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Influence of Hyaluronidase and Steroids on Permeability of Synovial Membrane. (17406)

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It has previously been reported[1] that hyaluronidase reversibly accelerated osmosis and abolished the semipermeable nature of membranes prepared from the excised urinary bladder of rabbits. Skeletal and cardiac muscle membranes were not affected. Adrenal cortical extract and estrone diminished permeability of bladder membranes and antagonized the effect of hyaluronidase. Desoxycorticosterone acetate enhanced the action of hyaluronidase.

The effect of hyaluronidase and steroids on membranes *in vivo* has not been studied heretofore. For this purpose we selected the synovial membrane of rabbits and humans. The synovial membrane and synovial fluid contain hyaluronic acid and are altered in arthritis.[2] The alteration has been attributed to a specific effect on the hyaluronic acid by hyaluronidases[2] and a structural change in the joint brought about by desoxycorticosterone intoxication as a disease of adaptation.[3] Arthritis has been treated successfully by the administration of Compound E or of adrenocorticotrophic hormone, which presumably releases this compound from the adrenal cortex.[4] It was therefore of interest to determine whether hyaluronidase and desoxycorticosterone weaken the normal synovial barrier by increasing its permeability and whether other adrenal steroids, either administered by injection or released endogenously by the alarm reaction, would strengthen the barrier and antagonize the effects of hyaluronidase.

The experiments on rabbits are reported in this paper. The data obtained on humans under the influence of various drugs and

1 Seifter, J., Baeder, D. H., and Dervinis, A., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 136.

2 Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

3 Selye, H., *J. Clin. Endocrin.*, 1946, **6**, 117.

4 Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet., Mayo Clinic*, 1949, **24**, 181.

diseases will be reported in a subsequent paper. by Fitch, D.R., Corn, O., Jallo, Jr., S. J., and Seifter, J.

Material and methods. Sixteen male white rabbits weighing between 3.5 and 5.0 kg were prepared for this study by a modification of the method reported by Curtis and Brunschwig for studying the human knee joint.[5] Each animal was anesthetized with 40 mg sodium pentobarbital per kg intraperitoneally, then placed on its dorsal surface and the inner aspect of each right hind leg was shaved at the talocrural articulation. A soft rubber catheter was inserted through the urethra into the bladder and retained there for the duration of the experiment. A sample of urine was removed immediately to show that no dye was present in the urine at the beginning of the experiment. Then by inserting a 26 gauge one-inch needle from the inner aspect of the ankle, 1.25 mg phenolsulphonphthalein (PSP) in 0.25 ml of 0.85% NaCl solution was injected into the cavity of the talocrural articulation. After insertion of the needle the syringe was aspirated to insure the presence of the needle in the joint cavity without having caused trauma and to check against the rupture of any small blood vessels. The joint was kept extended at all times throughout the experiment. Every 5 minutes after injection the urine was tested for the presence of dye. At its first appearance and every 15 minutes thereafter the amount of dye in 5 ml urine was measured in a Klett colorimeter by the method of Dandy and Rowntree.[6] If its disappearance from the urine was rapid, measurements were taken more often. The bladder was emptied after each sample of urine was withdrawn for a reading. All animals were standardized in the manner just described from one to 15 days before any other compound was administered. All instillations were made into the right hind talocrural articulation. The maximum number of tests in each rabbit was 5.

The doses and channel of administration of each compound used are listed in Table I. Compounds injected directly into the joint cavity were administered along with the dye. All intramuscular and intravenous injections were made 30 to 90 minutes before PSP or hyaluronidase was instilled into the joint cavity. The hyaluronidase used was prepared from bull testes and assayed 8400 turbidity reducing units per mg of nitrogen. The urinary spreading factor was isolated in our laboratory from normal urine and has been shown not to be hyaluronidase. Ten mg of the urinary spreading factor had the same activity as 150 turbidity reducing units of hyaluronidase in causing the spread of dye in the skin of rabbits. Colchicine was administered as the alarming stimulus to 2 rabbits in order to cause endogenous release of adrenal corticoids.

Results. The effects of hyaluronidase and steroids on the permeability of the synovial membrane are shown in Table I. Dye first appeared in the urine of the control animals on an average of 14 minutes after instillation of PSP into the synovial cavity. The urinary concentration of dye at this time was 49 γ per ml. The peak concentration was reached within 30 minutes with an average of 126 γ per ml. Excretion was complete on an average of 40 minutes, at which time the concentration was 3 γ per ml. The ranges of variation for the time of initial appearance and for the initial concentration were surprisingly small. The time to reach the peak, the concentration at 30 minutes, and the duration of excretion showed similar constancy.

