ANAPHYLACTIC SHOCK BY AZODYES. II

BY K. LANDSTEINER, M.D., AND J. VAN DER SCHEER

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, October 5, 1937)

As mentioned previously, the fact that antibodies, contrary to common belief, are capable of reacting not only with proteins but with many types of substances of simple composition was first established by means of the so called inhibition reaction (1). With the use of a physicochemical method confirmatory results were obtained by Marrack and Smith (2), and Haurowitz and Breinl (3). (See also (4).) Although further support was not needed, it was of interest to observe that certain azodyes of simple composition give direct precipitin reactions like protein antigens and produce anaphylactic shock in guinea pigs sensitized with corresponding azoproteins (Landsteiner and van der Scheer (5, 6)), a result of some significance for the question of allergic reactivity to simple substances in general.

A recent publication by Fierz-David, Jadassohn and Zürcher (7) has prompted us to carry out an additional investigation. These workers made a significant observation on one of the dyes used in our experiments, namely the coupling product of diazotized aminosuccinanilic acid with resorcinol. On coupling resorcinol in two steps, first with one molecule of the diazo compound in acid solution and then with a second molecule at alkaline reaction, they obtained a dye1 which gave the same analytical figure as ours2 prepared by coupling in alkaline solution with two molecules of the diazonium compound, but which showed somewhat different properties. In distinction to the F-substance the L-dye shows a violet hue in alkaline solution and is decomposed under conditions which leave the other dye practically unchanged. Thus the authors found that when a

1 Hereafter designated by F.
2 Designated by L.
solution of the L-dye was kept at 37°C. in a 1.4 N Na₂CO₃ solution containing R-salt for 10 days, a large proportion of the succinanilic residues had combined with R-salt, the recovered dye containing only 13.04 per cent N; a noticeable change was found after only 20 minutes. On account of this result, the authors suggested that in our experiments anaphylactic shock had been elicited not by the dye itself but by an azoprotein formed by interaction with serum protein following intravenous injection. As proof for their explanation the authors stated that they had been unable to obtain contractions of the uteri of sensitized (with dye) guinea pigs, using the dye in Schultz-Dale experiments, while the same experiment made with succinanilic acid azoprotein gave typical positive results. The failure of the dye to cause a positive Dale reaction was explained by reasoning that in this experiment there is no occasion for the formation of azoproteins in the bath (i.e. the bath does not contain serum proteins). Consequently no anaphylactic effect would occur, according to their concept.

In our opinion, the explanation offered by Fierz-David, Jadassohn and Zürcher for the anaphylactic shock which we induced with dyes meets with certain difficulties from the outset. An obvious objection is that the splitting off of azo groups from the L-dye proceeds slowly, especially at serum pH, so that an amount of azoprotein sufficient for producing shock can hardly be formed in the few minutes elapsed between injection and anaphylactic shock, especially considering that only a small amount of the dye is injected. Another objection arises from experiments of theirs in which the L-dye was mixed with serum and the mixture kept for 24 hours at room temperature before performing the Dale test. Whereas uniformly positive results were to have been expected from the point of view of Fierz and his colleagues, actually only one out of five uteri reacted. And then, there is a convincing counter-argument in that Klopstock and Selter (8) were unable to shock sensitized guinea pigs with solutions of diazonium compounds despite the high reactivity of these substances, whereas

3 The interpretation of the authors nevertheless may well hold for the sensitization by the dye which they obtained, since in this case there would be sufficient time for the supposed formation of azoproteins.

4 This result is understandable if one takes into account that on introduction
they were successful in sensitizing the animals with the diazonium compounds. Also, Fierz, Jadassohn and Stoll (10) succeeded in sensitizing but not in producing anaphylactic reactions with sodium atoxyl-diazoaminosulfoanthranilate, which they suppose to lead to the formation of azoproteins in the animal body.

**EXPERIMENTAL**

In order either to confirm or disprove the conclusions at which we had arrived previously, it was our first concern to repeat the precipitin tests with the stable dyes prepared by the method of Fierz. The result was unequivocal in that these dyes made from aminosuberanilic and aminosuccinanilic acids were specifically precipitated by the corresponding rabbit immune sera even more quickly and intensely than the less stable L-dyes. This at once excludes the explanation of azoprotein formation as being responsible for the success of the precipitin tests, and, at the same time, it renders this idea improbable also for the anaphylactic effect, because any substance which gives a precipitin reaction with immune sera would generally be expected to induce anaphylactic shock. As a matter of fact, the stable dyes proved to be effective in anaphylaxis experiments as well.

