the fastest (most basic) to the slowest migrating proteins.

The Biologic Activity of the Four Basic Proteins of Rabbit Neutrophilic Granules

Permeability-Inducing and Histamine-Releasing Activity.—The capacity of each isolated basic protein to induce an increase in vascular permeability of

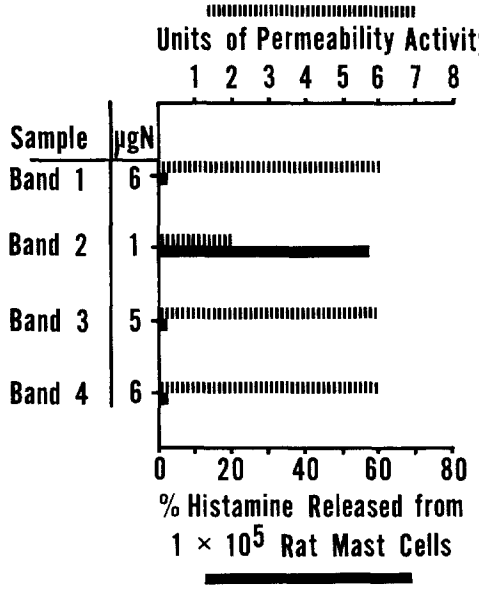

FIG. 5. Permeability-inducing and histamine-releasing activity of purified basic proteins from rabbit neutrophilic granules. Solid bars, histamine-releasing activity; broken bars, permeability-activity.

rabbit skin and to release histamine from isolated rat peritoneal mast cells was determined employing known amounts of the isolated fractions. The results of this study are shown in Fig. 5. All four fractions had roughly comparable permeability-inducing activity on a basis of equal amounts of protein. However, only one of these, band 2, was found to release histamine from mast cells. As little as one permeability-inducing unit (P.F. unit) of band 2 (0.5 μg N) could release about 40% of the total available histamine from 1×10^5 isolated rat mast cells, while 6 P.F. units of band 4 (6.0 μg N), 6 P.F. units of band 1 (6 μg N), and 6 units of band 3 (approximately 5 μg N) did not release histamine from the same number of mast cells.

The dose response curve for histamine release by band 2 protein was found

to be linear. When 1×10^5 isolated mast cells were allowed to react with 1 P.F. unit of band 2 (i.e., 3.4×10^{14} molecules assuming molecular weight of this fraction to be 5000, see below), about 15 molecules of histamine were released per molecule of the protein.

TABLE I

The Effect of Antihistamine on the Permeability-Inducing Activity of Isolated Basic Proteins of Rabbit Neutrophils

Test sample	Permeability index*						
	Control		Antihist.-treated‡		Normal	Antihist.-treated§	
	Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2		Rabbit 1	Rabbit 2
Band 1	—	21	—	16	8	—	8
Band 2							
sample I	18	20	1	4	12	6	1
sample II	—	8	—	0	—	—	—
Band 3	—	12	—	12	15	—	15
Band 4							
sample I	15	15	12	15	20	20	20
sample II	—	24	—	18	—	—	—
Histamine (1.2 μg)	30	24	1	0	20	0	1
48/80 (10 μg)	—	8	—	0	16	0	12
Kallidin (5 μg)	30	30	30	30	27	30	27

* Mean diameter (mm) of the lesion × strength of the reaction. Strength of reaction: strong, 4; moderate, 3; weak, 2; trace, 1. Reactions read 20 min after intradermal injection of 0.1 ml of test sample.

‡ 10 μg of chlorpheniramine maleate injected intradermally into the test site 30 min prior to the test.

§ Chlorpheniramine maleate (20 mg/kg) injected intravenously 3–4 hr prior to test.

Mast cell degranulation was studied on rat mesentery and it was found that only band 2 at a concentration of 2 P.F. units (1 μg N) degranulated about 50% of the mast cells in a 1 × 1 cm area of rat mesentery. No specific degranulation was observed when 2 P.F. units (2 μg N) band 4 were incubated with a similar section of mesentery.

