sera provide definite proof that positive complement fixation can be demonstrated when concentrated Lansing virus prepared as described[2] is used as the antigen. These latter results further support the importance of the Lansing virus or an antigenically related strain as one responsible for the human disease.

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Influence of Pyribenzamine and Antistine upon the Action of Hyaluronidase.

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It is the general belief that the antianaphylactic and anti-allergic properties of Pyribenzamine and similar substances are due primarily to their ability to nullify the pathologic effects of histamine. This belief is based upon the following premises: First, that the symptoms of anaphylaxis and certain allergic diseases are produced by histamine, which is released during the antigen-antibody reaction; and secondly, that the most outstanding property of Pyribenzamine and related substances is their antihistaminic power (Mayer, Huttrer and Scholz,[1] Mayer[2]).

Many clinical observations on the activity of Pyribenzamine and similar substances in various forms of allergic manifestations appear to sustain this explanation. The effectiveness of antihistaminic therapy depends in a large measure upon the type of allergy against which it is used, and antihistaminics are most effective in conditions in which histamine release is suggested by experimental findings, namely in anaphylaxis, serum disease, urticaria or hay fever. On the other hand, if the activity of these substances is due solely to the inhibition of histamine, then they should be ineffective in allergic dermatitis or eczema, since none of the clinical symptoms of contact dermatitis or eczema appear to have any resemblance to the known histamine symptoms. To date, all attempts to produce these forms of skin reactions by the administration of histamine have been unsuccessful.

But in spite of these facts, there are numerous clinical reports citing the effectiveness of the various antihistaminics in contact dermatitis and atopic eczema (Feinberg,[3] Chobot[4]), and one of us has shown that Pyribenzamine exerts a considerable prophylactic activity in experimental contact dermatitis of guinea pigs (Mayer[5,6]). Since the effectiveness in these epidermal sensitizations is difficult to explain on the basis of inhibition of histamine, it appears more likely that antihistaminic substances act in these cases by virtue of a pharmacodynamic property different from their action upon proven histamine effects. All antihistaminic substances developed thus far possess, in addition to their antihistaminic power, various degrees of antispasmodic and local anesthetic activities. It is possible that the curative effect in dermatitis and eczema may be explained as being due to the local anesthetic property, although several observations contradict this view.

Recent investigations on the mechanism of epidermal inflammations due to the action of chemical or physical influences have pointed to the importance of certain amines, other than histamine, and of substances such as leukotaxine, pyrexin and necrosin (Menkin[7]). Although the significance of these substances

1 Mayer, R. L., Huttrer, G. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

2 Mayer, R. L., *J. Allergy*, 1946, **17**, 153.

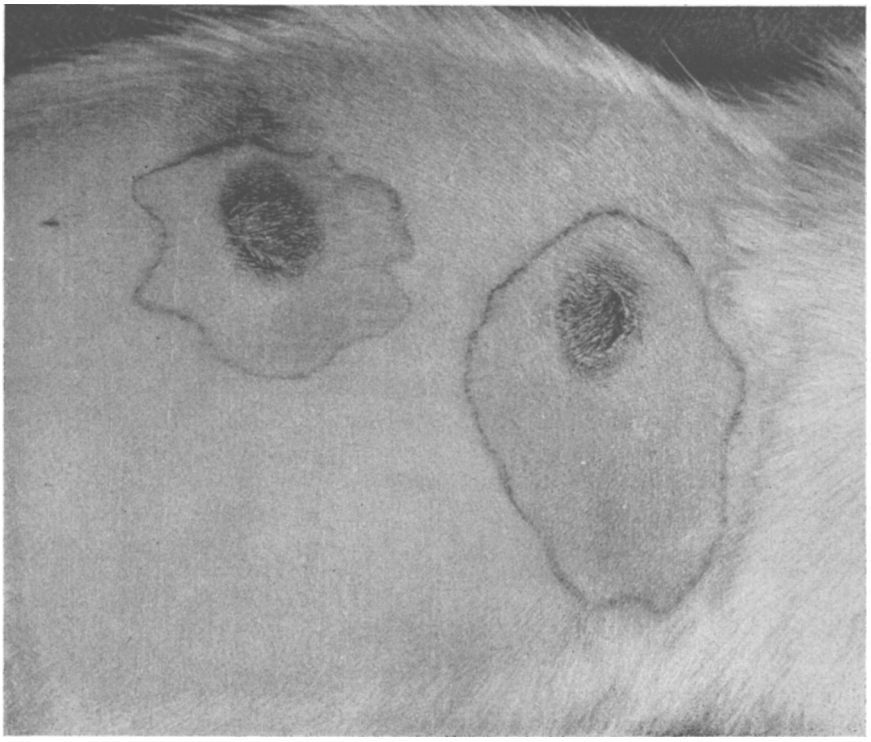
3 Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

