OBSERVATIONS ON THE FORMATION OF WHEALS

IV. The Influence of Calcium Concentrations on Histamine Wheals [1]

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The principal lesion of the type of allergy manifested by asthma, hay fever, and urticaria is a localized edema. This is typified in allergic urticaria wherein each wheal represents a local edema, and also in the wheal produced by a positive skin test to allergens. These wheals result from the contact between the introduced allergen and its specific antibody in the tissues. There is considerable evidence to show that when this contact occurs, histamine or a histamine-like substance is formed (1). It is this substance that causes the edema. Furthermore, when histamine itself is injected intradermally the resulting wheal is clinically and morphologically identical to an allergic wheal (2), a fact which holds true for both human and dog skin. Since it is possible, therefore, to simulate allergic edema with histamine, a method is offered for study of the mechanism of this lesion under controlled conditions.

In the course of experiments which we have previously reported, indications of the existence of a substance in normal skin which is capable of influencing the size of histamine wheals were noted (3). In investigating this point, it was found that saline extracts of dog skin, when added to the histamine solution used for injection, greatly enhanced the size of the resultant wheals.

The extract was prepared as follows: a dog was anesthetized with amytal, and a section of skin removed from the abdomen, (previously dehaired with sodium sulphide). The skin was chopped and extracted in physiologic saline solution (1 gram skin to 9 cc. saline), and histamine acid phosphate added to a concentration of 1 to 10,000. After standing one to two hours at room temperature, 0.02 cc. of the supernatant fluid was injected intradermally.

The injections were made on the uninjured side of the abdomen of the same dog, and on the outer surface of the thigh. At the same time,

[1] A preliminary report of this work appeared in the Proc. Soc. Exp. Biol. and Med., 1930, xxviii, 468.

similar injections were made of a 1 to 10,000 histamine [2] solution, to which no skin extract had been added, and of a 1 to 10 skin extract without histamine. Uniformly consistent results were obtained, and the wheals produced by the histamine-skin solution were invariably larger than those from the histamine alone. In fact, they frequently exceeded in size wheals

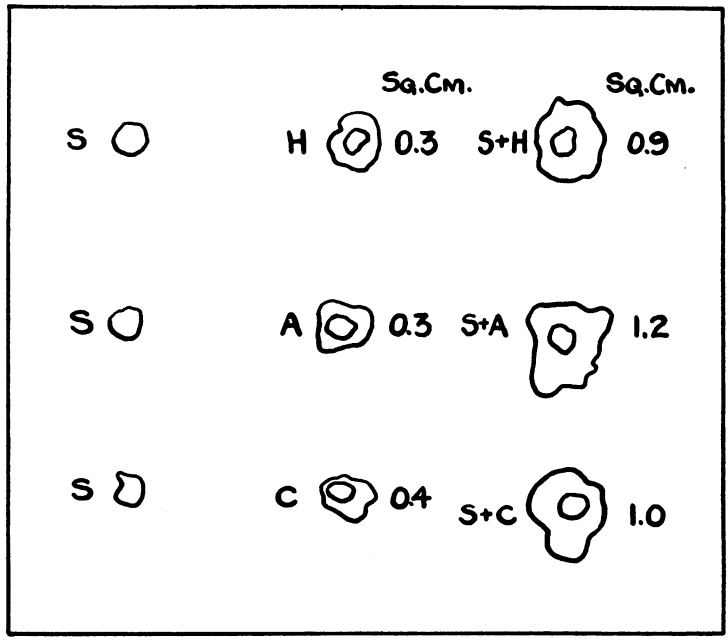

FIG. 1. THE EFFECT OF SKIN EXTRACT ON HISTAMINE, ATROPINE AND CODEINE WHEALS.

S = 0.02 cc. of skin extract 1 to 10 dilution.

H = Wheal from 0.02 cc. of histamine acid phosphate 1 to 10,000 dilution.

A = Wheal from 0.02 cc. of atropine sulphate 1 to 1,000 dilution.

C = Wheal from 0.02 cc. of codeine sulphate 1 to 1,000 dilution.

$S+$ = Wheal from 0.02 cc. of skin extract 1 to 10 dilution made up to a concentration of 1 to 10,000 histamine, and 1 to 1,000 atropine and 1 to 1,000 codeine.

