added 1 ml of 0.5% trypsin in Ringer's solution that had been freshly prepared from a filtered 2% crude trypsin stock(2,3). The culture tubes containing trypsin were returned to the roller drum and incubated for 1 hour at 37°C, at which time dissolution of the clot was usually complete. Simms-Hanks mixture (10-12 ml) was then added and the tubes were rotated in an angle centrifuge at 2500 rpm for 5 minutes. The supernate was discarded and the cells were washed once again in the same manner and recentrifuged. The sediment was suspended in 1.5 ml of 50% chick embryo extract and the cell clumps dispersed by gentle suction back and forth through a small bore capillary pipette. With the same pipette 2 drops of the cell suspension were spread lengthwise over the inner surface of a tube which had previously been coated with chicken plasma. When clotting was complete, 1.5 ml of nutrient fluid was added and the tubes were placed in the roller drum.

Results. Initial proliferation of fibroblasts was unusually vigorous and distinct growth originating from small clumps and single cells was observed in 24 hours. By 6 to 7 days all the tubes containing transplants were suitable for virus inoculation. The yield from one tube of original culture averaged 15 subcultures, which required an estimated working time of approximately 30 minutes for the entire procedure. A stock of primary cultures may be prepared when the testicle is first obtained and at selected intervals subcultures can be made thus insuring a continuous supply of young fibroblasts over a period of a month or longer. In a few experiments with human tissue, successful subcultures have been propagated from material kept in the roller drum for as long as 3 months.

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Effect of Hyaluronidase on Inflammation.* (20107)

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Investigations with hyaluronidase have shown that the spreading properties of the enzyme permit more effective application of certain drugs(1). The effect of this enzyme on local inflammation due to irritating drugs, however, was not known; and these experiments were designed to study this aspect and to evaluate the applicability of hyaluronidase as an adjuvant in therapy with irritating agents.

Two strong irritants were used: the oil-soluble allylisothiocyanate and the water-soluble paraphenylenediamine. Gross and microscopic observations on animals treated with these irritants showed that in the presence of hyaluronidase, the local inflammatory

TABLE I. Effect of Intradermal Paraphenylenediamine in the Presence and Absence of Hyaluronidase (End of 3 Hr). 6 rabbits per exp.

Treatment*	Reaction area† No. reacting	Reaction area† Avg area of dye (mm² × 10²)
.9% NaCl + .9% NaCl	1	.02
.9% NaCl + H	5	.23
4% P + .9% NaCl	6	11.98
4% P + H	6	21.96

*H = Hyaluronidase. P = Paraphenylenediamine.

† None of the rabbits showed petechiae. Only the rabbits (6) inj. with 4% P + .9% NaCl showed edema.

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TABLE II. Effect of Intradermal Allylisothiocyanate in the Presence and Absence of Hyaluronidase (End of 3 Hr).

Treatment*	No. rabbits used	Reaction area: No. reacting	Reaction area: Avg area of dye ($mm^2 \times 10^2$)	Intensity of inflammation: No. showing petechiae	Intensity of inflammation: Avg area of petechiae ($mm^2 \times 10^2$)	Intensity of inflammation: No. showing edema
M + .9% NaCl	13	3	.04	0	0	0
M + H	13	10	.92	0	0	0
2% A in M + .9% NaCl	16	16	21.3	16	2.8	16
2% A in M + H	16	16	39.4	16	4.6	2
.25% A in M + .9% NaCl	13	13	8.1	6	.42	13
.25% A in M + H	13	13	16.8	8	.89	1

* M = Mineral oil. A = Allylisothiocyanate. H = Hyaluronidase.

response was modified.