4 Chobot, R., *J. Allergy*, 1947, **18**, 76.

5 Mayer, R. L., *J. Invest. Dermat.*, 1947, **8**, 67.

6 Mayer, R. L., *Ann. Allergy*, 1947, **5**, 113.

7 Menkin, V., *Arch. Path.*, 1946, **41**, 376.



Tail Head

FIG. 1.

Rat No. 32, right side, before Pyribenzamine. Tail: India ink plus saline. Head: India ink plus 1% hyaluronidase.

Note: Dark spot upper center is due to the subsequent injection of Pyribenzamine.

is somewhat uncertain, Cullumbine[8] has investigated the influence of antihistaminic substances on the action of leukotaxine; he could not detect any anti-inflammatory activity.

On the other hand, various inflammatory processes produced by strongly invasive micro-organisms have been associated with the release, from these organisms, of a specific enzyme, namely hyaluronidase (Duran-Reynals[9]). So far, very little is known concerning the role which this enzyme plays in non-bacterial inflammations of the skin. Since relatively large concentrations of hyaluronidase occur in the normal skin, this may indicate a more general function. (Meyer *et al.*,[10] Meyer[11]) Certain of the observations described below indicate that hyaluronidase may be associated with certain phases of skin inflammations, especially of allergic non-bacterial nature.

We have directed our investigations in this direction and have studied the influence of Pyribenzamine and antistine upon the activity of hyaluronidase both as a spreading factor and as a possible factor responsible for allergic inflammation of the skin.

Experimental. A. Spreading of India ink in rats. The effect of Pyribenzamine and antistine upon the diffusion of India ink in the skin of albino rats in the presence and absence of hyaluronidase was investigated according to the method described by Duran-Reynals[8] and Cahen and Granier.[12] One hundred and twenty male and female rats, each

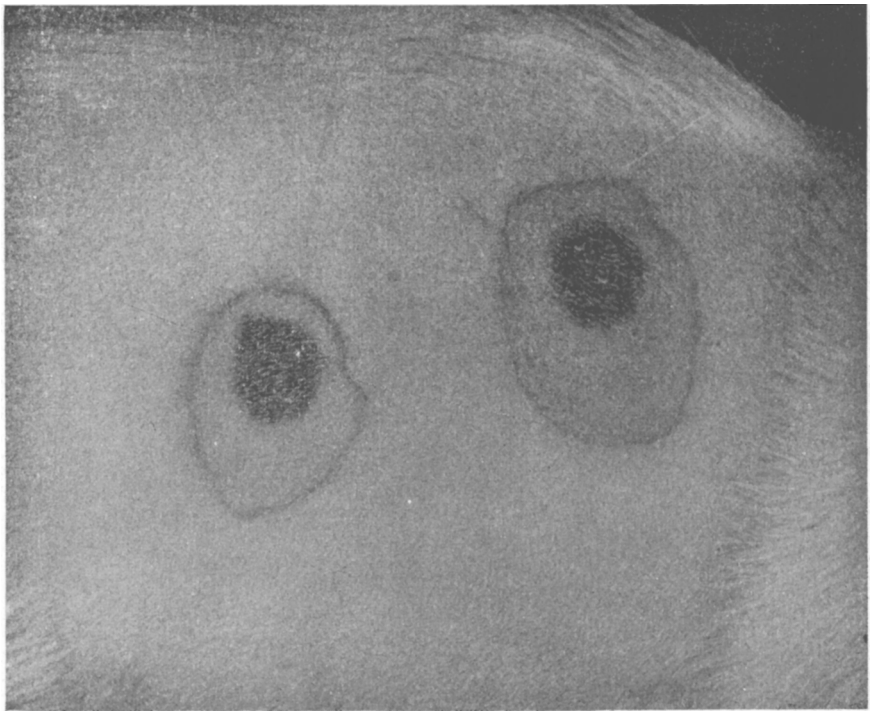
8 Cullumbine, H., *Nature*, 1947, **159**, 841.