Effect of Band 2 Protein on Rabbit Platelets.—It was found that 10 μg of band 2 protein neither released histamine from the platelets nor aggregated the platelets.

Effect of Antihistamine Treatment and Platelet Depletion on the Vascular Permeability Induced by the Vasoactive Basic Proteins.—The neutrophilic permeability factors were tested in rabbits pretreated with antihistamine

TABLE II

The Effect of Platelet Depletion on the Permeability-Inducing Activity of Isolated Basic Proteins of Rabbit Neutrophils

Test sample	Permeability index*		
	Normal	Platelet-depleted‡	
		Rabbit 1	Rabbit 2
Band 2	12	8	20
Band 4	15	15	20
Histamine (1.2 μg)	20	16	16
48/80 (10 μg)	16	10	14
Kallidin (5 μg)	27	27	33

* Mean diameter (mm) of the lesion × the strength of the reaction. Strength of reaction: strong, 4; moderate, 3; weak, 2; trace, 1. Reactions read 20 min after intradermal injection of 0.1 ml of test sample.

‡ Platelets were depleted by intraperitoneal injection of anti-platelet sheep antiserum 20 hr prior to test.

TABLE III

*Time Required to Elicit Permeability Reactions in the Skin of Neutropenic Rabbits**

Time of injection‡	Permeability index§				
	Band 1	Band 2	Band 4	G-50 Fr II	G-50 Fr II
min					
60 before	0	0	0	0	0
30 before	0	0	0	0	0
15 before	0	0	1	1	0
5 before	6	8	18	18	12
5 after	6	8	18	18	12

* Rabbits were made neutropenic by injecting 1.75 mg/kg of nitrogen mustard 3 days prior to testing.

‡ Time of injection of test sample relative to intravenous injection of Evan's blue dye.

§ Mean diameter (mm) of the lesion × the strength of reaction. Strength of reaction: strong, 4; moderate, 3; weak, 2; trace, 1.

(chlorpheniramine maleate) and in rabbits depleted of platelets. The results are shown in Table I. The amount of each protein administered was selected to yield moderate blueing reactions in normal rabbits, i.e., reactions that would

be readily modified by treatment with antagonists. As noted, the permeability-inducing activity of band 2 as well as that of histamine and compound 48/80 was almost completely inhibited, while the activity of other permeability factors was not affected by antihistamine treatment in the same rabbits. Similarly, there was no change in the reaction produced by kallidin in these rabbits compared with normal rabbits. Depletion of platelets from the circulation in two rabbits did not inhibit the permeability-inducing activity of band 2 and band 4 protein (Table II). The permeability-inducing capacity of histamine and kallidin remained unaffected in these rabbits.

TABLE IV

*Amino Acid Composition of Vasoactive Basic Proteins of Rabbit Neutrophilic Granules**

	Band 1	Band 2	Band 3	Band 4
Lysine	16.4	1.6	7.7	8.6
Histidine	5.7	2.5	1.6	2.0
Arginine	10.3	18.2	2.1	6.6
Aspartic acid	4.3	2.8	6.8	13.8
Threonine	3.7	5.6	4.4	5.9
Serine	8.0	7.7	19.0	5.9
Glutamic acid	5.7	4.1	13.9	6.8
Proline	5.1	3.7	4.0	4.8
Glycine	10.7	12.5	19.3	7.5
Alanine	12.6	4.8	6.0	9.5
Half cystine	—	14.9	—	4.0
Valine	5.2	5.2	2.9	5.2
Methionine	—	—	—	0.7
Isoleucine	2.9	3.2	3.0	5.2
Leucine	5.5	7.6	5.3	7.9
Tyrosine	2.1	0.5	1.9	4.7
Phenylalanine	1.9	5.1	2.0	1.2
Total	100.1	100.0	99.9	100.3

* Moles/100 moles recovered.