Figures = Increase in size of wheals.

In this, and in the following figures, the measurements were obtained by marking with ink the outline of the original bleb due to the injection, and the outline of the resulting wheal at the end of fifteen minutes. Tracings were made of these outlines at once and measured with a planimeter. The difference between the two areas represents the size of the wheal, which is expressed in square centimeters.

[2] Hereafter by 1 to 10,000 histamine solution is meant a 1 to 10,000 histamine acid phosphate solution.

produced by a histamine solution 10 times as strong as that used. The skin extract alone gave no wheals. Similar results were obtained when either atropine sulphate or codeine sulphate was used instead of histamine acid phosphate as the wheal producing agent.

Table 1 compiled from the protocols of consecutive experiments is representative of the results obtained in a large series of such tests.

TABLE 1

The effect of skin extract on histamine wheals

Histamine*	Histamine and skin extract†
sq. cm.	*sq. cm.*
0.2	0.8
0.2	0.7
0.3	0.9
0.4	0.9
0.3	0.8
0.2	0.8
0.5	0.9
0.5	1.0
0.2	0.6
0.3	0.9
0.2	0.7
0.3	0.6
0.4	0.7
0.3	0.8
0.2	0.8
0.3	0.9
0.4	1.0

* Increase in size of wheals from 0.02 cc. histamine acid phosphate, 1 to 10,000 dilution measured in square centimeters fifteen minutes after injection.

† Increase in size of wheals from 0.02 cc. of skin extract 1 to 10 with histamine acid phosphate added to a concentration of 1 to 10,000 measured in square centimeters 15 minutes after injection. Each pair of figures represents the same experiment. No wheals occurred from the control injections of skin extract alone.

These results might be explained on the basis of Lewis' theory regarding the presence of histamine, or a histamine-like substance, (H-substance) in skin (1) were it not for the fact that the skin extract alone is incapable of producing a wheal, no matter how concentrated or diluted. Moreover histamine color tests by the method of Hanke and Koessler (4) were used to determine the amount, if any, of histamine present, since Harris and others have extracted histamine from the skin (5). These tests showed that histamine was present only in extracts which had decomposed slightly. When such extracts were freed from nitrogenous matter (by mercury precipation), including any histamine or histamine-like substance possibly present, their wheal enhancing activity was unimpaired. Consequently, the augmenting substance extracted from skin is evidently neither histamine nor a histamine-like substance.

These results induced us to undertake a detailed chemical analysis of skin extract to determine if possible the exact nature of the augmenting substance. The various steps in the separation were, briefly, as follows:

One part of chopped dog skin was soaked in nine parts of distilled water for at least one hour, and then filtered. Two volumes of 95 per cent alcohol were added to one volume of filtrate and the precipitated proteins removed by filtration. The filtrate from this step was evaporated to dryness, at temperatures below 56° F., and the resulting residue extracted with water. A saturated mercuric acetate solution was added to this watery extract, and this caused a white flocculent precipitate which was filtered off, washed and suspended in water. Hydrogen sulphide was passed through to precipitate the mercury. This precipitate was discarded and air passed through to free the acid solution from hydrogen sulphide. Thirty per cent mercuric sulphate in 10 per cent sulphuric acid, and barium carbonate were next employed (according to the method of West and others (6)) to free the solution from practically all remaining nitrogenous compounds and phosphates. Then, sulphuric acid was added slowly until all traces of barium were removed and the solution made slightly acid. The mercury and hydrogen sulphide were removed as before, and the filtrate from this step dialysed through thick collodion sacs. The activity was found to remain on the inside of the sac. This solution was evaporated and the slight residue extracted successively with hot chloroform, hot alcohol and hot water. The chloroform and alcohol portions were evaporated and water solutions made from each of the residues. The chloroform extracted all fatty material and the activity was found only in the water extracts from the alcohol and water portions. The solutions when evaporated were found to contain a few crystals of a distinct type which appeared again and again in various extracts at this stage. These crystals resembled those of calcium sulphate. Crystals to a weight of 3.6 mgm. were obtained from 1,000 cc. of an active extract representing 100 grams of tissue, and after quantitative analysis were found to consist largely of calcium sulphate.