Guinea pigs weighing 250 to 300 gm. received two intraperitoneal injections, a week apart, of 1 cc. of a 1 per cent solution of azoprotein prepared from diazotized p-aminsuccinanilic acid and horse serum as described previously. The animals were tested 3 weeks after the last injection with a solution (6) of the para-aminsuccinanilic acid resorcinol dye made according to the method prescribed by Fierz. The results are presented in Table I. Of seven animals injected intravenously with 1 mg. of the dye, four showed typical anaphylactic shock and died after 5, 7, 8 and 23 minutes, respectively, and the remaining animals showed definite anaphylactic symptoms of varying degree. No effect was observed upon injection of 2 mg. of the dye into normal guinea pigs.

---

of the diazonium compounds into the bloodstream this substance becomes mixed with a large excess of serum proteins and consequently an azoprotein containing enough azo groups to be reactive would not be formed (9), even if the reactions were to proceed at a velocity higher than that observed *in vitro*, which we have no reason to assume.

---

5 For the technique see (5).
Passive sensitization experiments were made by injecting guinea pigs of approximately 300 gm. weight intraperitoneally with precipitating serum from a rabbit which had been immunized by intravenous injection of suberanilic acid azoprotein (made with horse serum) and which reacted strongly with an antigen prepared from $p$-aminosuberanilic acid and chicken serum. Three guinea pigs sensitized with 1 cc. of the immune serum were injected intravenously the next day with 0.33 mg. of the suberanilic acid dye (made according to the method of Fierz) in 1 cc. Of these animals one died in acute

shock in 3 minutes, one had severe and one medium anaphylactic symptoms. Three animals injected with 2 cc. of the immune serum died in 3 to 5 minutes on intravenous injection of 1 mg. of the dye the next day.

Likewise three out of four guinea pigs passively sensitized with 2 cc. of rabbit immune serum to succinanilic acid azoprotein died of anaphylactic shock within 4 minutes when they were reinjected intravenously the next day with 2 mg. of $F$-succinanilic acid dye.

Injection of either dye into non-sensitized animals caused no symptoms.

| Animals Tested by Intravenous Injection of Solutions of Resorcinoldisazo-$p$-Succinanilic Acid Prepared According to the Method of Fiers-David and Coworkers |
|---|---|---|---|
| Animals sensitized with azoprotein made from $p$-aminosuccinanilic acid | Normal animals |
| Guinea pig No. | Amount of dye injected | Subsequent change in body temperature | Result, symptoms | Guinea pig No. | Amount of dye injected | Subsequent change in body temperature | Result, symptoms |
| | mg. | °C. | | | | | |
| 1 | 2 | -1.0 | Medium to severe | 10 | 2 | +0.3 | Negative |
| 2 | 1 | -1.0 | Slight | 11 | 2 | -0.7 | " |
| 3 | 1 | -1.8 | Medium | 12 | 2 | -0.8 | " |
| 4 | 1 | -2.0 | Slight | | | | |
| 5 | 1 | -2.9 | Slight to medium | | | | |

† means death of animal.
TEXT-FIG. 1 A. Anaphylactic contraction to F-resorcinoldisazo-$p$-succinanilic acid dye (suc.) in a guinea pig sensitized by injections of succinanilic acid azoprotein. X designates washing. Histamine (hist.) was added to a concentration of 1:20,000,000.

TEXT-FIG. 1 B. Specificity test. Passive sensitization with antiserum for succinanilic acid azoprotein, the uterus responding not to F-resorcinoldisazo-$p$-phenylarsenic acid dye (as.), but to suc.
**Text-Fig. 2 C.** Specificity test, converse to Text-fig. 1 B. Passive sensitization with antiserum to $p$-aminophenylarsenic acid azoprotein, reaction to as. but not to suc.

**Text-Fig. 2 D.** Specificity test. Passive sensitization with antiserum to tartranilic acid azoprotein, tested with as. and with $F$-resorcinoldisazo-$p$-tartranilic acid.
In view of these results it remained to be investigated whether Schultz-Dale effects could not indeed be produced with the azodyes under discussion without the presence of serum proteins.