Time Required for the Permeability Proteins to Bring About Vascular Injury.— The permeability-inducing proteins, together with crude cationic proteins obtained from the column of DEAE cellulose, were injected into the skin of two neutrophil-depleted rabbits at various time intervals. The first injection was given 60 min before and the last 5 min after the intravenous injection of the Evan's blue dye. The results of this experiment are summarized in Table III. The permeability reaction was found to reach its maximum within 5 min in the case of band 2 and within 15 min in the case of band 4. None of the sites injected 15 min before the injection of the dye showed a reaction.

Physicochemical Studies of the Basic Proteins

Amino Acid Analysis of the Isolated Proteins.—Amino acid compositions of the four proteins are shown in Table IV. It is apparent from the amino acid compositions that these four proteins are distinct entities.

TABLE V

Number of Residues of Amino Acid per Molecule of Isolated Protein

	Band 1		Band 2		Band 4	
	Calculated on basis of 4800 mol. wt.		Calculated on basis of 5000 mol. wt.		Calculated on basis of 11,000 mol. wt.	
	Actual value	Nearest whole figure	Actual value	Nearest whole figure	Actual value	Nearest whole figure
Lysine	6.20	6	0.61	1	3.17	8
Histidine	2.16	2	0.99	1	2.01	2
Arginine	3.90	4	7.16	7	6.02	6
Aspartic acid	1.63	2	1.09	1	13.48	13
Threonine	1.42	1	1.97	2	5.71	6
Serine	3.04	3	3.02	3	5.76	6
Glutamic acid	2.16	2	1.60	2	5.76	6
Proline	1.91	2	1.50	2	4.31	4
Glycine	4.04	4	4.93	5	7.34	7
Alanine	4.77	5	1.90	2	9.11	9
Half cystine	—	—	5.86	6	3.89	4
Valine	1.96	2	2.03	2	5.02	5
Methionine	—	—		—	0.50	1
Isoleucine	1.09	1	1.24	1	5.01	5
Leucine	2.09	2	3.00	3	7.50	7
Tyrosine	0.76	1	0.20	1	4.40	4
Phenylalanine	0.71	1	2.02	2	1.29	1
Total		38		41		94
Calculated mol. wt. from residue	4826		5283		11,955	

Molecular Weights of the Isolated Factors.—Molecular weights of band 1, 2, 3, and 4 proteins were estimated from the volume of elution from a column of Sephadex G-50 that was previously calibrated with several different proteins of known molecular weight (Fig. 2). The elutions were carried out at pH 4.0 in 0.1 M NaCl and 0.02 M sodium acetate buffer. The molecular weight of band 4 appeared to be approximately 9000 and that of bands 1, 2, and 3 about 4000 to 5000. Band 4 was also subjected to analytical ultracentrifugal analysis using the method of the approach to sedimentation equilibrium. The molecular weight derived from this study was found to be 12,300. Molecular weights of

the isolated proteins were also computed from the amino acid composition. The results of these computations and assignment of the number of residues of each amino acid in the protein are shown in Table V. The molecular weight of band 4 obtained from the amino acid composition was 11,950, a value which is in reasonable agreement with that obtained by ultracentrifugal analysis (12,300). Molecular weights of band 1 and band 2 proteins as obtained from the amino acid composition were 4826 and 5283, respectively, which are within the range shown by gel filtration studies.

DISCUSSION

The data presented indicate that four different proteins are present in the lysosomal granules of rabbit neutrophils having the capacity of increasing vascular permeability in rabbit, rat, and guinea pig skin. All other fractions of rabbit neutrophils obtained from chromatography on DEAE cellulose, including those containing cathepsins D and E, failed to induce increased vascular permeability upon intradermal injection. In addition, other neutrophilic cationic proteins were found not to be vasoactive in the concentrations employed. We have thus tentatively concluded that these four proteins represent the major vasoactive basic proteins of rabbit neutrophils. They have been called band 1, 2, 3, and 4 proteins according to their migrational characteristics in analytical polyacrylamide gel electrophoresis. In rat neutrophilic granules at least three vasoactive basic proteins have also been identified.[2] In aggreement with Scherer and Janoff (16), one of these from rat neutrophils releases histamine from isolated rat mast cells.[2]