We learned that calcium salts would not dialyse through a thick collodion membrane and consequently a technique was employed which produced thinner sacs (7). When these thin sacs were used for dialysis of the extracts, the activity was found in the dialysate. Quantitative calcium determinations showed also that most of the calcium was in the solution outside the sac.

Several simplifications were introduced into the method of preparing the extracts. (1) Since the sodium chloride was needed only to make the solutions isotonic, it was added just before testing on the dog. This allowed us to make up the extracts and carry them through the various stages of purification in distilled water instead of in saline. (2) In earlier experiments an incubation period of several hours, after addition

of the histamine solution, appeared to be necessary to obtain the full augmentation effect. With the later, purified extracts this period could be omitted, and the histamine added just before testing. Control tests were run on each extract at every stage of the process of separation. These were made by injecting intradermally the usual 0.02 cc. of the extract before adding any histamine. If the control gave a slight wheal (probably due to decomposition products of nitrogenous matters) the corresponding test, (the extract with added histamine), was discarded.

Parallel experiments were run on other tissues of the dog, and their extracts subjected to the same process of analysis. The heart, liver, lungs, muscle (both smooth and striated) were found to have more or less activity. Blood serum from both dog and man, gave just as consistent results as skin. Since blood serum was both easy to procure and to manipulate we employed it extensively. From these extracts, also, calcium sulphate crystals were isolated, and evidently the calcium present in these solutions was responsible for their augmenting effect.

As it now seemed clear that the augmenting effect was due to the calcium present in the extracts, quantitative calcium determinations were run on all the fractions obtained from the various steps of the analysis. It was found that the later, purer portions of the extract had a higher calcium content than the earlier impurer fractions. This led to the discovery that the reagents employed to precipitate the proteins present, such as mercuric acetate and barium carbonate, contained considerable calcium in impurities. Since the amount of calcium necessary to produce augmentation of histamine wheals is very minute, this gave a considerable source for error. Even the amount of calcium in ordinary filter papers was sufficient to influence the results of the experiment.

However, these steps had enabled us to eliminate practically everything but calcium from the extract, without destroying its augmenting effect. On repeating the analysis and using ashless filter papers, silica dishes for evaporating solutions, etc. active extracts were still obtained. Extracts prepared in this way had an average calcium content of 3 parts per million, a decrease of approximately 60 per cent, since no calcium impurities were added to the original extract.

Although it seemed that this amount of calcium was far too low to account for the results, solutions of pure calcium sulphate in corresponding dilutions were tested. Solutions of this salt at dilutions varying from 1 to 10,000 to 1 to 10,000,000 were found to have the same augmenting effect as the skin extract. The dilution of 1 to 100,000 usually gave the greatest augmentation. Calcium sulphate solutions in these dilutions produced no wheal except in the presence of histamine.

At an early stage in these experiments, ashing of the skin extract had been tried as a method for removing the organic material, but it was found that this procedure completely destroyed its activity. If the ash con-

tained a high calcium concentration a solution prepared from it remained inactive. But when this solution was diluted to the optimum calcium concentration (1 to 100,000) the augmentation effect was again apparent.