Method. The trypan blue irritation test procedure described by Menkin(2) was modified and used in this study. This test is based on the observation that intravenously injected colloidal dyes seek out and stain injured skin areas as a result of increased vascular permeability(3-5). Forty-six albino rabbits were used. The restrained animals were injected intradermally on the clipped abdomen with 0.3 ml of either allylisothiocyanate in mineral oil or aqueous paraphenylenediamine. Within 1 minute after the injection of the irritants, 0.1 ml of hyaluronidase (900 turbidity reducing units†) was introduced into some of the injection sites; the remaining sites were injected with 0.1 ml of physiological salt solution (Tables I and II). The vehicles with and without hyaluronidase were injected as controls. Fifteen minutes after the intradermal injections were completed, 10 mg/kg of trypan blue was injected into the marginal ear vein of each rabbit and observations on the injection sites were made periodically for 3 hours. The area of spread of extravasated dye at various time intervals after injection was obtained using the formula D x d x Pi/4 (assuming an elliptical shape for the bleb) where D is the longest diameter and d the shortest diameter. Other animals similarly treated were observed and sacrificed at the end of 24, 48, 96 and 192 hours. At sacrifice, the injection sites were excised and prepared for microscopic evaluation. Hematoxylin-eosin stain and Beyer's trichrome stain were used on the sections.

Results. Tables I and II summarize the gross observations on rabbits following intradermal injections of either paraphenylenediamine or allylisothiocyanate in the presence and absence of hyaluronidase. The most striking feature is the absence of swelling (edema) at the sites where the irritants were administered with hyaluronidase; however, the area of extravasation of dye at these sites is approximately 2 times greater. While petechial spots characteristically appear only after injections of allylisothiocyanate, they are more numerous and appear earlier when the injections are supplemented with hyaluronidase. The gross appearance of these lesions is shown in Fig. 1 (A,B).

A number of days after the initial exposure to the irritants the reaction areas are still identifiable grossly; and the differences between the sites that received the irritants with and without hyaluronidase are easily recognized. The lesions resulting from the irritants administered without supplemental hyaluronidase have a firm consistency. Elongated masses limited to the under surface of the skin extend in several directions from the injection sites and are glistening and gelatinous in appearance. The areas exposed to the irritants administered with supplemental hyaluronidase are, in contrast, soft and pliable.

The microscopic appearance of the injection sites at the end of 24 hours is shown in Fig. 2 (A-F). Sections A (physiological salt solution) and D (mineral oil) show the changes due to injection of the vehicles. In general, there is minimal separation of the epidermis, fatty connective tissue and muscle layers. Vacuoles are present in the corium of the mineral oil section only and show the dispersion of the oil droplets.

† Wydase,® Wyeth.