9 Duran-Reynals, F., *Bact. Rev.*, 1946, **6**, 197.

10 Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

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

12 Cahen, R. L., and Granier, M., *Yale J. Biol. and Med.*, 1944, **16**, 257.



Tail Head*

FIG. 2.

Rat No. 32, left side, after 37.5 mg of Pyribenzamine. Tail: India ink plus saline. Head: India ink plus 1% hyaluronidase.

* This photograph has purposely been reversed in order to permit a comparison of Fig. 1 and 2.

weighing between 120 and 220 g, were treated simultaneously at 2 sites on the right flank; one site being injected intradermally with a mixture of 0.05 cc of Higgins India ink and 0.10 cc physiological saline, and the other site with a mixture of 0.05 cc of ink and 0.10 cc 1% hyaluronidase solution. The hyaluronidase preparation* used in these experiments contained 150 turbidity-reducing units per milligram. Twenty hours later the areas were outlined in ink, traced on cellophane sheets, and measured with a planimeter.

Having thus determined the *in vivo* activity of the hyaluronidase preparation, the animals were subcutaneously injected on the right side 24 hours after the India ink injections with a solution of Pyribenzamine or antistine in doses of 15, 37.5 and 75 mg per kg of body weight respectively; the highest dose representing 1/3 to 1/5 of the LD^{50} for rats (Mayer *et al.*[13]).

Twenty minutes after the administration of the antihistaminic, the intradermal injections of India ink plus saline, and India ink plus hyaluronidase were repeated on the corresponding areas of the left flank. The areas of spreading were measured as outlined above.

The results of this India ink test, given in Table I, show that the antihistaminic substances Pyribenzamine and antistine strongly reduced both the extent of the normal spreading of injected India ink, as well as the spread produced in the presence of hyaluronidase. Doses of 15, 37.5 and 75 mg/kg of Pyribenzamine restricted the area of spreading pro-

* We are indebted to Dr. E. Schwenk, of the Schering Corp., who kindly supplied the material.

[13] Mayer, R. L., Hays, H. W., Brousseau, D., Mathieson, D., Rennick, B., and Yonkman, F. F., *J. Lab. Clin. Med.*, 1946, **31**, 749.

duced by hyaluronidase by 6%, 21% and 44% respectively over that of the control areas. Similar doses of antistine restricted the spreading by 6%, 20% and 51% respectively. Fig. 2 shows the decrease of the hyaluronidase spreading effect after injection of 37.5 mg/kg Pyribenzamine, in contrast to the hyaluronidase effect on the same animal before Pyribenzamine treatment, as shown in Fig. 1.

Morphine sulfate when tested according to the method of Cahen and Granier[12] in doses of 25 and 32 mg/kg, restricted the increase of spreading by 46% and 47% respectively.

B. *Effect of Antihistaminics Upon the Action of Hyaluronidase in Experimental Sensitizations.* 1. *Action of Hyaluronidase upon Allergic Inflammation.* We sensitized 18 guinea pigs to paraphenylenediamine by treatment with 10% paraphenylenediamine ointment according to a method previously described (Mayer[14]) and challenged them 3 weeks later with an intradermal injection of 0.10 ml of 0.75% paraphenylenediamine solution.

The paraphenylenediamine used for the challenge was freshly recrystalized and solutions prepared in the absence of oxygen, in order to minimize the formation of oxidation products which could destroy the hyaluronidase. Since the solutions were slightly tinted in spite of these precautions, 10% horse serum was added immediately before the injections as a further precaution, the serum proteins combining with and simultaneously reducing the small amount of oxidation product present. Although it is known that certain sera inhibit the action of hyaluronidase (McClean[15]), Hechter and Scully[16] have recently shown that serum interferes only after incubation; in our experiments no significant diminution of the spreading effect took place.