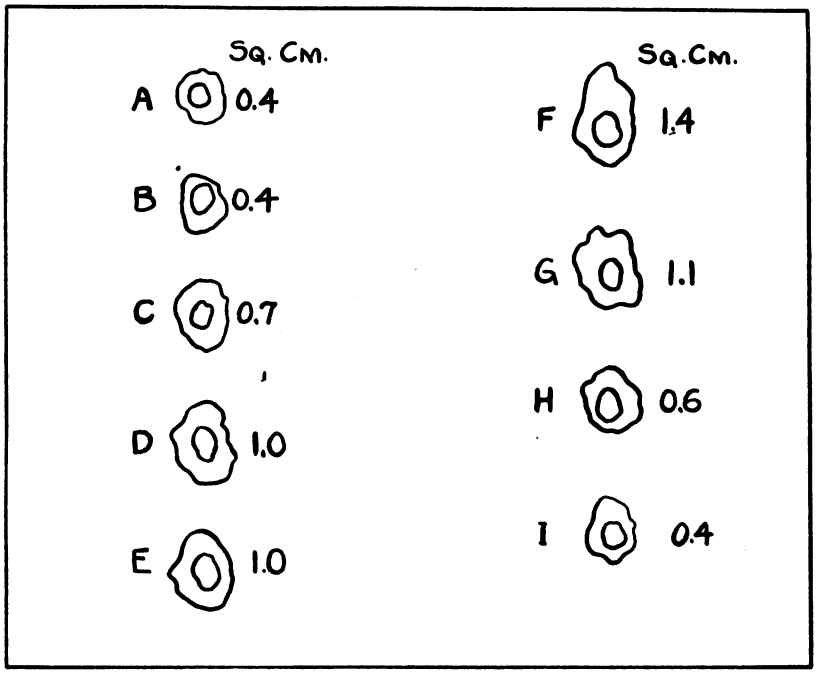

FIG. 2. THE EFFECT OF CALCIUM CONCENTRATIONS ON HISTAMINE WHEALS

A = Histamine acid phosphate solution 1 part to 10,000
B = $CaSO_4$ 1400 parts per million*
C = $CaSO_4$ 700 parts per million
D = $CaSO_4$ 100 parts per million
E = $CaSO_4$ 50 parts per million
F = $CaSO_4$ 10 parts per million
G = $CaSO_4$ 7 parts per million
H = $CaSO_4$ 0.8 part per million
I = $CaSO_4$ 0.08 part per million

At times ashing was substituted for the mercury precipitation of proteins. In weak solutions this improved their augmenting effect, since no calcium was lost in this procedure.

These results showed that the lack of augmenting power in an extract, or in a fraction of such an extract obtained during its analysis, might be

* All $CaSO_4$ solutions were made up to contain histamine acid phosphate 1 part to 10,000. Dilutions refer to calcium sulphate $CaSO_4 \cdot 2H_2O$. The calcium forms approximately one-fourth of the total.

due to an excess of calcium, rather than to its absence. Henceforth, all skin tests on the dog were made in triplicate, (*a*) at the usual concentration of one gram of skin to 9 cc. saline, (*b*) at one-tenth this concentration, and (*c*) at varying strengths of 3 to 7 times the usual concentration.

Although there was little doubt in our minds that calcium was responsible for all the augmenting effect, various other possibilities were considered. We found that strong solutions of phosphates also possessed

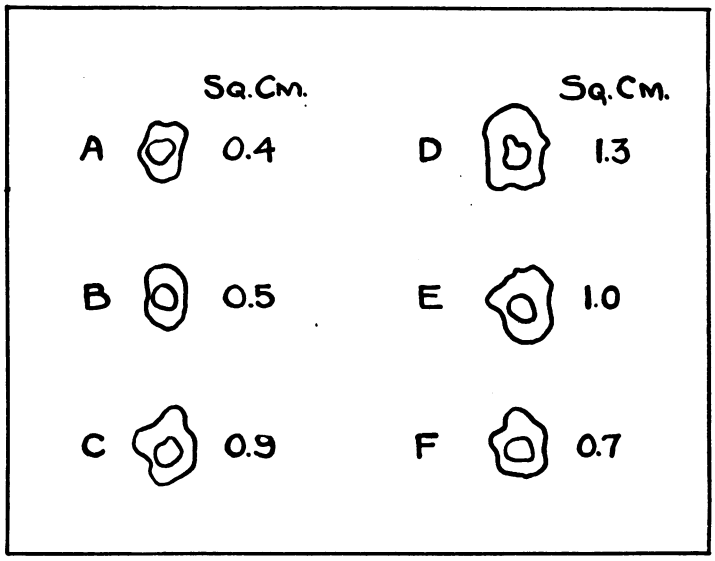

FIG. 3. THE EFFECT OF CONCENTRATIONS OF SKIN EXTRACT ON HISTAMINE WHEALS.

A = Histamine acid phosphate solution 1 part to 10,000
B = 1 part skin extract diluted 1.5 times with saline*
C = 1 part skin extract diluted 5 times with saline
D = 1 part skin extract diluted 10 times with saline
E = 1 part skin extract diluted 100 times with saline
F = 1 part skin extract diluted 200 times with saline

considerable augmenting power. In none of our extracts, however, was the phosphate present in sufficient concentration to have this effect. The phosphate contents were determined colorimetrically according to the method of Fiske and Subbarow (8), and were always much lower than the concentrations of sodium phosphate which are capable of augmenting histamine wheals.