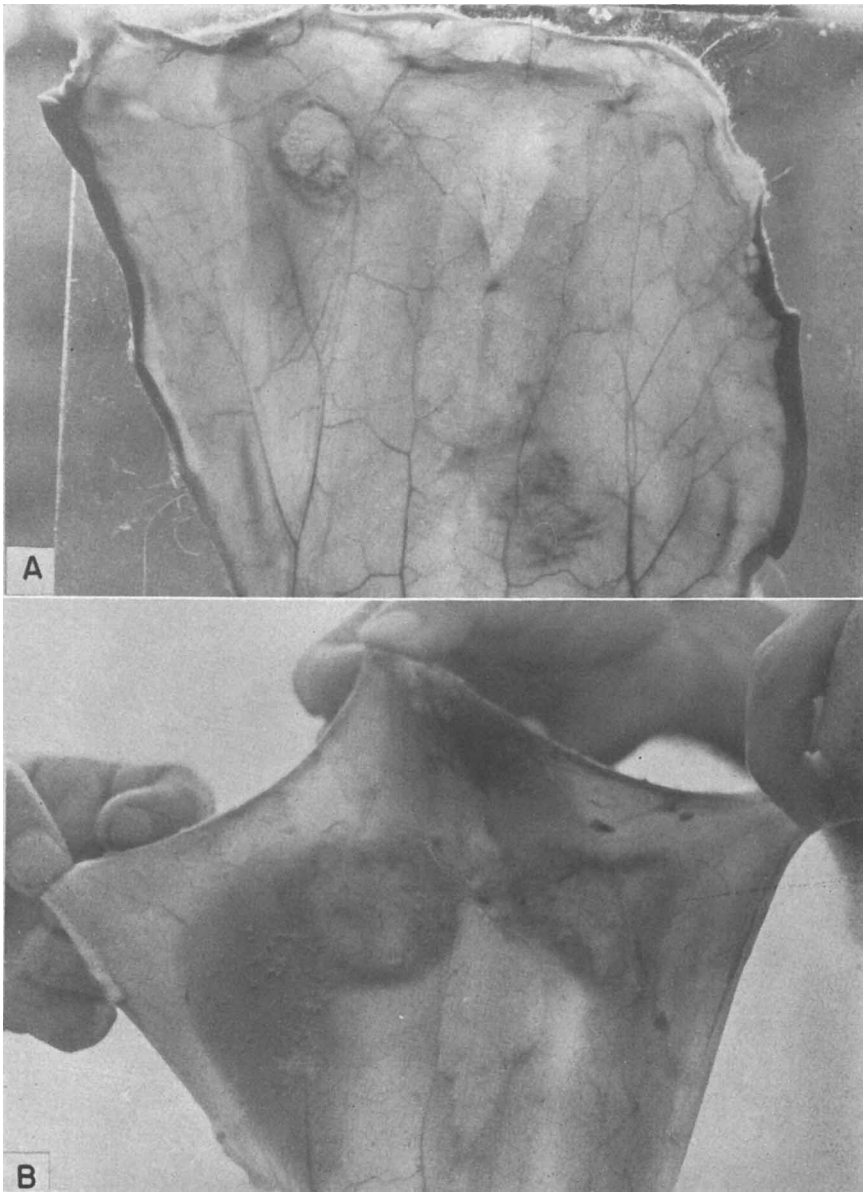

FIG. 1. (Gross appearance of lesions). A: 4% paraphenylenediamine; abdominal skin viewed by transmitted light (end of 24 hr). Upper left, with physiological saline (note extravasation of dye with suggestion of swelling lateral to lesion); lower right, with hyaluronidase. B: 2% allylisothiocyanate in mineral oil; abdominal skin viewed by transmitted light (end of 24 hr). Left, with physiological saline (note the gelatinous thickening of tissue lateral to the lesion with extravasation of dye in this area); right, with hyaluronidase.

The swelling seen grossly at the sites of injection of the aqueous irritant, paraphenylenediamine, or the oil-soluble irritant, allylisothiocyanate, is seen microscopically as distortion of the tissue architecture with separation of the fibers and cellular elements (sections B,E). The oil droplets (vacuoles) conspicuous in the tissue exposed to allylisothiocyanate (section E) further contribute to the over-all distortion. At the same magnification both sections almost completely fill the entire microscopic field. In comparison,