The challenge injections with antigen alone and with antigen-hyaluronidase mixtures were performed in the same manner as described above for the India ink experiments in rats. The areas of inflammation and infiltration, varying from 6 mm to 25 mm in diameter, were measured and at the same time, the intensity of infiltration and redness noted by symbols as indicated in Table II.

TABLE I.
Effect of Pyribenzamine and Antistine upon the Spreading of India Ink in the Presence and Absence of Hyaluronidase.
20 rats tested in each experiment.

Exp.	Dosage (mg/kg)	Reduction of spreading area: India ink alone	Reduction of spreading area: India ink plus hyalurondase
	Pyribenzamine	%	%
1	15	14.05 ± 4.21*	6.25 ± 6.39
2	37.5	18.70 ± 5.20	21.10 ± 3.77
3	75	47.85 ± 2.53	43.95 ± 3.73
	Antistine		
4	15	13.65 ± 5.73	11.10 ± 4.77
5	37.5	31.75 ± 5.22	20.00 ± 4.23
6	75	28.10 ± 4.99	37.00 ± 3.22

* ± values represent probable error of the mean.

The results obtained indicate that hyaluronidase increases the spread of the allergic epidermal reaction in sensitized guinea pigs, as it increases the spread of India ink in rats. The average challenge reaction in sensitized animals measured 2.3 mm² when the antigen was injected alone, and 16.9 mm² when it was combined with hyaluronidase. Simultaneously, there was a marked increase in the *intensity* of the allergic inflammation which was even more significant than the increase in the size of the reaction. The intensity increased from a + reaction to a +++ or ++++ reaction in the presence of hyaluronidase, in spite of the fact that the greater spread, as proven by the increased area of inflammation, had resulted in a marked dilution of the antigen concentration at the site of reaction.

2. *Influence of Pyribenzamine Upon the Hyaluronidase Effect in Allergic Inflammations. a. Sensitization to paraphenylenediamine.* Twenty-four hours after the spreading test was performed these animals received 15 mg/kg Pyribenzamine subcutaneously on the same flank. Twenty minutes later they were retested on the opposite flank with paraphenylenediamine and a paraphenylenediamine-hyaluronidase horse serum mixture in the same manner. Since the challenge reaction is in this case an epidermal inflammation of the contact dermatitis or tuberculin type, which

14 Mayer, R. L., *Arch. f. Dermatol. u. Syph.*, 1931, **163**, 223.

15 McClean, D., *J. Path. and Bact.*, 1942, **54**, 284.

16 Hechter, O., and Scully, E. L., *J. Exp. Med.*, 1947, **86**, 19.

TABLE II.
Influence of Hyaluronidase on the Challenge Reaction in Experimental Epidermal Sensitizations to Paraphenylenediamine (PP) in Guinea Pigs.

		Average size and intensity of challenge reaction			
		Without hyaluronidase		With hyaluronidase	
	No. of animals	Size in mm²	Intensity of inflammation	Size in mm²	Intensity of inflammation
Controls	4	0.317 ± 0.124	(+)	1.60 ± 0.14	+
Sensitized animals	18	2.3 ± 0.74	+	16.9 ± 0.52	+++ to ++++

Signs: (+) to ++++ represent varying degrees of inflammation as measured by the intensity of redness and degree of infiltration.

TABLE III.
Influence of Pyribenzamine upon the Allergic Reaction in Guinea Pigs to Paraphenylenediamine in Presense and Absence of Hyaluronidase.

		Average size and intensity of local challenge reaction			
		Without hyaluronidase		With hyaluronidase	
	No. of animals	Size in mm²	Intensity of inflammation	Size in mm²	Intensity of inflammation
Controls	4	0	0	0.75 ± 0.48	0
Sensitized animals	18	0.27 ± 0.14	0	2.7 ± 0.54	(+)

15 mg per kg body weight of Pyribenzamine was given twice subcutaneously, namely, 15 to 20 minutes before and 4½ hours after the challenge injection of the antigen.

develops gradually, reaching a maximum only after 12 to 16 hours, an additional dose of Pyribenzamine (15 mg/kg) was injected 4½ hours later.