One factor which had been constant throughout these experiments was the amount of sodium chloride which was added to produce isotonicity in

* All skin extract solutions were made up to contain histamine acid phosphate 1 part to 10,000.

the solutions just before testing on the dog. At this stage glucose was substituted for sodium chloride in order to exclude any effect which the saline solution may have had. The same results were obtained as when saline was used.

As the histamine used was in the form of an acid phosphate, this was replaced by an equivalent amount of histamine di-chloride to determine whether or not the negative radical had any effect. With histamine di-chloride in dilution of 1 to 16,600, comparable results were obtained, and the augmention was just as great and just as consistent as when histamine acid phosphate in dilution of 1 to 10,000 was used.

It was possible to inhibit the augmenting effect of skin extract on histamine in various ways. If the pH of the solutions was much more acid, or alkaline, than that of blood, the augmentation was decreased. Therefore, the pH was always adjusted to approximately 7.2 (using phenol red as the indicator), before testing on the dog, to insure obtaining the maximum augmentation.

The addition of strong soap solutions completely destroyed the activity of the extracts, and even cut down to a considerable degree the size of the standard histamine wheal. Sodium citrate solutions had a similar effect, possibly due to their power of changing the diffusibility of calcium (9). As stated above, too high a calcium concentration prevented augmenting effect. This held true also when calcium was added to an extract that was already active.

A 10 per cent solution of calcium chloride (10) will of itself induce a wheal not unlike that from histamine. This, however, is so far beyond the concentrations used in these experiments that the augmenting effect of calcium on histamine cannot be considered as merely an added effect.

SUMMARY

From these experiments, it appears that calcium possesses the power of enhancing the size of wheals due to histamine (or to atropine or codeine), but only in a definite zone of concentration. The optimum concentration for different dogs, however, varies considerably. In some the 1 to 10,000 dilution gave greater augmentation than the 1 to 1,000,000 dilution; in others this effect was reversed. In most instances the optimum dilution was 1 to 100,000. Outside of these limits, calcium seems incabable of increasing the size of wheals. The explanation for this is not clear. Further experiments are being carried out to study the behavior of wheals under conditions that are supposed to influence the concentration of calcium in the fluids and tissues of the body.

BIBLIOGRAPHY

1. Lewis, T., The Blood Vessels of the Human Skin and Their Responses. London, 1927.
2. Torök, L., and Lehner, E., Arch. f. Dermat. u. Syph., 1921, cxxxii, 401. Zur Anatomie und Pathogenese der Urticaria.
3. Alexander, H. L., Weaver, W. K., and McConnell, F. S., Proc. Soc. Exp. Biol. and Med., 1930, xxvii, 486. Observations on the Formation of Wheals. III. The Participation of an Unidentified Tissue Substance.
   Weaver, W. K., McConnell, F. H., and Alexander, H. L., Proc. Soc. Exp. Biol. and Med., 1931, xxviii, 468. Observations on the Formation of Wheals. IV. Influence of Calcium Concentrations on Histamine Wheals.
   Alexander, H. L., and McConnell, F. S., J. Allergy, 1930, ii, 23. Variability of Skin Reactions in Allergy.
4. Hanke, M. T., and Koessler, K. K., J. Biol. Chem., 1920, xliii, 543. Studies on Proteinogenous Amines. VIII. Quantitative Colorimetric Estimation of Histamine.
5. Harris, K. E., Heart, 1927, xiv, 161. Observations Upon a Histamine-like Substance in Skin Extracts.
6. West, E. S., Scharles, F. H., and Petersen, V. L., J. Biol. Chem., 1929, lxxxii, 137. The Determination of True Sugar in Blood.
7. Greenberg, D. M., and Gunther, L., 1930, lxxxv, 491. On the Determination of Diffusible and Non-Diffusible Serum Calcium.
8. Fiske, C. H., and Subbarow, T., J. Biol. Chem., 1925, lxvi, 375. The Colorimetric Determination of Phosphorus.
9. Schelling, D. H., and Maslow, H. L., J. Biol. Chem., 1928, lxxviii, 661. Effect of Sodium Citrate, Acetate, and Lactate on the Ultrafiltrability of Serum Calcium.
10. Sollmann, T., and Pilcher, J. D., J. Pharm. and Exp. Therap., 1917, ix, 309, Endermic Reactions. I.