of 6.80–7.0. We used the following ninhydrin solution with good results: A fresh 1% ninhydrin solution in water is mixed with an equal volume of a sodium veronal–HCl buffer of pH 7.0. This pH is obtained by mixing 5.36 volumes of 0.1 *M* sodium veronal with 4.64 volumes of 0.1 *N* HCl.

Table 1 illustrates the results we obtained with pure amino acids.

The sensitivity of the ninhydrin reaction is different depending on the amino acid (*11, 12*), histidine being the least sensitive. The lower quantities of amino acid mentioned in the table give values greater than expected; the reaction is quantitative only above these lower limits. For glycine the Lambert-Beer law is followed only above, and including, 5 γ; for alanine, valine, glutamic acid, and phenylalanine the lower quantitative limit (inclusive) is 10 γ, whereas for histidine this limit is 25 γ. Fig. 1 illustrates this, showing that for the quantities employed, optical density is directly proportional to concentration.

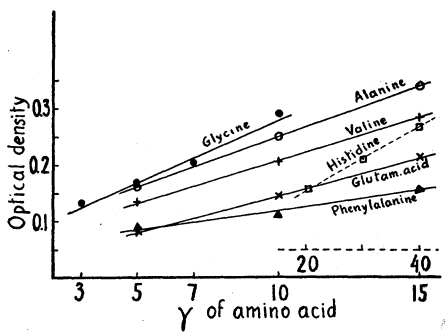

FIG. 1.

In order to determine an amount of amino acid below the lower quantitative limit, a known quantity of amino acid is added to the paper spot with a fine capillary pipette (e.g., 0.005 ml and 10 γ of amino acid), and afterwards the reaction is performed as usual, the piece of paper with the spot having previously been dried. For the quantities of histidine normally found in proteins it is generally necessary to add a small amount of this compound to the respective spot in paper chromatographies corresponding to about 200–400 γ protein, to obtain a quantitative color reaction.

We hope that the procedure will prove to be useful also in the hands of other workers. The reproducibility of the method lies in general within an error of 3–5%, and duplicates usually give errors inferior to this.

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Evidence for the Heparin Nature of the Nonspecific Hyaluronidase Inhibitor in Tissue Extracts and Blood Serum[1]

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The possible role of the nonspecific (nonantibody) hyaluronidase inhibitor of blood serum in the animal body, and its remarkable changes in concentration in infectious diseases, cancer, liver and kidney damage, and many conditions of stress (*1*), led to an earlier study (*2*) of its anatomical origin. A wide variety of tissues from the rabbit was investigated as possible sources of the serum inhibitor, but it could be demonstrated in none of the tissue extracts obtained. This might be explained by the very low concentration of inhibitor in these tissues. It was further found that certain steroids and hemoglobin derivatives were inhibitors *in vitro*, but none of these could be the serum factor in question since the latter is heat-labile and its effect is decreased in the presence of phosphate. It is well known that isolated polysaccharides of the heparin group are potent inhibitors (*3–9*), and, as our work developed, evidence accumulated suggesting that the native heparin-protein complex (*10*) probably present in low concentrations in normal serum (*11*) might exert a hyaluronidase inhibition. It should be mentioned that certain mucoproteins of human serum prepared by the perchloric acid method of Winzler *et al.* (*12*) were devoid both of anticoagulant (*13*) and hyaluronidase inhibitor activities (*14*).

The first suggestion as to the chemical nature of the nonspecific hyaluronidase inhibitor was provided by the finding that most of the serum inhibitor migrated with the albumin in the electrophoretic field (*15*), a finding later confirmed (*16*). It was previously known that purified commercial heparin added to plasma migrated with an electrophoretic mobility intermediate between that of heparin and albumin (*17*). This seems also to be the case for one of the components obtained by electrophoresis of the native heparin complex obtained from ox liver capsule extracts (*18*).