1. *Effect of hyaluronidase and of urinary spreading factor.* Instillation of 150 turbidity reducing units of hyaluronidase and PSP into the joint resulted in more rapid absorption and elimination of the dye. The first appearance of dye was in 9 minutes, with a urinary concentration of 119 γ per ml, which was the peak. The duration of excretion was 20 minutes. Intravenous injection of 120,000 turbidity reducing units of hyaluronidase per kg of body weight caused the dye to first appear in the urine in 8 minutes at a concentration of 110 γ per ml, which was the peak. Excretion was completed in 25 minutes. Intravenous injection of 12,000 turbidity reduc-

[5] Curtis, G. M., and Brunschwig, A., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 358.

[6] Dandy, W. E., and Rowntree, L. G., *Ann. Surg.*, 1914, **59**, 587.

TABLE I.
Influence of Hyaluronidase and Steroids on Permeability of Synovial Membrane *in vivo*.

Procedure	No. of rabbits	Min. from instillation of PSP i.j. to its appearance in urine: First Avg	First Range	Peak	Dur'n of excretion Avg	Dur'n of excretion Range	Gammas PSP in 1 cc of urine: Onset Avg	Onset Range	Peak Avg	Peak Range	End Avg	End Range
Control	16	14	13–16	30	45	43–48	49	48–50	126	123–127	3	3
150 TRU Hydase* i.j.	6	9	8–10	9	20	19–21	119	118–119	119	118–119	4	4
12,000 TRU Hydase/kg i.v.	2	12	12–13	30	46	46–47	52	52	102	102	4	4
120,000 TRU Hydase/kg i.v.	1	8	—	8	25	—	110	—	110	—	7	—
U.S.F. (non-Hydase)	2	14½	14–15	30	47	—	48	48–49	126	125–127	35	3–4
1 u. A.C.E. i.j.	3	23	23–24	30	86	86–87	30	30–31	68	68	49	49–50 (60 min.)
100 u. A.C.E. i.m.	4	22	19–22	30	139	138–140	40	40–41	49	49	21	21 (130 min.)
1 u. A.C.E. i.j. + Hydase i.j.	3	20	19–20	30	73	72–73	42	42	54	54–55	41	41 (70 min.)
100 u. A.C.E. i.m. + Hydase i.j.	4	17	16–18	30	135	135–136	46	46–47	58	58–59	25	25 (130 min.)
5 mg DOCA i.m.	2	10	10	10	19	19–20	112	112–113	112	112–113	4	4
5 mg DOCA i.m. + Hydase i.j.	2	9	9–10	9	19	19–20	111	111	111	111	5	5
Alarm reaction†	2	More than 2¼ hr					No dye in urine in 2¼ hr; All present in joint cavity at autopsy					
Acute adrenalectomy	2	14½	14–15		46	46	Specimens contaminated by hematuria					
Acute adrenalectomy + Hydase i.j.	1	10	—	10	21	—	118	—	118	—	—	4
2 mg Estrone i.m.	2	15	15	15	243	242–244	69	69–70	69	69–70	4	4
2 mg Estrone i.m. + Hydase i.j.	2	19	19–20	19	246	246	70	70	70	70	5	5

i.j. = In talocrural articulation.
TRU = Turbidity reducing units.
i.m. = Intramuscular inj.
PSP = Phenolsulphonphthalein.
USF = Urinary spreading factor.

* Testicular hyaluronidase, Wyeth.
A.C.E. = Adrenal cortical extr. (alcoholic), Upjohn Co.
DOCA = Desoxycorticosterone acetate in oil.
† Induced by 3 mg Colchicine/kg body wt subcutaneously.

ing units of hyaluronidase per kg resulted in no significant change from the normal excretion pattern.

Instillation of urinary spreading factor in an amount equal to the spreading activity of 150 turbidity reducing units of hyaluronidase did not alter the normal excretion pattern.

2. *Effect of adrenal cortical extract* (ACE). Instillation of one unit of ACE into the joint or intramuscular injection of 100 units delayed the initial appearance of dye in the urine to 23 minutes at a concentration of 30 to 40 γ per ml. The peak of 49 to 68 γ per ml was attained within 30 minutes. Excretion was prolonged so that the concentration at 60 minutes was 49 γ per ml and 21 γ in 130 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the joint of ACE treated rabbits did not significantly alter the excretion pattern obtained with either channel of administration of ACE.