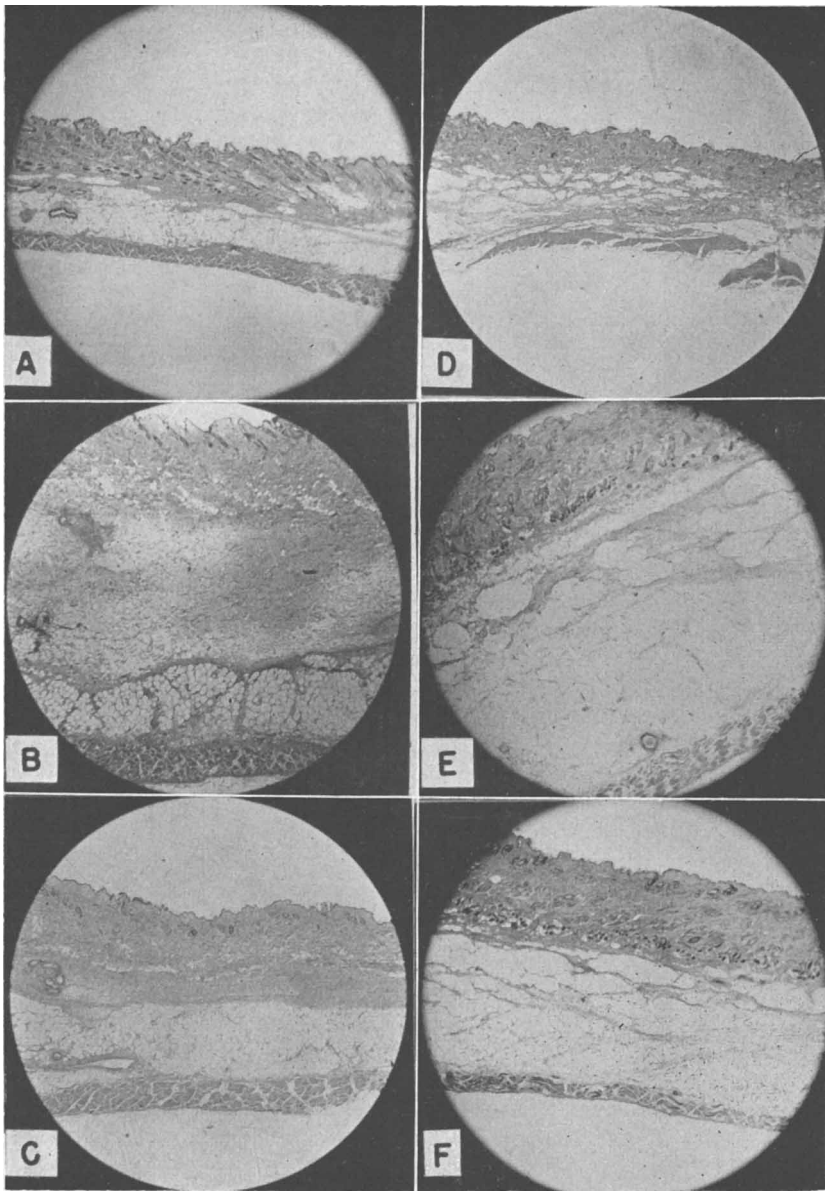

FIG. 2. Microscopic appearance of rabbit skin 24 hr after intradermal inj. (× 15). A: physiological saline; B: 4% paraphenylenediamine + physiological saline; C: 4% paraphenylenediamine + hyaluronidase; D: mineral oil; E: 2% allylisothiocyanate in mineral oil + physiological saline; F: 2% allylisothiocyanate in mineral oil + hyaluronidase.

sections taken after administration of the irritants with supplemental hyaluronidase (sections C,F) are considerably thinner, show less tissue distortion and less separation of fibers. The oil droplets due to the allylisothiocyanate, however, are still conspicuous (section F).

Table III summarizes the chief microscopic findings as they relate to injury and repair of the skin following contact with the irritants for various lengths of time. Here the separation of tissue elements, fibrosis and degree of extravasation of erythrocytes are scored on a 1 to 5 basis. In brief, it can be said that there is no microscopic evidence that the degree of vasodilatation, number of polymorphonu-

TABLE III. Summary of Microscopic Findings* (Avg 6 Rabbits per Group).

Treatment†		Response of tissue (by hr) — Separation of elements 3	24	192	Hemorrhage 3	24	192	Fibrosis‡ 192
4% P	+ S	3.5	2.7	.5	1.8	2.5	2.3	3.3
	+ H	2.3	3.0	.6	2.7	2.2	2.5	2.8
2% A in M	+ S	4.5	3.7	1.2	3.3	3.0	2.0	3.0
	+ H	3.0	2.3	.2	3.8	3.5	2.5	2.3

* Scores are on the basis of 1 to 5.

† P = Paraphenylenediamine. A = Allylisothiocyanate. S = Physiological saline. H = Hyaluronidase. M = Mineral oil.

‡ Fibrosis occurred in all animals between 24 and 192 hr.

clear leukocytes, number of lymphoid cells or the number of spindle cells occurring at the irritation sites are affected by the use of hyaluronidase. Fibrosis is scored at 8 days and does not appear to be significantly less where hyaluronidase is used with the irritants. On the other hand, however, where hyaluronidase is used in conjunction with the irritants, sections of these sites taken after 3 hours show more extravasated erythrocytes than those sites where the irritants are administered alone; even after 8 days the sites show evidence of hemorrhage.

Discussion. This problem was undertaken for practical purposes. The local irritating properties of certain drugs sometimes preclude their parenteral use; but under certain conditions the tissue response to the presence of irritants can be altered so that less injury and discomfort results.

The non-toxic mucolytic enzyme hyaluronidase seemed to be the agent of choice for two reasons. Because of its power to hydrolyze the hyaluronic acid barrier found in various tissues of the body, hyaluronidase promotes the diffusion of irritants. Furthermore, the action of hyaluronidase on the structures surrounding and supporting the capillary wall, while not altering the endothelium *per se*(6), results in increased filtration across the affected area. These factors operating together at the site of the injury serve both to lower the local concentration of the irritants and aid in their removal; in this way the damaging effects on the surrounding cells are reduced.

