phagocytic states in an effort to ascertain the mechanism of their altered immune responses. RE hyperfunction, induced by glucan, was associated with an enhanced vascular removal of the foreign red cells due exclusively to an increased liver uptake. Conversely, RE depression, induced by methyl palmitate administration, was associated with decreased vascular clearance due to a failure in phagocytosis by both liver and spleen. The present studies not only indicate that the previously observed alterations in hemolysin formation in mice with altered RE activity is associated with both a change in vascular clearance and organ distribution of the foreign red cell, but also demonstrate the ability to control the fate of colloids and particulate antigens by purified chemicals which influence the behavior of macrophages.

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## Use of Heparin in Distinguishing Plasma Kallikrein from Pf/dil. (30262)

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The plasma permeability globulins, kallikrein and Pf/dil, have recently been isolated from human serum and shown to be distinct from each other(1,2). Plasma kallikrein is a gamma globulin while Pf/dil is a beta globulin. The activity of both is inhibited by soybean trypsin inhibitor, but not by antihistamines. Both plasma kallikrein and Pf/dil are apparently members of the plasma kinin-forming system(3). Kallikrein releases kinin from both heated and unheated plasma by acting directly on kininogen. Pf/dil will release kinin only from unheated plasma apparently by activating plasma kallikrein or some other substance which then releases kinin.

The polyanion, heparin, is known to be a potent inhibitor of a number of different enzymes(4). Elder and Wilhelm demonstrated that heparin would inhibit the activation of Pf/dil in human serum(5). Ratnoff and Miles showed that heparin could inhibit the induction of permeability activity in diluted plasma by active Hageman factor although heparin did not inhibit the permeability activity of active Hageman factor itself(6).

The present paper deals with the ability of heparin in sufficiently high concentration to inhibit the permeability activity of both Pf/dil and kallikrein. Since about 6 times as much heparin is required to inhibit kallikrein as Pf/dil, one can determine the amount of each in an unknown solution from the per cent inhibition produced by a given amount of heparin. Using this observation, the nature of the permeability activity of diluted serum was investigated to determine the possible presence of kallikrein in addition to Pf/dil.

*Materials and methods. Sodium Heparin USP* obtained in the powdered form from Fischer Scientific Co., Fair Lawn, N. J., Lt no. 733700; 144,000 units per gram.

*Diphenhydramine hydrochloride USP* obtained in liquid form from Parke, Davis and Co., Detroit, Mich., 10 mg/cc.

*Soybean Trypsin Inhibitor* obtained in the powdered form from Worthington Biochemicals, Freehold, N. J.

*Assay of permeability activity.* The assay was carried out in 250-500 g blued Hartley guinea pigs as described by Miles and Wilhelm(7) with the following alterations. All animals were given 20 mg/kg of diphenhydramine intraperitoneally one to two hours before use. This procedure eliminated "hyperreactivity" in the guinea pigs—a problem encountered in Hartley animals we have obtained from both our sources (Nat. Inst. of Health and Fort Detrick, Md.). "Hyperreactive" refers to blued guinea pigs which were otherwise normal, but gave lesions over 4.0 mm in mean diameter when injected intradermally with 0.3 cc of 0.15 M NaCl. "Normal" animals gave lesions less than 4.0 mm in diameter. As would be expected, the pretreatment of the animals with antihistamine had no effect on the size lesion produced by the permeability factors studied or on their inhibition by heparin.

The volume of test solution injected intradermally in most of the studies was 0.2 cc. This volume was most convenient in studying serial dilutions of the permeability factors. In the studies on permeability activity of diluted serum, 0.3 cc volumes were injected as the activity was relatively weak. It was determined that the amount of inhibition of a given solution of kallikrein or Pf/dil by heparin was the same whether 0.2 cc or 0.3 cc injection volumes were used.

Lesions were measured 15 minutes after injection of the test solution. The size of a lesion was taken as the mean of the longest diameter and that perpendicular to it.

