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The Effect of Hyaluronidase on Some Physiological and Pathological Processes

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There is considerable evidence that hyaluronidases secreted by pathogenic bacteria may initiate some of the pathological changes during the course of infection by attacking hyaluronate-containing tissues. Evidence for a physiological or pathological role of endogenous hyaluronidase, however, is lacking since there is no reliable assay or isolation procedure for detecting the small amounts of hyaluronidase capable of profoundly altering the ground substance. Nor has it been possible to isolate with certainty from mammalian tissue other than the testicle significant amounts of hyaluronidase. Nevertheless, many experimental pathologists assume that the ground substance is regulated by hyaluronidases and that the collagen group of diseases may result from excess activity of the enzyme. In this paper no effort is made to review the literature on hyaluronidase or to reconcile the contradictions in it. The experimental results may be critically influenced by the quality of enzyme used. We had available for our investigations highly purified hyaluronidase free from irritants and histamine-like substances. It had low antigenicity and was practically non-toxic. In most species it had no cardiovascular, capillary or renal effects when administered in large single doses.

We have used hyaluronidase and steroids to study physiological and pathological changes in the ground substance. From experiments on membranes in vitro (1) it was assumed that the hyaluronate in the ground substance is regulated by a buffer consisting of hyaluronidase, stimulatory steroids and inhibitory steroids. In these experiments hyaluronidase and desoxycorticosterone increased permeability and cortisone decreased it even in the presence of hyaluronidase or DCA. Cortisone appeared to render the substrate incapable of attack by hyaluronidase and had no direct effect on the enzyme. The action of steroids therefore

appears to be locally on the ground substance. This has recently been confirmed by *Hollander* who instilled compound F directly into the joints of arthritics and obtained the usual alleviation (2).

Effects of hyaluronidase and steroids on permeability can also be demonstrated by other methods, but none of these is suited for quantitative assay. The method of *Curtis* and *Brunschwig* (3) for studying the permeability of human joints and that of *Tani* (4) and *Shinkawa* (5) for studying the permeability of joints in rabbits was adapted for measuring quantitatively the effect of various agents on synovial permeability (6). A measured amount of a standard solution of phenolsulfonphthalein was instilled into the talocrural articulation. A retention catheter inserted into the urinary bladder was flushed at intervals of 5 to 10 minutes and the washings analyzed for PSP content.

The permeability of synovial membrane as measured by this method is influenced by numerous factors. Improper injection and passage of the catheter into the seminal vesicles are frequent sources of error. Among the other factors influencing permeability are: massage of the injected joint, application of pressure above it, application of heat, cold or irritants, systemic administration of vasodilators or vasoconstrictors, and intra-articular injection of cations capable of reaction with hyaluronate. In more than 5000 control tests performed on 1658 rabbits, the standard deviation was $\pm$ 4.86% and the standard error $\pm$1.93%. In measuring the effects of cortisone the standard deviation was $\pm$ 1.21% and the standard error $\pm$1.08%. Similar results were obtained with ACTH and most of the steroids tested (7). According to these statistical data, the permeability effect of a steroid can be determined accurately on 2 to 3 rabbits provided that the steroid affects the ground substance directly and not through the adrenals or pituitary.

In a typical standardization experiment, color appears first in the urine in the 15 minute sample and reaches a maximum concentration in 30 minutes and then rapidly falls off (table below). Approximately 90% of the injected dye can be accounted for during the 50 to 60 minutes of active excretion. Injection of 150 turbidity reducing units of

Effect of various agents on permeability of rabbit synovial membranes

Agent	Onset (min.)	Peak (min.)	Duration (min.)	% PSP excreted
Control	15	30	40–60	90
Hyaluronidase	10	10	20	75
DCA	10	10	20	75
Cortisone	25	90	280	30
ACTH	30	120	300	10

hyaluronidase simultaneously with PSP results in a threefold increase in the rate of excretion of dye. The steroids tested were dissolved in either oil or an organic amide and injected intramuscularly at a dose of 1 mg/kg 30 minutes before PSP was injected into the joint. In such experiments the effect of desoxycorticosterone was almost identical with that of hyaluronidase (table 1). Alarm reaction, ACTH and cortisone, on the other hand, markedly suppressed permeability of the synovial membrane, even in the presence of DCA or hyaluronidase. In order to overcome the effects of 1 mg of cortisone it was necessary to administer 5 mg of DCA. The suppression of PSP excretion by alarm reaction, ACTH and cortisone is not a renal effect, since most of the dye that was not excreted through the kidney could be recovered from the synovial cavity. Furthermore, cortisone-treated rabbits had no impairment of renal clearance of intravenously injected PSP, and bilateral nephrectomy in normal rabbits had no effect on clearance of PSP across the synovial membrane.