3. *Effect of desoxycorticosterone acetate* (DOCA). Intramuscular injection of DOCA decreased the time for initial appearance of dye in the urine to 10 minutes at a concentration of 112 gammas per ml, which was also the peak. Excretion was completed in 19 to 20 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the synovial cavity of DOCA treated rabbits did not further hasten absorption and excretion.

4. *Effect of alarm reaction.* PSP was not absorbed from the synovial cavity of rabbits in colchicine alarm reaction, and all the dye could be recovered from the synovial cavity more than two hours after its instillation.

5. *Effect of adrenalectomy.* The pattern of excretion begun 30 minutes after adrenalectomy was essentially the same as in the intact controls. Hyaluronidase had no greater effect in such an adrenalectomized rabbit than in intact rabbits.

6. *Effect of estrone.* Dye first appeared in the urine of estrone treated rabbits in 15 minutes at a concentration of 69 gammas per ml, which was the peak. The duration of excretion was prolonged to 243 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the joint of estrone treated rabbits did not significantly alter this pattern.

Discussion. The surprising uniformity of rate of absorption from the joints of normal rabbits and the constant effect of hyaluronidase lends great weight to the results obtained when small groups of animals were subjected to the other experimental procedures under test and when small changes were obtained. The more rapid absorption of PSP from the joint of rabbits receiving hyaluronidase is undoubtedly due to increased permeability of the synovial membrane. Presumably this is a specific effect on the hyaluronic acid of this membrane since a non-hyaluronidase spreading factor did not alter its permeability. The large amount of hyaluronidase that was needed by intravenous injection to produce the same effect as a small amount applied locally is accounted for by the presence of hyaluronidase inhibitors in the blood.

The delayed and prolonged absorption of PSP from the joint of rabbits receiving adrenal cortical extract is undoubtedly due to decreased permeability of the synovial membrane. ACE probably altered the state of polymerization of the hyaluronic acid of the membrane since hyaluronidase was no longer effective in increasing its permeability in ACE treated animals. Inhibition of hyaluronidase activity by ACE has been observed in skin[7] and isolated membranes.[1] Estrone treatment also prolonged but did not significantly delay the onset of absorption of PSP. Since hyaluronidase did not increase the permeability of the synovial membrane in the estrone treated rabbits, it is assumed that estrone also altered the hyaluronic acid of the membrane. Increased resistance to hyaluronidase by estrone has been observed in skin[8] and isolated membranes.[1]

The synovial membrane was more permeable to PSP during DOCA treatment than in the control period. The effect was identical with that produced by hyaluronidase. It was maximal and could not be augmented by the instillation of hyaluronidase into the joint. It is significant that the total adrenal steroids had the opposite effect of one of them. The

7 Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, in press.

8 Sprunt, D. H., and McDearman, S., *Endocrinology*, 1940, **27**, 893.

state of permeability of synovial membranes could be governed by the balance between DOCA and some other adrenal steroid. A similar opposition of effect was noted in isolated membranes[1] and in human hypertensives.[9] Furthermore, DOCA aggravates rheumatoid arthritis,[10] while Compound E alleviates this disease.[4]

The synovial membrane of rabbits in the alarm reaction phase of the adaptation syndrome was completely impermeable to PSP. The dye could be recovered from the joint more than 2 hours after its instillation. Isolated urinary bladder membranes prepared from rabbits in the alarm reaction are impermeable to the passage of fluid when used to separate M/1 sucrose from distilled water, while such bladders prepared from normal rabbits act as semipermeable membranes under the same conditions.[1] Such semipermeable membranes become impermeable to the further passage of fluid if serum from a rabbit in the alarm reaction is added to them.[1]

Adrenal steroids are released during the adaptation syndrome in response to physical, chemical, and psychic stresses. A protective role is attributed to these in the alarm reaction stage.[3,11] Their role during the stage of adaptation is not clear, but the diseases that are thought to arise during this stage are attributed to DOCA intoxication. It might well be that protective steroids possibly of the Compound E type are predominant during the alarm reaction phase and that possibly DOCA types dominate the adaptation phase. Alleviation of adaptation disease could then be obtained by administration of Compound E types or by the administration of adrenocorticotrophic hormone. It is of interest in this respect that DOCA does not affect the blood pressure of normotensive individuals but aggravates hypertension, which is considered to be a disease of adaptation,[6] and that the aggravation is nullified by ACE.

The synovial membrane permeability was not altered shortly after adrenalectomy, nor was the response to hyaluronidase abnormal. Depletion of adrenal steroids could not have occurred in this short period. Changes in permeability will probably be seen in the animals under study at various intervals after adrenalectomy.

