group followed by the lung cancer group with 48%. The extent of the cancerous growth did not appear to influence the incidence of positive results since 27 (32%) of the 85 patients with localized cancers had positive results, 43 (49%) of the 88 with regional spread had positive results and 26 (38%) of the 68 with distant metastases, had positive results.

Deletion from the data of non-cancerous disease patients with allergy, arthritis, acute tuberculosis or syphilis, temperature over 99.6°F and those with trauma, surgery or blood transfusion in the 10 days prior to the test, decreased the incidence of positive results in both groups by approximately the same extent, *i.e.*, 6%, thereby throwing additional doubt on the cancer specificity of the reaction.

*Conclusion.* (1) The Penn-Hall serofloccculation test was not found to be a satisfactory cancer selective procedure. Although it was seldom positive in normal individuals (5% of 129), it was positive in only 43% (96 of 241) of cancerous patients. Incidence of false positive results in 1608 non-cancerous patients was 19%. If patients with allergy, arthritis, acute tuberculosis or syphilis, temperature over 99.6°F and those with trauma, surgery or blood transfusion in prior 10 days were omitted as Penn and Hall suggested, the false positive results and the correct positive results were equally reduced by about 6%. (2) Positive results were more frequent among patients who had elevated blood sedimentation rates or who had inflammatory disease processes. As we evaluated, it is highly improbable that the reaction is cancer specific, a finding agreeing with the results of other investigators(3,4,5,6).

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## Artifacts in Mast Cell Metachromasia Produced by Hyaluronidase Preparations.* (23795)

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In the course of recent experiments it was observed that, under specific conditions of staining with toluidine blue, mast cells of the hamster were rendered non-metachromatic by previous treatment with commercially prepared bovine testicular hyaluronidase. This finding is in direct contrast to that of other workers(3.5.15,17,18). Through a series of investigations it was possible to show that the observed effect was not the result of hyaluronidase activity but was instead an artifact.

*Procedure.* The tissue of the hamster studied most extensively was the cheek pouch, both in its normal state and when undergoing hyperplasia and neoplasia in response to applications of 9, 10-dimethyl-1,2-benzanthracene. Observations were also made on skin, mesentery, tongue and uterus. Tissues were fixed in Bouin's fluid, absolute ethyl alcohol and formalin, after which paraffin sections were prepared. Serial sections of each tissue were incubated 12 hours in hyaluronidase so-

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lutions§ containing 25 T.R.U./ml prepared both in distilled water and 0.12 M sodium chloride while the adjacent control section was exposed to same temperature and same solution without hyaluronidase. These tissues, along with a freshly hydrated section, were stained after the method of Moore and Schoenberg(14) for 20 minutes in 1 x $10^{-4}$ M solution of recrystallized toluidine blue, prepared in acetate and phosphate buffers of ionic strength 0.0025. Buffers were prepared for each pH unit from 2 to 7 inclusive according to the method of Miller and Golder(13). Tissue sections were equilibrated in buffer alone for 2 hours prior to staining. After staining, sections were washed in buffer and read while wet. Hyaluronidase solutions inactivated by heat were also used and as a check on effectiveness of heat as an enzyme inactivator, turbidimetric assay for hyaluronidase activity was carried out according to the method of Tolksdorf *et al.*(16). After incubation in sodium chloride solution of the enzyme, tissues were washed in sodium chloride solution followed by distilled water. Tissue sections exposed to aqueous solution of hyaluronidase were routinely washed in distilled water alone. On one occasion the latter were washed in 0.12 M sodium chloride and on another they were exposed 30 minutes to 3 changes of phosphate buffer solution at pH of 7.5 and ionic strength of 0.5.

*Observations.* Mast cells stained metachromatically at all pH's used. At pH 2 and 3, pretreatment of tissues with hyaluronidase in distilled water abolished all staining of mast cells irrespective of method of fixation.

At pH 4, the mast cells were metachromatic in hyaluronidase treated tissues but appeared distinctly lighter than control slides stained at same pH. There appeared to be no effect of exposure of tissues to water and elevated temperature alone. At pH 5, 6 and 7, exposure to a hyaluronidase solution had no effect upon metachromatic staining properties of the mast cells.

§ A comparison was made of the activity of 3 commercially available hyaluronidase preparations obtained from Nutritional Biochemicals Corp., Cleveland, O., Worthington Biochemical Corp., Freehold, N. J., Wyeth Laboratories, Philadelphia, Pa.