The marked antipermeability action of cortisone was assigned a value of 100% and the activities of other steroids were rated according to this. Since the inhibitory effect of cortisone is nearly complete, activities apparently greater have no statistical significance. Of more than 100 steroids examined by this method, only 12 had antipermeability action equal to that of cortisone by direct effect on the synovial membrane. The most important of these are Δ^5-pregnene-3(β),21-diol-20-one, allopregnane-3(β),21-diol-20-one, allopregnane-3(β),17(α), 21-triol-20-one, allopregnane-21-ol-3,20-dione, Δ^4-pregnene-17(α)-ol-3,20-dione, pregnane-3(β),17(α)-diol-20-one, pregnane-3(β)-ol-20-one. All but two are saturated pregnanes and the majority are allopregnanes. Some of these compounds may be considered structurally related to desoxycorticosterone. It is noteworthy that most steroids had some degree of antipermeability activity but that only desoxycorticosterone and possibly Reichstein's compound S increased permeability. According to the ground substance buffer postulated above, permeability could be suppressed by increasing cortisone and antihyaluronidase activity and by compounds that could act as competitive inhibitors of DCA. A position isomer of desoxycorticosterone was found to possess marked antipermeability action.

The acute experiments do not indicate the changes in the ground substance that could result from prolonged administration of hyaluronidase or steroids. A single injection of hyaluronidase or DCA produces sufficient depolymerization and edema of the ground substance to simulate one phase of initial injury of collagen disease. According to *Ehrich* (8), mucoid degeneration is characterized by accumulation in the

connective tissue of acid mucopolysaccharide, notably hyaluronic acid, in depolymerized form. It was of interest to see whether continuous depolymerization would result in collagen disease and whether antipermeability steroids could alleviate the pathological changes. Daily injection of 100 TRU of hyaluronidase for 8 consecutive days into the knee joint of rats resulted in a mild to marked proliferative response of fibroblasts and endothelial cells of the synovia (9). This was associated with mild leukocytic infiltration and exudation of fibrin into the capsular space. Some rats also developed a few focal granulomas consisting of undifferentiated mesenchymal cells. This was associated with or preceded by fibrinoid degeneration. Both cortisone and 21-acetoxypregnenolone had a beneficial effect on this type of arthritis, cortisone being far more effective, particularly in suppressing fibrinous exudation into the synovial cavity.

Hyaluronidase also produces marked changes in the ground substance of embryonic bone in tissue culture (10). Repeated immersion of early embryonic femurs for relatively short periods in hyaluronidase solution resulted in dwarfing and decreased density of the epiphysis and shaft. There was no retardation in the onset of bone formation, although the effect of hyaluronidase on the cartilage appeared to simulate the natural process of systemic erosion. Apart from the dwarfing effect, hyaluronidase markedly altered the compact zone of the matrix in the future synovial region. Normally this zone consists of a compact band of cells with intensely basophilic staining interstitial ground substance. In hyaluronidase-treated femurs the band was wider due to the greater interstitial spaces, and stained weakly basophilic. The interstitial ground substance was thinned and presented the edema or mucoid degeneration phase of the initial lesion of collagen disease.

Hyaluronidase and steroids can also be used to demonstrate the important role of the ground substance in the development of experimental sensitivity lesions. The Arthus reaction in the skin, and the allergic arteritis and valvulitis caused by horse serum in rabbits can be depressed or prevented by stimulation of the adrenals (11). The beneficial effects may be due to either suppression of the edema by the antipermeability effects of some of the steroids or they may be due to depression of antibody formation by lymphocytolytic action. It is possible to evaluate the roles of permeability and immune reactions on the pathological changes in experimental serum disease by comparing the effects of hyaluronidase, a steroid which has high antipermeability action but no lymphocytolytic action, and cortisone which is both antipermeable and lymphocytolytic.