*Isolated Kallikrein and Pf/dil.* These partially purified proteins were prepared by DEAE cellulose chromatography of normal human serum or of Cohn Fraction I + III, 1, 2, 3 as described by Kagen *et al*(1).

*Permeability factor of diluted human serum.* This material was prepared as described by Elder and Wilhelm(5). Fresh blood was drawn and allowed to clot at room temperature for one hour. The clot was rimmed and the blood centrifuged at 4000 rpm at 0° for 20 minutes. The serum was drawn off and made up into a series of dilutions in 0.15 M NaCl from 1/1 to 1/2000. These dilutions were allowed to stand at 5°C for 42 hours, then incubated at 37° for one hour, and assayed for permeability activity. Maximum activity was located in 2 dilutions—that of 1/1 (lesion size—11.0 mm corresponding to Pf/Nat of Elder and Wilhelm(5)) and that of 1/1000 (lesion size—6.7 mm corresponding to Pf/dil of Elder and Wilhelm(5)). Minimum activity was located in the 1/100 dilution (lesion size—4.4 mm). The activity of the 1/1000 dilution was completely inhibited by soybean trypsin inhibitor 50 $\mu$g/ml. This dilution was used for further studies on the permeability activity of diluted serum.

Our findings differed somewhat from those of Elder and Wilhelm(5) in the dilution at which Pf/dil activity was maximal. However, since they found that the behaviour of individual sera was variable and since the activity of our diluted serum resembled theirs in other

respects we did not regard the difference as significant.

*Effect of heparin on isolated kallikrein and Pf/dil.* In the inhibition studies, solutions of permeability globulin in 0.15 M NaCl were made up to contain 20 effective blueing units per cc. One effective blueing unit was taken as the amount of permeability globulin in 0.2 cc of solution which would produce a lesion 6.0 mm in diameter. This unit differs from that of Miles and Wilhelm which was the amount of activity in 0.1 cc producing a lesion 6.0 mm in diameter. Serial 2-fold dilutions of these solutions in 0.15 M NaCl were made up with an equal concentration of heparin in each dilution. An identical control series was set up without heparin. The control series was then injected in random order in duplicate into one side of a guinea pig's back followed by the heparinized series in the same manner into the other side. The concentration of heparin used in different experiments was varied between 100 units and 2200 units per ml.

*Permeability activity of heparin solutions alone.* The concentrations of heparin used in these studies were admittedly high. Below 600 units/ml, solutions of heparin in 0.15 M NaCl produced no lesions in the blued guinea pig. Between 600 and 1800 units/ml occasional animals developed 9.0-10.0 mm blue lesions. Above 1800 units/ml 7 out of 15 animals developed these lesions. Inhibition studies were carried out in those animals which did not develop these lesions. The nature of the lesions was not clear. This effect cannot be attributed to a change in pH. The pH of the solutions studied varied from 7.72 at the lowest concentration of heparin to 7.40 at the highest. Control solutions of 0.15 M NaCl with 0.1 M sodium phosphate without heparin did not produce lesions greater than 4.0 mm in diameter in this pH range.

*Effect of heparin on mixtures of kallikrein and Pf/dil.* Solutions of kallikrein and Pf/dil of equal potency (20 effective blueing units per cc) were prepared. Varying volumes of these solutions were mixed to give final solutions with equal total permeability potency, but with varying percentages of permeability activity due to kallikrein and Pf/dil. The effect of heparin 500 units/ml on these mixtures was then determined in the same manner as that used with the isolated permeability globulin solutions.

*Effect of heparin on permeability activity of diluted serum.* For this study serum was diluted 1/1000 and allowed to develop full permeability activity as described above. The activated solution was then placed in 2 test tubes. One was kept as a control and heparin was added to the other to give a final concentration of 500 units/ml. Since the solutions contained only slightly more than 5 effective blueing units per cc, serial dilutions of the control and heparinized samples could not be performed and, as noted below, per cent inhibition could only be estimated.