Female guinea pigs of 170 to 200 gm. were sensitized by two intraperitoneal injections, with an interval of 4 days, of 0.5 cc. of a 1 per cent solution of succinanilic acid-azoprotein. The uteri of these animals were tested 3 to 4 weeks later in a Dale apparatus by adding 0.02 to 0.1 mg. F-succinanilic acid-resorcinol dye to a bath of 20 cc. capacity (cf. 11); the horns were suspended in Dale solution with the calcium chloride reduced to one-half (NaCl 0.9 per cent, KCl 0.042 per cent, CaCl₂ 0.006 per cent, NaHCO₃ 0.05 per cent). The reactions were strongly positive in three out of five animals, the horns remaining in maximal contraction for 2 to 3 minutes. In the other animals one medium and one negative reaction were observed. After the uteri had relaxed and the bath had been changed in the usual way, desensitization could be demonstrated by the failure to respond to a second dose of the dye (Text-fig. 1 A). Another lot of guinea pigs sensitized by three instead of two injections as above with intervals of 4 and 3 days gave definitely poorer results since only 2 of the 6 uteri reacted, one of them with submaximal contraction.

The method of passive sensitization offered promise of more uniform results. Six female guinea pigs weighing between 180 and 220 gm. were passively sensitized by intraperitoneal injection of 2 or 2.5 cc. of a succinanilic acid rabbit immune serum, as above. When the uteri were tested the following day by the Dale method with the F-succinanilic acid-resorcinol dye, positive reactions were obtained in all cases, with maximal sustained contractions in four and submaximal contraction in the remaining two instances. The test dose for the most part was 0.01 mg. of dye but even quantities as small as 0.002 mg. of dye produced maximal effects. Specificity tests were performed by first adding to the bath another dye such as resorcinoldisazo-p-suberanilic acid or resorcinoldisazo-p-phenylarsenic acid made by the method of Fierz and coworkers. No reactions were observed with the heterologous substances, and subsequent addition of F-succinanilic acid resorcinol dye elicited the usual typical contraction; similarly, the succinanilic acid dye did not cause contraction of uteri sensitive to other azodyes (Text-figs. 1B and 2C). With the uteri
of normal guinea pigs none of the dyes caused an effect with quantities even of 0.5 mg. in the 20 cc. bath.

Similar reactions were secured in passive sensitization with a rabbit immune serum for suberanilic acid azoprotein, the tests being made with the corresponding dye.

These striking results suggested trying analogous experiments with some other azodyes for which immune sera prepared with the corresponding azoproteins were available. Positive effects were found with azodyes made according to the method of Fierz from resorcinol and \( p \)-aminophenylarsenic, \( p \)-aminotartranilic acids and \( m \)-aminobenzoyl glycine, while the dyes made from \( m \)-aminobenzenesulfonic and \( m \)-aminobenzoic acids were ineffective. Also here the specificity of the reactions could be demonstrated unambiguously (Text-fig. 2 D). It was further observed that the positive cases were those in which addition of Ca salts to a 0.01 per cent solution of the dye caused precipitation within a few hours at the most, whereas in the negative cases precipitation appeared very slowly (visible on the next day), or not at all. Since the bath in which the uteri are suspended contains calcium, one may surmise that this property of the dyes may be of influence in obtaining positive results; however the number of instances investigated is relatively small. With the concentrations obtaining in the tests, no precipitation was observed in the bath during the experiments; only with the suberanilic acid compound, if the concentration was high enough, a turbidity was soon seen and later a precipitate appeared.

SUMMARY

From the experiments presented, it follows that the specific precipitation and the production of anaphylactic shock with certain azodyes, as described previously, is due to these substances themselves and is not dependent upon formation of azoproteins by interaction of the dyes with proteins in the test tube or the animal body.

Besides these, some other azodyes which in our tests did not give precipitation with corresponding immune sera were also found, in very small quantities, to induce anaphylactic contraction of the uterus of sensitized guinea pigs.

*On the possible relation of the precipitability of dyes by means of metal salts to the degree of dispersion, vide (12).*
K. LANDSTEINER AND J. VAN DER SCHEER

BIBLIOGRAPHY