The findings reported in this paper may be of particular significance in considering the pathogenesis and treatment of rheumatoid arthritis. This disease appears to be a deficiency in the function of hyaluronic acid of the synoviae. We have demonstrated that similar abnormal function of lowered resistance and increased permeability can be induced by either hyaluronidase or DOCA, both of which could permit exudation into joints. The etiology of rheumatoid arthritis has been attributed to hyaluronidases attacking the synovial structures[2] and to what may be DOCA intoxication due to various stresses.[6] We have furthermore shown that the effect of hyaluronidase is antagonized by ACE and endogenously released adrenal steroids by increasing the resistance of the synovial barrier. We have also demonstrated that DOCA acts in opposition to the other steroids present in ACE. These findings are to be considered in explaining the mechanism of the beneficial effect of Compound E, adrenocorticotrophic hormone, and possibly colchicine and salicylates in the treatment of rheumatoid arthritis and rheumatic fever.[12,13]

Summary and conclusions. 1. The permeability of the synovial membrane as measured by speed of absorption and excretion into the urine of phenolsulphonphthalein (PSP) instilled into the joint was found to be surprisingly constant in a group of 16 normal rabbits.

2. Hyaluronidase markedly increased permeability of the synovial membrane. The effect was maximal and was not augmented by desoxycorticosterone acetate (DOCA).

3. Adrenal cortical extract decreased permeability of the synovial membrane and an-

[9] Perera, G. A., and Pines, K. L., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 443.

[10] Degennes, L., Mahoudeau, D., and Bricaire, H., *Bull. Soc. Med. Hosp. Paris*, 1947, **63**, 532.

[11] Long, C. N. H., *Fed. Proc.*, 1947, **6**, 461.

[12] Forman, C., Seifter, J., and Ehrich, W. E., *J. Allergy*, 1949, **20**, 273.

[13] Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet., Mayo Clinic*, 1949, **24**, 277.

tagonized the effect of hyaluronidase. This effect of adrenal steroids was more pronounced when they were released endogenously by the alarm reaction. Estrone also decreased the permeability of synovial membrane.

4. Desoxycorticosterone increased maximally the permeability of synovial membrane to the same extent as hyaluronidase and could not be augmented by hyaluronidase.

5. It is suggested that the normal permeability of the synovial membrane is in part controlled by the balance between adrenal steroids of the DOCA type and of the Compound E type.

6. These findings are consistent with current views of the etiology of rheumatoid arthritis either by hyaluronidases or from exposure to stressing stimuli. They are also consistent with the therapeutic effects of Compound E or of adrenocorticotrophic hormone in the treatment of arthritis.

Addendum: Since completion of this work cortisone acetate and adrenocorticotrophic hormone (ACTH) were made available to us. One mg of cortisone acetate per kg and 0.5 mg of ACTH per kg were almost as effective in decreasing permeability of the synovial membrane as the alarm reaction. With both compounds only 25 to 30% of the dye was excreted in 4½ hours. The remainder was recovered from the synovial cavity. On this basis, the dose of adrenal cortical extract and estrone listed in Table I has cortisone activity of less than 20%, and the normal membrane balance of DOCA:cortisone an activity of 4.5%.

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Development of Adrenal Cortical Adenomas in Ovariectomized Mice Injected with "Physiologic" Doses of Sex Hormone.* (17407)

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Since the development of adrenal cortical adenomas in gonadectomized mice has been inhibited by the implantation of pellets of estrogenic hormone,[1] it might be inferred that withdrawal of gonadal secretion is of primary importance in precipitating the development of the castration-induced adenoma of the mouse adrenal cortex. The objective of the experiments being reported was to determine the effect of the administration of regulated, "physiologic" amounts of estrogenic or androgenic hormone on the induction of adrenal cortical tumors in susceptible ovariectomized mice.

Mice of the NH strain were used in these experiments. NH females develop adrenal cortical tumors spontaneously following ovarian regression, and precociously if gonadectomized.[2] Adrenal cortical adenomas are present in most NH mice of both sexes 5 months after removal of the gonads.

Four NH males and one female which had been gonadectomized at 23 days of age received (by subcutaneous injection beginning 2 weeks after operation) 0.05 microgram of estradiol dipropionate‡ in sesame oil at intervals of from 5 to 9 days. This treatment with estrogenic hormone provided for constant estrus, as determined by vaginal smear. These

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1 Woolley, G., and Little, C. C., *Cancer Research*, 1946, **6**, 491.

2 Kirschbaum, A., Frantz, M. F., and Williams, W. L., *Cancer Research*, 1946, **6**, 707.

‡ The synthetic sex hormones were supplied by Ciba Pharmaceutical Products through the courtesy of F. E. Houghton.