The results of this experiment are given in Table III. The area of the challenge reaction to the antigen alone this time was found to be to an average size of 0.27 mm², against 2.3 mm² before the Pyribenzamine injection. This reaction became even smaller than the unspecific, non-allergic reaction to the paraphenylenediamine injection in non-sensitized control animals. The average size of the challenge reaction produced in the presence of hyaluronidase was reduced from 16.9 mm² to 2.7 mm², a value close to that observed in the animals having received antigen alone (Table III). Thus the pretreatment with Pyribenzamine almost completely suppressed the allergic reaction regardless of whether the antigen was injected with or without hyaluronidase.

b. Sensitization to 2,4-dinitrochlorobenzene. In a previous study it was shown that antihistaminic substances prevent or diminish the epidermal manifestations of experimental sensitizations of guinea pigs to 2,4-dinitrochlorobenzene (Mayer[14]). This type of sensitization differs from that produced by paraphenylenediamine in the clinical aspect of the challenge reaction, as well as in the histological changes produced. It was therefore of interest to investigate whether the challenge reaction in sensitizations to this nitro compound was also reinforced by hyaluronidase, as was the case in sensitizations to the diamine.

Ten guinea pigs were sensitized to 2,4-dinitrochlorobenzene by intradermal injection of 0.02 mg of antigen, and after 27 days all animals had become strongly allergic to the substance. They were then challenged intradermally with 0.1 ml containing 0.02 mg of antigen alone and with a mixture of 0.02 mg of antigen and 0.5 mg of hyaluronidase in a similar manner, as described for paraphenylenediamine. However, in this case no horse serum was added to the antigen-hyaluronidase mixture, since, according to Landsteiner[17] 2,4-dinitrochlorobenzene does not react with protein at room temperature.

In this case, however, hyaluronidase neither increased the size nor the intensity of the

[17] Landsteiner, K., *The Specificity of Serological Reactions*, Cambridge, Mass., 1945.

challenge reaction. It is not known yet whether this negative result is due to a destruction of hyaluronidase, or to a blocking of its action by the nitro compound, or whether this type of epidermal sensitization does not intrinsically respond to hyaluronidase.

C. Role of the Local Anesthetic Activity of Antihistaminics. Pyribenzamine and the other antihistaminic substances of similar chemical constitution have local anesthetic activity equal to or superior to that of procaine. It was therefore of interest to determine whether a local anesthetic substance with low antihistaminic activity, such as procaine, influenced the spreading effect of hyaluronidase in the same way as Pyribenzamine.

Twenty rats were tested as outlined above and the influence of procaine upon the spread of India ink investigated at two different levels, namely 75 mg procaine per kg of body weight, which corresponded to the highest doses of Pyribenzamine used in the earlier experiments and 225 mg per kg of body weight, representing a 3-fold increase in the local anesthetic action.

Seventy-five mg/kg of procaine had no significant influence upon the spread of India ink either in the absence or presence of hyaluronidase. With 225 mg per kg procaine, the spread of India ink was decreased by 11% in the absence of hyaluronidase, and 18% in experiments performed in the presence of hyaluronidase. The activity of this high concentration of procaine could be due to an unspecific cell injury, since it was highly irritating locally and produced deep necrosis.

According to these results, procaine has a slight influence upon the normal spread of India ink, as well as on the spreading effect of hyaluronidase. It is, however, much less active than Pyribenzamine or antistine in this respect.

Discussion. The present experimentation was performed to determine (a) whether hyaluronidase plays a role in allergic, nonbacterial inflammation of the skin and (b) whether antihistaminic substances have an anti-hyaluronidase activity.