Physical and Chemical Properties of the Four Vasoactive Proteins

Electrostatic Charge.—Although precise information regarding the isoelectric point of the purified materials could not be obtained due to the low yield of the proteins, the electrophoretic migration of these proteins clearly indicates that they are highly basic in nature. This is confirmed by the high ratio of basic to acidic amino acid residues in band 1 and band 2 proteins. In the case of band 3 and band 4 proteins, the ratio of basic to acidic amino acid residues is found to be less than unity. However, the high ammonia content of the acid hydrolysates of these proteins suggests that some of the carboxylic acids in these proteins were masked by amide groups.

Amino Acid Composition.—It is apparent from the amino acid compositions that these neutrophilic lysosomal permeability factors represent four distinct protein entities (Table IV). Band 1, 2, and 3 proteins appear to be of comparable molecular size and electrostatic charge, but marked differences exist in their amino acid composition. In respect to the relative quantities of lysine and arginine, bands 1, 3, and 4 are lysine-rich, while band 2 contains predominantly

[2] Ranadive, N. S., and C. G. Cochrane. Unpublished data.

arginine. Since band 2 is the only permeability factor which shows histamine-releasing and mast cell degranulation activity and is found to be exceptionally rich in arginine content, it is presumed to be the same factor reported by Janoff and coworkers (2) as mastocytolytic factor (MCF). The differences in amino acid composition reported by Seegers and Janoff (2) and Zeya and Spitznagel (17) and the composition obtained by us for band 2 protein may well be attributed to the purification exerted by the final step of isolation employed in the present studies, i.e., separation on polyacrylamide gel electrophoresis. This yielded a preparation having a single band on analytical polyacrylamide gel electrophoresis.

In these studies of amino acid composition, tryptophan analysis was not done and the correction for the partial loss of cystine, serine, and tyrosine during hydrolysis (18) was not applied. These determinations, however, should not change the amino acid composition by more than 3 or 4 residues. It is also evident from the amino acid composition of band 4 that methionine gives an unacceptable minimal molecular weight value, which may be attributed to the loss of this amino acid during analysis (18, 19). Similarly, low tyrosine in band 2 can be attributed to loss during hydrolysis or to the presence of a small amount of impurity. In addition, since the amount of protein employed in the analysis of band 3 protein was small, the amino acid composition obtained must be considered as approximate. Nevertheless, the purity of the other proteins and thereby the reliability of their amino acid compositions were supported by determinations of their minimal molecular weights from the amino acid compositions. As noted in the next section, the values obtained were in good agreement with those obtained by gel filtration and ultracentrifugal analysis.

Molecular Weights.—The molecular weight of band 4 was determined by the analytical ultracentrifugation method and yielded a value of approximately 12,000. The molecular weight determination from amino acid composition was calculated to be 11,955. On the basis of this, it was anticipated and has been experimentally established that this protein is nondializable through Visking tubing (molecular exclusion of 6000).

The molecular weights of band 1, 2, and 3 proteins were found by gel filtration to be approximately 5000. Computations from the amino acid compositions of band 1 and 2 proteins gave molecular weights of 4826 and 5283, respectively, values in close agreement with the gel filtration results. The disparity in the molecular weight of band 2 protein and MCF of Seegers and Janoff (2) may possibly be owing to the different marker proteins used to calibrate the gel filtration columns, since other conditions were quite similar.

Biologic Activities of the Four Vasoactive Proteins of Neutrophilic Lysosomes

Each of the isolated cationic proteins induced an increase in vascular permeability, but only one, band 2 protein, appeared to increase vascular perme-

ability through the release of histamine. Band 2 protein induced the degranulation of rat mast cells in vitro, bringing about the release of histamine; and its permeability-inducing capacity in vivo was inhibited by pretreatment of rabbits with antihistamine. The source of histamine in rabbits was probably tissue mast cells or perhaps circulating basophils, since band 2 protein failed to release histamine from rabbit platelets, and since platelet depletion of rabbits failed to inhibit the permeability reaction. By contrast, the three other cationic proteins, bands 1, 3, and 4 proteins, did not release histamine from isolated mast cells in quantities up to 5–7-fold greater than the amount of band 2 protein that released 40% of the contained histamine. In addition, pretreatment of rabbits with antihistamine did not inhibit the permeability effect of these three proteins.