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From recent work on the chemical purification of the inhibitor it was observed that protamine,[2] which is known to precipitate the polysaccharide part of the native labile heparin complex from serum (*18*), also precipitates the inhibitor. Details of the use of protamine in the inhibitor purification will be given elsewhere (*19*). It was also found that peptone shock in rabbits caused not only the usual rise in the concentration of heparin in the blood serum, but also an elevation of the inhibitor concentration (*20*). If the heparin increase was blocked by intravenous injection of India ink prior to peptone shock, following the method of Volkert (*21*), no rise in inhibitor concentration was noted (*20*).

TABLE 1

PERCENTAGE INHIBITION* OF TESTIS HYALURONIDASE AND HEPARIN CONCENTRATION OF TISSUES RICH IN MAST CELLS

	Liver capsule (cow), %	Endometrial caruncular† (heifer), %	Swim bladder‡ (whitefish), %
(*A*) Inhibition by 1.5 ml tissue extract§	4	14	20
(*B*) Inhibition by 0.04 ml human serum	11	19	25
(*A*) + (*B*)	26	49	53
Heparin in extract (mg %)	1.5	4.6	5.1

* Inhibition determined by the method previously used (*2*).
† Obtained through the courtesy of A. F. Weber, Department of Veterinary Medicine, University of Minnesota.
‡ Obtained through the courtesy of A. J. Walstad, Booth Fisheries Corp., Bayfield, Wis.
§ Extract prepared by grinding tissue in a Wiley mill, extracting pulp with 0.9% NaCl (5 ml/g tissue) for 2 hr at 8° C, centrifuging, and filtering supernatant through a sintered glass funnel.

Since mast cells contain a heparin-lipoprotein complex provisionally considered to be associated with the microsomal fraction of their cytoplasm (*18*), tissues particularly rich in these cells were investigated as possible sources of inhibitor. These proved to be the only exceptions observed to the previously reported absence of inhibitor in a variety of tissues (*2*). Thus, in Table 1, it may be seen that the three tissues chosen for their high mast-cell content all yielded extracts containing inhibitor, and when these extracts were combined with human serum, the mixtures had more than the additive amount of inhibitor. The basis of this mutual enhancing effect is not known. The colorimetric Azure A method (*11*) employed for the heparin analysis is far from precise, but it is capable of a certain degree of semiquantitation. It will be noted in the table that the order of inhibition and approximate heparin content of the tissue extracts were parallel.

It should be pointed out that radiation sickness, which is accompanied by a hemorrhagic condition, has been shown to be associated with an increase of serum heparin (*22–24*). It has been found recently that total-body x-irradiation of rabbits resulted in an elevation of the serum inhibitor concentration (*25*).

Finally, in connection with other investigations by B. Sylvén, O. Snellman, and co-workers, samples were repeatedly prepared by means of differential centrifugation of fresh ox liver capsules. These contained various amounts of the native heparin-lipoprotein complex which possesses very high metachromatic and anticoagulant activities. The electrophoretic mobility of the active fraction was 5.9×10^{-5} u (*18*). In these separations acetate and veronal buffer media were employed, and the samples were shipped from Stockholm to Minneapolis by air in the frozen state in thermos bottles packed with solid CO_2. On arrival the material was still frozen. The amounts of heparin present in the different samples had been assayed by the thrombin method of Jaques and Charles (*26*). After thawing, the liquids were assayed for inhibitor activity. In three different preparations, 33% inhibition was given by 1.5 ml of each of the solutions, containing the equivalent of 45, 60, and 40 μg commercial heparin (Vitrum, Sweden), respectively.

The evidence presented shows that the native heparin-lipoprotein compound obtained from tissues rich in mast cells is a hyaluronidase inhibitor. It is possible that this or part of this heparin compound is responsible for the nonspecific hyaluronidase inhibition given by serum. Final proof will depend on the isolation of this serum factor and its comparison with the isolated tissue heparin complex.

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