Hyaluronidase administered twice daily intravenously in doses of 6000 TRU/kg depressed the Arthus-reaction in the skin elicited by intradermal injection of horse serum in sensitized rabbits, probably by more rapidly dispersing the injected antigen (12). Cortisone also depressed the Arthus reaction, probably by depressing the antigen-antibody effects since equi-antipermeability doses of 21-acetoxypregnenolone had no effect. By increasing the permeability of the ground substance of the heart and arteries for antigen and antibody, hyaluronidase caused a greater frequency and severity of carditis and arteritis. There was less glomerulonephritis due to the lessened load on the glomeruli. When a high antihyaluronidase titer appeared in the blood, on the seventh to tenth day, the enzyme had no further effect on the development of lesions. Cortisone, on the other hand, prevented practically all vascular changes and carditis but not glomerulonephritis, probably due to the lymphocytolysis which depressed formation of antibodies. Antipermeability action appears to have played an insignificant role since equi-antipermeability doses of 21-acetoxypregnenolone were without effect. The effect of larger doses of the latter steroid was not investigated.

Sections of the kidneys taken from the hyaluronidase-treated rabbits had unusual changes in the ground substance when stained by the Ritter-Oleson technique (13). In normal rabbits the mucopolysaccharides of the interstitial ground substance stains blue. The basement membrane is sharply defined and stains red. The red stain is due to active carbonyl groups present in altered ground substance. The kidneys from rabbits treated with large doses of hyaluronidase had pink staining interstitial ground substance probably due to the depolymerizing action of hyaluronidase. This alteration in chemistry of the ground substance may be considered equivalent to the transformations occurring in mucoid degeneration. The basement membrane retained the red color but appeared to be thicker. It is of interest that a normal human kidney of a young man resembled that of the normal rabbit, but contained more blue staining ground substance. A section of a kidney taken from an aged woman resembled those of hyaluronidase-treated rabbits. It would appear that the aging process is accompanied by a decreased capacity for polymerization of new ground substance and is analogous to depolymerization by hyaluronidase.

As a result of permeability studies with hyaluronidase the ground substance is considered to be a mechanical barrier to spreading. No thought has been given to the possibility that the depolymerized hyaluronate released by the enzyme could actively enter into the dis-

persion. Recently *Butt* (14) has demonstrated that hypodermic injection of hyaluronidase is followed by the appearance of protective colloids in the urine. He has successfully applied this observation for the treatment of renal calculi in patients. Urinary sediment of people not susceptible to kidney stones when examined ultramicroscopically is free from crystalline material but has numerous amorphous particles covered with a jelly-like substance. Sediment of the urine from patients with kidney stones shows an abundance of crystalline material and an absence of jelly-like substance. Injection of hyaluronidase promptly alters the pathologic urine so that it resembles the normal. The addition of hyaluronidase directly to the urine does not produce such changes and therefore it is suggested that the injection of the enzyme releases the protective colloid from the tissues (15). The observation that potassium hyaluronate acts as a powerful peptizing and dispersing agent when added to pathologic urine suggests that the effect of hyaluronidase is due to the release of hyaluronic acid (16).

From the preceding it is conceivable that calculous disease may result from inhibition of mucopolysaccharides of the ground substance and is therefore another manifestation of dysfunction of the collagen system. Some years ago *Ehrich* and *Seifter* (17) assigned to the alarm reaction a role in the causation of post-operative parotitis and salivary stone formation. More definite evidence for the suppression of the peptizing activity of hyaluronate by stress has been obtained in humans by *Butt*, *Beischer* and *Seifter* (18). The urine of subjects under stress and those receiving ACTH and cortisone sediments rapidly and appears to be devoid of protective colloidal activity.

Some collagen diseases undergo remission during pregnancy and it is assumed that this benefit is brought about by the increased production of steroids. We have considered the possibility that the remission may be brought about by the increased production of hyaluronate that occurs during pregnancy. It was of interest, therefore, to investigate whether hyaluronic acid administered by intravenous injection enters into the ground substance or whether it is treated as foreign material by the reticuloendothelial system. Purified potassium hyaluronate prepared from bovine vitreous humor was injected into rabbits three times daily for 14 consecutive days. There was no evidence that it was disposed of as foreign material. On the contrary, the kidneys had increased amounts of blue staining ground substance, indicating that the hyaluronate had been incorporated into it (19). It would be of interest to determine whether hyaluronate thus assimilated participates in the function of the ground substance or whether it interferes with the hyaluronate normally present.

The answer to this question might explain some of the pathology that occurs during infection with hyaluronate-producing bacteria.

Summary

1. From a pharmacological point of view the ground substance is a labile material highly sensitive to the action of hyaluronidase and adrenal steroids.

2. The action of hyaluronidase and steroids following injection is directly on the ground substance and the same effect may be obtained by local application.

3. Some bacteria may alter the function of the ground substance by the hyaluronidase which they secrete, and others may possibly inhibit it functionally by the production of atypical hyaluronates.

4. The ground substance is not an inert barrier but actively participates in the reactions which occur in it.

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