The results indicate that hyaluronidase markedly influences the epidermal challenge reaction in guinea pigs sensitized to paraphenylenediamine, its presence increasing the size and intensity of inflammation. Furthermore, the fact that antihistaminic substances markedly decrease the effect of hyaluronidase upon the spread of India ink and upon the allergic reaction to paraphenylenediamine constitutes, we believe, reasonable indication that hyaluronidase does play a role in certain nonbacterial, especially allergic inflammation, as it does in certain bacterial processes. The direct proof for this theory, however, is still lacking since it has not been shown that hyaluronidase is liberated during the allergic inflammation of the skin (and other organs) and that, if its amount is increased during this inflammation, it is etiologically related with some phase of the allergic reaction. So far, certain technical difficulties have been encountered in the interpretation of experiments performed to elucidate this point.

The present investigation has furthermore disclosed that antihistaminic substances such as Pyribenzamine and antistine possess, in addition to their antihistaminic activity, other properties which may permit action in various manifestations thus far not associated with histamine-release. The strong anti-hyaluronidase effect, which results in a reduction of the size as well as the intensity of an allergic inflammation, may explain the activity of Pyribenzamine and similar substances in epidermal sensitizations such as eczema or contact dermatitis. It might also explain the effect antihistaminics seem to exert in certain rheumatic manifestations, where, according to Guerra, salicylates act by a similar mechanism.

It is not yet known whether this anti-hyaluronidase effect is a specific pharmacologic property of Pyribenzamine and antistine, comparable, for instance, to their specific antihistaminic power. It is quite possible that the effect is the result of a nonspecific process. On the other hand, the local anesthetic power may, to a certain extent, be associated with this activity—a question which could be decided by the use of antihistaminic substances devoid of local anesthetic power.

Conclusions. Experiments are presented which show that (a) hyaluronidase increases the intensity of allergic skin inflammation of

TABLE IV.
Influence of Procaine upon the Spreading of India Ink in the Presence and Absence of Hyaluronidase. 10 rats tested in each experiment.

Exp.	Dosage (mg/kg)	Average size of spreading		Reduction of spreading area	
		Without hyaluronidase	With hyaluronidase	Without hyaluronidase	With hyaluronidase
				%	%
1	0	5.92	8.9		
	75	5.8	8.4	3.8 ± 6.4	5.3 ± 4.5
2	0	6.1	8.8		
	225	5.3	7.0	11.1 ± 3.4	18.1 ± 3.3

the epidermal type and (b) that antihistaminic substances such as Pyribenzamine and antistine counteract the spreading effect of India ink and hyaluronidase, as well as the latter's effect upon the allergic skin inflammation. The significance of these findings for the activity of antihistaminics in epidermal sensitizations is discussed.

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Isolation and Properties of Snake Erythrocyte Nuclei.

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The separation of nuclei from avian erythrocytes has been attempted by Warburg and by Miyake using the freezing and thawing technique.[1,2] However, the nuclei obtained by this method are not well separated and usually agglutinate in a mass. Laskowski was able to prepare a stable suspension of free nuclei of chicken blood using lysolecithin in neutral phosphate buffer solution for the hemolysis with subsequent washing of the nuclei with isotonic saline.[3] The poison glands of bees were ground with the lecithin emulsion which was then incubated for 24 hours at 37°C and finally filtered through a Berkefeld filter. The lysolecithin so prepared was added to the blood and after the hemolysis the nuclei were separated by centrifugation. This technique yields good preparations but the lysolecithin is not easily obtained under standard conditions. The procurement of the bee poison and the time required are disadvantageous conditions when large amounts of blood are used for chemical analysis.

The well known hemolytic action of tyrothricin, an antibiotic obtained from *Bacillus brevis* and commercially available,* has been used in our experiments with very satisfactory results. Snake erythrocytes after rapid hemolysis liberate the nuclei which are then washed several times in 0.9% saline and 5% citric acid solution. Microscopic examination of the nuclei suspension shows that they are not agglutinated and are almost free from the cytoplasm (Fig. 1). They are easily stained with methylene blue and Giemsa. The nuclei in suspension appear microscopically smaller and more compact than in the intact erythrocytes. This observation was also reported by Laskowski for chicken blood preparations.

1 Warburg, O., *Z. physiol. Chemie*, 1910, **70**, 413.

2 Miyake, M., *Keijo J. Med.*, 1933, **4**, 247.

3 Laskowski, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 354.

* Tyrothricin 2% solution, Parke, Davis & Co.