*Calculation of per cent inhibition.* The per cent inhibition of a solution of permeability globulin by a given amount of heparin was determined as follows. On the same graph, the logarithm of the concentration of permeability globulin in the serial dilutions was plotted against the diameter of the lesion produced by these dilutions with and without heparin. Since the actual concentration of permeability globulin was unknown, the highest was taken as 100%, the next 50%, etc. From these plots, 2 parallel straight lines resulted when inhibition occurred. If no inhibition occurred, a single straight line resulted. Per cent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = \frac{I - C}{I} \times 100$$

I and C represent the antilogs of the log concentrations of permeability globulin in the inhibited and control solutions producing the same size lesions as determined from the graph.

Per cent inhibition of a given solution by a given amount of heparin was taken as the average inhibition value obtained from a minimum of 3 different animals. The standard deviation of any given measurement of per cent inhibition was $\pm$ 10%.

*Results. Effect of heparin on isolated kallikrein and Pf/dil.* A graph of per cent inhibition of the activated enzymes kallikrein and Pf/dil given by varying concentrations of

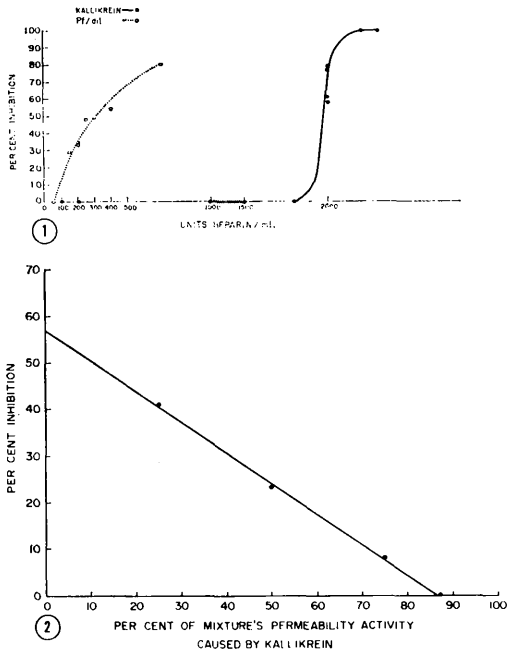


FIG. 1. Heparin inhibition of plasma kallikrein and Pf/dil.

FIG. 2. % inhibition of kallikrein-Pf/dil mixtures by heparin 500 units per ml *vs* % kallikrein in mixtures.

heparin is shown in Fig. 1. Fifty per cent inhibition of Pf/dil occurred at 320 units/ml of heparin. Fifty per cent inhibition of kallikrein occurred at 1950 units/ml of heparin.

*Effect of heparin on mixtures of kallikrein and Pf/dil.* The effect of heparin 500 units/ml on mixtures of kallikrein and Pf/dil is shown in Fig. 2. As can be seen, there was a straight line relationship between per cent kallikrein in the mixture and per cent inhibition obtained. The value for per cent inhibition of 100% Pf/dil by 500 units/ml of heparin obtained from Fig. 2 (56%) agrees within experimental error with that obtained from Fig. 1 (67%).

*Effect of heparin on permeability activity of diluted serum.* The average lesions obtained from 6 animals with the control solution had a mean diameter of 6.4 mm; the average lesions for the heparinized solution (500 units/ml) had a mean diameter of 3.3 mm. From a standard dilution curve for Pf/dil, this degree of inhibition would equal at least 57%. The exact amount of inhibition which occurred is impossible to ascertain as the size of the inhibited lesion is not significantly larger than that produced by 0.15 M NaCl (2.0-4.0 mm).

*Discussion.* The above studies demonstrate the ability of heparin to inhibit both isolated kallikrein and Pf/dil. About 6 times as much heparin is required to inhibit kallikrein as Pf/dil. Previously Elder and Wilhelm were unable to demonstrate inhibition of Pf/dil by heparin(5). However, the concentrations of heparin used were apparently lower than those used in the present experiments.

