STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

V. Sensitization to Diazomethane and Mustard Oil

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In continuation of studies on the production of hypersensitiveness to simple chemical compounds in animals, experiments have been carried out on sensitization to two non-aromatic substances, diazomethane and allylisothiocyanate.

While it would be rather pointless at present to extend anaphylactic experiments to a great variety of proteins which in general behave much alike, in spite of differences in their sensitizing activity (Doerr) and the apparent absence of this capacity in peculiar proteins such as gelatine, with simple chemical compounds, on the other hand, new problems are offered with each different group of substances as regards the possibility of inducing sensitization, the search for appropriate methods to attain this end, and the mode of action of the substances in the animal body. Indeed, it may be stated again, with a considerable number of substances which cause severe allergic disease in man experimental sensitization has not been surely achieved either in animals or in human beings, and this holds true even for cases where the formation of antigenic conjugates might be expected on chemical grounds. The two compounds investigated in the present article are readily capable of forming conjugates.

1. Diazomethane

Diazomethane is a yellow gas of the formula CH_2N_2. It is widely used in preparative chemistry on account of its high reactivity, especially for the introduction of methyl groups in acids, alcohols, and nitrogen compounds. Its toxicity was noticed by its discoverer von Pechmann (1) and the substance has since proved troublesome to laboratory workers. In part the effects were found to be attributable to a condition of hypersensitiveness as stated by Arndt (2).

According to this author, a person may be able to work with the compound for some time without untoward effects. Afterwards, however, hypersensitiveness may develop, even if precautions are taken, so that for such persons it is almost impossible to work with diazomethane without being subjected to severe attacks of asthma and fever. Experimental sensitization with the substance seems not to have been reported.

Our first attempts to sensitize guinea pigs were made with repeated applications of a solution of diazomethane in dioxane since ether solutions commonly used in chemical work evaporate too rapidly. The results were positive in part but were inconsistent. Several modifications of the procedure were tried and these experiments suggested that solvents having irritating properties in themselves give better results. In particular it seemed that dioxane containing peroxides such as is frequently met with was more suitable than pure dioxane. Positive results were also observed when cottonseed oil was employed as solvent.

The following method was finally found satisfactory in preliminary experiments and was then applied to a rather larger group of animals.

Diazomethane was prepared in the usual manner from nitrosomethylurethane but dioxane was substituted for ether as solvent and the gas was absorbed in cooled pure dioxane. The solution made in this way, containing 20 to 25 mg. CH_2N_2 per cc., was used in the tests. For sensitizing the solution was diluted with an equal volume of dioxane 0.01 molar with respect to organic peroxide (determined iodometrically), obtained by concentration *in vacuo* of commercial dioxane.

The solution of dioxane was applied to the skin of the haunch, the hair being removed before each application by means of an electric clipper. 10 drops were allowed to fall from a capillary pipette onto the skin and this was repeated twice at intervals of about 15 minutes, totalling 6 to 8 mg. diazomethane per day per animal. Obviously, a large part of the substance evaporated since the site became dry after a short time. As the treatment continued, the treated site became pink and rough and layers of scales developed. The skin beneath the scales remained unbroken in most cases. In order to secure contact of the solution with the skin the scales were removed when necessary by clipping after softening with olive oil. After the animals had received twelve such treatments within 2 weeks, they were tested 3 weeks later by applying a saturated solution of diazomethane in pure dioxane, as mentioned above, for 2 or 3 successive days, on the flank.

A lot of 40 male albino guinea pigs weighing between 330 and 450 gm. were treated in the manner described. The 38 surviving animals were tested by applying diazomethane solutions on 3 successive days. Of ten control animals similarly treated three had a very faint pink color on the site of application 24 hours after the third treatment, the others were practically negative. Of the experimental animals 60 per cent showed definite reactions of varying degrees. The reactions consisted in erythema ranging from faint pink to pink color. The test sites often were somewhat elevated, and thickening could be detected upon pinching up a fold of skin. The best reactors, 12 in number, were selected and subjected to a second course of 8 treatments on the opposite haunch, followed 3 weeks later by test applications given on the unused flank on 2 successive days (controls 23 to 32). The reactions observed in these animals after the first and the second course are tabulated (Table I). From the table it appears that a second course of applications had distinctly increased the degree of sensitization. When treatment of the sensitized animals was continued on one site, employing diazomethane without peroxide, the marked thickening and scaling of the skin appeared much more quickly than in non-sensitized animals even when in the latter there was the added effect of peroxides.

In the advanced stage microscopical examination showed that the epithelial layer was very much thickened (up to five times its normal depth), in proliferation, with increased keratin layer and papilli extending into the dermis.