The closely similar physical properties of band 1, 2, and 3 proteins that have distinctly different biological properties may offer an important means of gaining an understanding of their mechanisms of action. Band 2 protein, which acts on mast cell membranes causing a release of granules and histamine, migrates electrophoretically between bands 1 and 3. While these latter proteins do not affect mast cell membranes, they, together with band 4 protein, do increase the permeability of vessels in vivo without the mediation of histamine. The differing biological activities of these physically similar proteins suggests that the basicity alone does not determine the characteristic biological activity. It therefore focuses attention on the biological functions of particular amino acid groupings within each of these closely related proteins. Attempts to identify such groupings are currently in progress. Preliminary studies on the mechanism of action of band 2 protein on mast cells suggest that certain amino acid groupings within the band 2 protein function as a specific activator. Contact between this protein and mast cells sets in motion a number of metabolic events within the cells that require ATP and certain serine esterase activities (20).

The chemical and biological heterogeneity of the most cationic protein components of neutrophil lysosomes is also apparent from the recent studies of Zeya and Spitznagel on the antibacterial activity of these proteins (21). Distinct variation in bactericidal specificity was noted among isolated components of the basic proteins.

These studies were initiated in great part to determine if basic protein permeability factors existed in neutrophils that could act independently of the action of histamine. Presumably, such proteins could play a significant role in renal glomerular and arterial injury where susceptible histamine reservoirs are absent. The results provide evidence that such factors indeed exist. It should be noted that band 4 protein, along with cathepsin E, has been identified immunologically in the urine during acute neutrophil-dependent immunologic glomerulonephritis (22). The permeability proteins now take their place along with cathepsins D and E, the histamine-releasing protein (band 2 or MCF),

slow-reacting substance (23), and the as yet unidentified constituents in neutrophils that reportedly release kinins from kininogens (24, 25), in the list of neutrophilic constituents known to be capable of injuring vascular structures. And of potential significance, recent studies have revealed a collagenase (26, 27) and an elastase (27) in human neutrophils that may also play a role in vascular injury.

SUMMARY

Four basic proteins that increase vascular permeability have been isolated in purified form from rabbit neutrophilic granules. These proteins are termed band 1, 2, 3, and 4 protein according to their electrophoretic migration in acrylamide gel. Molecular weights of band 1 and 2 protein derived from amino acid composition were 4800 and 5300, respectively. These values are in good agreement with those obtained for these proteins by gel diffusion techniques. The molecular weight of band 3 protein was also in the range of 5000 by the latter technique. The molecular weight of band 4 protein determined by ultracentrifugal analysis and amino acid composition was 12,000.

Although all four proteins had the capacity to induce immediate increase in vascular permeability, only band 2 protein was found to release histamine from isolated rat peritoneal mast cells. Furthermore, it has been shown that the permeability-inducing activity of band 2 protein can be inhibited by pretreating rabbits with antihistamine. Band 2 protein did not release histamine from rabbit platelets and depletion of rabbit platelets from the circulation had no influence on the permeability-inducing activity of this protein.

Band 1, 3, and 4 proteins did not release histamine from isolated rat peritoneal mast cells and their capacity to increase vascular permeability remained unaffected by treatment of rabbits with antihistamine.

These investigations suggest that the histamine-releasing activity of band 2 protein is a specific phenomenon and is associated with particular amino acid grouping or spacial configuration of the molecules. By the same token, the increase in vascular permeability induced by the nonhistamine-releasing band 1, 3, and 4 proteins represents a specific phenomenon (or phenomena) not particularly related to the over-all charge of these molecules.

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