The swelling due to edema and pressure exerted thereby on underlying structures are also eliminated. The edema, which in the absence of supplemental hyaluronidase cannot easily escape or move about, diffuses rapidly away from the site of injury; and this diffusion continues, according to Hechter(7), as long as edema furnishes pressure and volume for hyaluronidase action. Moreover, elimination of the edema pocket results in minimal distortion of the tissue architecture. This may be of some importance in terms of healing and scar tissue formation.

The picture is somewhat altered when the irritant is oil-soluble. The exudate formed at the site of injury forms an emulsion with the oily irritant; and the oil droplets make their way between the structural elements of the tissue to contribute to the distortion of the architecture. Although hyaluronidase facilitates the diffusion of the oily irritant and the exudate, it is questionable whether this is an advantage. Elimination of the edema phase of inflammation by dispersing the oily irritant and the exudate results in a larger area of tissue in intimate contact with the concentrated irritant. And since oil is absorbed at a slow rate, as indicated by the persistence of oil droplets in the connective tissue for 8 or more days, this allows the irritant to act on this greater area during this time.

The phenomenon of hemorrhage seen in the sections represents the vascular damage resulting from the action of the chemical irritants upon the capillary wall. Although hyaluronidase itself does not produce any signs of local tissue injury(1), its use in conjunction with either allylisothiocyanate or paraphenylenediamine hastens the extravasation of erythrocytes from the damaged vessels. Hyaluronidase accomplishes this by softening the material surrounding and supporting the damaged capillary wall thereby permitting minute

vascular ruptures and petechial hemorrhages to occur earlier and more prominently.

Summary. 1. Hyaluronidase modifies the inflammatory response of tissue due to certain oil and water-soluble irritants. 2. Hyaluronidase affects the vascular response. The spread of trypan blue in the tissues representing increased vascular permeability, appears earlier and is greater when the irritants are administered with hyaluronidase; and where the nature of the irritant is such as to produce hemorrhages, the areas of these hemorrhages are also greater and occur earlier. 3. Hyaluronidase does not alter the solubility or absorption of an oil-soluble irritant. 4. Use of hyaluronidase with oil or water-soluble irritants eliminates edema from the classical picture of inflammation. This may have practical applicability where prevention of edema resulting from contact with irritants is important. 5. These studies also indicate that hyaluronidase is a useful tool for investigating the edema aspect of the inflammatory reaction.

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Use of "Granuloma Pouch" Technic in the Study of Antiphlogistic Corticoids.* (20108)

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The "granuloma pouch" technic has been devised as a procedure for the quantitative analysis of factors regulating inflammation and wound healing. Its principle is that by injecting a given amount of air into the loose subcutaneous tissue of the rat, an almost perfectly symmetrical, spherical or ellipsoid air space, of any desired size, can be created. This acts as a mold for the subsequent formation of a granulomatous membrane, into which the internal surface of this cavity can readily be transformed, by injection of some irritant (*e.g.,* croton oil, formalin, kaolin) into the air space. The thickness and structure of the granuloma pouch, as well as the quantity and constitution (cellular and chemical constituents) of the fluid, which gradually replaces the air, can be largely determined at will by the selection of appropriate irritants. The salient features of this technic and its principal applications have been outlined elsewhere(1,2).

In the present communication, we would like to report upon experiments demonstrating the striking changes which occur, in the granuloma pouch, upon topical administration of hydrocortisone acetate.

Experimental observations. In the *1st experiment,* 16 female Sprague-Dawley rats, weighing 128 to 151 g, were subdivided into 2 equal groups. In all the animals of both groups, the dorsal region was depilated with barium sulfide. Immediately afterwards, the 8 rats of one group were treated with hydrocortisone micro-crystals. The suspension was made up by diluting the original preparation of Hydrocortone Merck (which contains 25 mg per ml) with the original suspension fluid (kindly supplied by Merck and Co., Montreal), in the amount required to obtain a concentration of 10 mg per ml. Of this suspension, 0.1 ml (*i.e.,* 1 mg) was then injected in the interscapular region. In order to obtain

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