A point of interest in the reported experiments concerns the nature of the sensitizing substance which is so highly reactive that it doubtless combines with substances of the animal body rapidly after administration and for this reason the spread of sensitization can hardly be ascribed to the distribution of the exciting substance itself but to transportation of some sort of conjugate[1] or, perhaps, of antibodies.

Although the profound change in the serological properties of proteins through methylation has been established in previous work (3), it is still undecided whether the sensitization effects described

[1] For the antigenic activity of methylated proteins, see Landsteiner (3).

TABLE I

Reactions of animals sensitized to diazomethane, after one and two courses of treatments, and of non-sensitized control animals. The tests were made on a fresh site of the skin by applying a diazomethane solution as described in the text on each of 3 (or 2) successive days. Readings were made the day following each application.

No.	Reactions after first course			Reactions after second course	
	First application	Second application	Third application	First application	Second application
1	pp., mac.	pp.–p.	pp.–p., th., sc.	pp.	dp., th.
2	vfp.	fp.–pp., mac.	pp.–p., sl.sc.	pp., mac.	p.
3	pp., mac.	pp.	fp.–pp., sc.	pp.–p.	pp.
4	fp.–pp., mac.	p., sl.th.	pp.–p., th., sc.	p.	dp., sl.th.
5	vfp.	pp.	p., sc.	pp.	p., sl.th.
6	pp., mac.	p., sl.th.	p., sc.	bp., sl.th.	dp., th.
7	fp., mac.	pp.–p.	fp.–pp., sc.	pp.–p.	pp.–p.
8	pp.	p., sl.th.	pp.–p., sc.	bp., sl.th.	bp., th.
9	fp.–pp., mac.	pp.–p.	p., sc.	p.	no application
10	fp.	p.	pp.–p., sc.	bp., th.	bp., th.
11	vfp., mac.	pp.–p.	pp., sl.sc.	pp.–p.	p., sl.th.
12	fp., mac.	pp.	p., sl.th.	p.	dp.
			Controls		
13	neg.	neg.	neg.	23 neg.	fp., mac.
14	neg.	neg.	neg.	24 neg.	vfp., mac.
15	neg.	vfp.	neg.	25 vfp., mac.	fp.
16	neg.	neg.	neg.	26 fp., mac.	fp.–pp.
17	neg.	vfp.	neg.	27 neg.	al.neg.
18	neg.	al.neg.	al.neg.	28 neg.	fp.
19	neg.	al.neg.	al.neg.	29 vfp., mac.	vfp.
20	neg.	vfp.	al.neg.	30 fp., mac.	fp., mac.
21	neg.	neg.	neg.	31 al.neg.	vfp., mac.
22	vfp.	al.neg.	vfp.	32 neg.	al.neg.

The following abbreviations are used: negative (neg.), almost negative (al. neg.), faint pink (fp.), very faint pink (vfp.), pale pink (pp.), pink (p.), bright pink (bp.), dark pink (dp.), slightly thickened (sl.th.), thickened (th.), macular or spotted (mac.), slight scaling (sl.sc.), scaling (sc.).

are simply due to the formation of antigenic methyl proteins. In some cases unquestionable though slight anaphylactic reactions were seen in guinea pigs sensitized to diazomethane upon injection of

methylated guinea pig proteins.[2] This phase ought to be studied more extensively. Also, it has not yet been investigated whether skin sensitivity to diazomethane can be produced by injection of methylated proteins. In the study of some other substances an effect of this sort was not obtained (4).

Skin tests with other methylating chemicals gave definite cross reactions in the case of nitrosomethylurethane.[3] In a few cases methylsulfate produced intense skin reactions in sensitive animals but the results on reapplication were irregular and therefore are only mentioned incidentally.

The high toxicity of dimethylsulfate which at times has caused deaths in factory workers is in part due to its local corrosive action, in part to a systemic action. General toxic effects (6) have likewise been observed with methylchloride which has been widely used as refrigerant. In the literature we found only one casual remark (7) to the effect that individual idiosyncrasy may play a part in the poisoning by methylchloride. From the preceding, however, some attention, in our opinion, ought to be paid to the possibility of sensitization by methylating substances other than diazomethane.

2. *Allylisothiocyanate (Mustard Oil)*

Hypersensitiveness to mustard oil has been reported in two cases, to our knowledge, a small number in view of its not uncommon use as "counterirritant." Lehner and Rajka (8) described increased reactivity of the skin to mustard oil in a patient who had received eleven daily rubbings on the same site. The authors remarked that the individual appeared to be more sensitive than normal persons from the beginning. In a second similarly treated case Tezner (9) claims that he obtained local, not general, sensitization of the skin, showing immediate but no delayed skin reactions.[4]

On account of these reports it was deemed of interest to investigate the possibility of sensitizing animals and also to repeat the test with human beings.

[2] It should be mentioned that in these animals some superficial sores had developed on the treated site.