The exact mechanism of inhibition is not clear. No protein precipitate was observed in any of our experiments using heparin as an inhibitor. Thus, the inhibition effect cannot be ascribed to a precipitation of the permeability globulins although precipitation of certain plasma proteins by high concentrations of heparin is a recognized phenomenon(8).

As demonstrated in Fig. 2, if all of a solution's permeability activity can be inhibited by soybean trypsin inhibitor, the differential effect of heparin on kallikrein and Pf/dil can be used to determine the amount of each in the solution. This method was used to reinvestigate the nature of the permeability activity of diluted serum. Since both kallikrein and Pf/dil can be isolated from human serum, it was thought possible that kallikrein in addition to Pf/dil was contributing to the permeability activity of diluted serum. However, the fact that heparin (500 units/ml) produces at least a 57% inhibition of this activity indicates that Pf/dil is the sole permeability factor present at the dilution tested.

This finding raises the question of what happens to kallikrein under these conditions. At present one can only speculate. Among the possibilities are that kallikrein is diluted out, that it becomes inactive, or that its inhibitors cannot be diluted out as can those of Pf/dil.

*Summary.* The ability of heparin to inhibit both kallikrein and Pf/dil was demonstrated. Since about 6 times as much heparin was required to inhibit kallikrein as Pf/dil, it was possible to determine the amount of each in an unknown solution from the per cent inhibition produced by a given amount

of heparin. Using this method, the nature of the permeability activity of diluted serum was investigated and found to be caused solely by Pf/dil.

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## Histoimmunological Study of Collagen During Tendon Fibrillogenesis. (30263)

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Although there is general agreement that the tropocollagen molecules originate in the fibroblast, the morphological evidence for intra- or extracellular formation of collagen fibers at the optical and cellular electron microscopic levels and the organization of the fibers is still controversial(1,2). Connective tissue extracts(3), an acetic acid extract of collagen(4), and also of soluble and insoluble fractions(5) are antigenic. These soluble and insoluble fractions in the collagen fibers of adult connective tissue have been visualized by immunofluorescence(6). Since, according to chemical studies of skin and granulomas, neutral salt soluble collagen has the properties of a precursor of both acid and insoluble collagen(7), a preliminary attempt is being reported to localize the soluble and insoluble fractions during collagen formation in the normal, the developing, and the injured tendon.

*Material and methods.* The purified neutral salt soluble and the insoluble fractions of collagen extracted from leg tendons of adult chickens were used as antigens. The preparation of these fractions, the schedule of the heterologous sensitization of adult rabbits, the periodic bleeding for gamma globulin determination, and the serological tests of the titers and the specificity of corresponding antibodies were previously reported(5) as were details concerning the separation of gamma globulin fractions, the conjugation with fluorescein isothiocyanate and its further purification(6). Subcutaneous areas of growing tendon adjacent to the knee joint of 8-10- and 14-16-day-old chicken embryos and of newborn, young (20 days), and adult chicken (3 months) were studied for development of normal tendon connective tissue. To examine the induced granulation tissue in adult chickens, the tendon of the knee joint was cut transversely under aseptic conditions, the skin was closed and the leg immobilized. The newly formed tissue was obtained from each animal at 4, 8, 14, 20 and 35 days. Tissue blocks from the growing tendinous areas were rapidly frozen and cut at 4 to 8 $\mu$ in the cryostat at $-20°C$, whereas other small pieces fixed in 4% formol or Carnoy fluid were stained with H & E, silver impregnation (Gridley method), toluidine blue (pH 5), and periodic acid Schiff (PAS) techniques for mucopolysaccharides and collagen fibers. Direct or indirect Coon's methods were applied to the unfixed or 95% alcohol or acetone treated frozen sections, using the labeled globulin fraction of the immune sera or the intact immune sera, followed by the fluoresceinated antirabbit globulin antiserum prepared in sheep. As control reactions in both

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