In contrast to above findings, tissues incubated in hyaluronidase solution prepared in 0.12 M sodium chloride and washed in sodium chloride after incubation, exhibited metachromatic staining of mast cells at pH 2 and 3. However, tissues incubated in distilled water preparation of hyaluronidase and then washed in 0.12 M sodium chloride revealed no staining of mast cells at these low pH values. If instead of 0.12 M sodium chloride, tissues were washed in phosphate buffer at pH 7.5 and ionic strength of 0.5, the mast cells stained metachromatic upon subsequent exposure to toluidine blue at pH 2 and 3.

Heating the aqueous enzyme solution 20 minutes in 60°C water bath did not alter its ability to prevent metachromasia of mast cells at pH 2 and 3 even though turbidimetric assay of the heated solution revealed an absence of hyaluronidase activity. However, when sodium chloride was added to a heated aqueous solution of hyaluronidase the mast cells exhibited metachromatic staining at pH 2 and 3.

The aqueous hyaluronidase solution which was subjected to a boiling water bath for 60 minutes became slightly opalescent. The mast cells of tissues incubated in this solution did stain when exposed to toluidine blue at pH 3 but staining was not as intense as that exhibited by mast cells subjected to sodium chloride hyaluronidase preparation.

*Discussion.* The experiments demonstrate that hyaluronidase activity *per se* was not responsible for loss of metachromatic staining of mast cells when exposure to heated aqueous enzyme preparations prevented all staining at pH 2 and 3 even though turbidimetric assay of the solutions revealed no active enzyme to be present. The fact that sodium chloride at the concentration used does not inhibit but rather enhances the activity of hyaluronidase (11) lends further support to the conclusion that loss of metachromatic staining of mast cells is not due to hyaluronidase activity *per se*. It appeared that some factor other than hyaluronidase activity, was responsible for the observed effect on mast cell staining.

Chauncey *et al.*(2) and Davidson and coworkers(4) have demonstrated numerous enzymes present as impurities in testicular hyal-

uronidase preparations. The possibility of some unknown "mucopolysaccharidase" in the preparations seems unlikely since sodium chloride was capable of abolishing the effect of the aqueous solution. Thus evidence points to the fact that it is some activity other than an enzymatic one which is responsible for rendering mast cells unstainable at low pH's.

It is known that anionic polyelectrolytes can combine with proteins at pH's sufficiently low to charge basic protein groups. If an anionic polyelectrolyte is metachromatic, such a combination may prevent its metachromatic staining. In fact, French and Benditt(6) and Kelly(9) have shown *in vitro* that basic proteins can suppress metachromasia and they do this more effectively at lower pH values. It is suggested that a masking of mast cell mucopolysaccharides by proteins present in the hyaluronidase preparations has occurred in the test system discussed here, creating an artifact which resembles enzymatic hydrolysis of the mucopolysaccharides. It is possible that hyaluronidase itself may be one of the masking proteins. There is evidence indicating that heparin is a competitive inhibitor of hyaluronidase(1,8,12). It forms with the enzyme salt-like bonds, which can be broken by increasing the ionic strength of the solvent (8). Since mast cells are thought to contain heparin(7,10), it may be postulated that the enzyme and heparin of mast cells formed a complex which prevented metachromatic staining of these cells. The pH dependency of the artifact and its failure to occur when a salt solution of hyaluronidase was used are consistent with this view. It is also possible that the masking effect may be due to basic nonprotein substances present in the hyaluronidase solution. The observation that mast cells could be stained at pH 3 if they were exposed for 30 minutes to a phosphate buffer solution at pH 7.5 and ionic strength of 0.5 after treatment with aqueous hyaluronidase, further suggests that some substances were removed by the relatively strong buffer and relatively high pH.

In the present day histochemical use of hyaluronidase preparations in particular and enzyme preparations in general, the observations presented here emphasize the need for cautious interpretation. The practice of running inactivated enzyme control sections is to be encouraged. Hyaluronidase prepared in salt solution is preferred over distilled water solutions of the enzyme.

*Summary.* 1) Commercially available hyaluronidase preparations appear to contain substances which are capable of preventing metachromatic staining of mast cells at pH 2 and 3 thus simulating enzymatic hydrolysis of stainable mucopolysaccharides. 2) Evidence suggests that staining is prevented by substances, perhaps proteins, which form a complex with mast cell mucopolysaccharides and prevent staining. It is possible that hyaluronidase itself may be one of the masking proteins. 3) The value of an inactivated enzyme control and use of salt solutions of hyaluronidase in histochemical studies is emphasized.

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