[3] On the use of this compound and of nitrosomethylurea as methylating agents, see (5).

[4] It may be mentioned that an increase in resistance to mustard oil on repeated administration to the skin of rabbits has been reported by Saudek (10).

Six persons from our laboratory were treated on 6 days each week for 3 weeks by allowing 1 drop of synthetic mustard oil to fall onto the skin of the forearm. This was followed by immediate hyperemia which faded soon. In five of these individuals there was no significant change except for a few slight transient reactions (delayed) in two individuals which may perhaps indicate a very low grade of sensitization. The sixth person, however, developed distinct hypersensitiveness. In this case, on the 13th application on the same site, an erythematous reaction began to appear after about 12 hours, and on the following day the site was intensely red, sharply demarcated, and slightly elevated, whereas before in this and the other individuals no reaction, or only a faint color was to be seen on the next day. The erythema began to fade on the 3rd day and gradually the site became brownish. When the sudden increase in reactivity was first observed, two other sites on the arms were tested and reactions developed similar to that described. On the next day, several other areas were tested (chest, back, both legs, and one arm) with positive results. Only minor differences in the intensity of the reaction were seen in the various regions. On testing fresh sites on the arm with drops of various dilutions of mustard oil in absolute alcohol, a definite though not intense reaction was still seen with a dilution of 1:20.

In order possibly to increase the sensitivity another course of 15 applications was given this individual 10 weeks later. Almost 3 weeks after the termination of the second course, the skin was still definitely hypersensitive, but the intensity of the reactions was somewhat diminished; a month later a new test site showed again a very distinct reaction.

Attempts to sensitize animals were made with guinea pigs by repeated superficial application and also by intracutaneous injection of mustard oil diluted with olive oil. In this species, in three monkeys, and three rabbits no definitely positive results were obtained. On the other hand, of three young hogs (Chester Whites) treated in similar manner as the human beings, two became distinctly hypersensitive.

One animal which had received eighteen superficial treatments on two sites, within 3 weeks, was tested on a new site by gently spreading 1 drop of mustard oil with a glass rod. It gave a pronounced reaction, whereas before sensitization this animal and also other normal pigs

showed no or very light erythema, only exceptionally a somewhat stronger reaction, on the day following an application of mustard oil. A second course seemed to increase the sensitivity.

Another male hog, after having received eight applications of a drop of mustard oil within 10 days on the same site, showed a strong reaction—pink elevated area seen on the following day—when tested on a new area with a drop of the substance. Further treatment of the animal resulted in some increase of sensitivity. A "titration" gave positive reactions with as little as a drop of a 5 per cent solution in dioxane.

In all instances, in contrast to the statement of Tezner, the skin sensitivity was not local but general, and the reactions were delayed, not immediate. In some of our tests the erythema at once following upon application of the substance seemed to be somewhat more pronounced and more lasting in sensitive than in non-sensitive individuals, but the differences were too small to be considered as significant. Furthermore, experiments made in view of the claims by Lehner and Rajka—of importance if correct—that they were able to demonstrate passive transfer of sensitivity with human serum in guinea pigs, gave negative results. When their procedure was used, normal animals also exhibited toxic symptoms not weaker than those in the "passively sensitized" ones.

As to the mode of sensitization with mustard oil, it may be pointed out that the substance reacts with amines and amino acids (11). Also, when we treated protein with mustard oil a reaction took place similar to that observed by Hopkins and Wormall (12) who were able to couple proteins with phenylisocyanate. Naturally, this does not permit one to decide whether a combination of isothiocyanate with protein or perhaps with some other substance in the body is responsible for the sensitization.

From the results described it would seem that swine are more apt to develop hypersensitiveness to mustard oil than guinea pigs which, however, can be readily sensitized to a number of other chemicals. This may indicate species differences in the reaction to various sensitizing simple compounds. Whether in general hogs offer an advantage in similar studies with other substances remains to be seen. Regarding the test with human beings it may be pointed out

that only one out of six became definitely sensitive; hence there appears to exist considerable individual variation in the ability of man to become hypersensitive to mustard oil, and therefore this substance would probably be of use in the study of hereditary disposition to drug hypersensitiveness.

SUMMARY

With the view of making new types of chemicals accessible for investigations on drug hypersensitiveness, methods have been devised for sensitizing animals with diazomethane and mustard oil, two non-aromatic compounds.

Guinea pigs have been sensitized to diazomethane, a substance of high reactivity and known to cause severe allergic effects in man.

With the second substance, allylisothiocyanate, likewise capable of forming conjugates with substances in the animal body, sensitization effects have been obtained in man and in hogs. Sensitization in human beings was successful with one out of six individuals treated.

The observations indicate species and individual differences as regards the ability to become sensitized to various chemical compounds.

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