

ASPIRIN SENSITIVITY—EXPERIMENTAL STUDIES*†

ALAN R. FEINBERG, M.D., AND SAUL MALKIEL, M.D., CHICAGO, ILL.

EXPERIMENTAL sensitization to salicylates has been the subject of previous investigations,¹⁻³ undoubtedly as a result of the relatively severe allergic reactions of human beings to drugs and especially to aspirin.⁴⁻⁸ Suzuki¹ reported that guinea pigs could be sensitized to aspirin by various routes, including the oral, and that he could differentiate in this way between Bayer, Japanese, and soluble aspirin. These results were not confirmed by Frei² who, in addition to these techniques, used "old" arsphenamine as an adjuvant. Sensitization of rabbits to aspirin was accomplished by Butler, Harington, and Yuill³ by using a conjugate of aspirin with horse serum globulin. A reduction of the antipyretic action of aspirin in rats with artificially produced pyrexia was afforded by passive transfer of these antibodies. This method of determining sensitivity was questioned by Marrack.⁹

Storm van Leeuwen and Drzimal¹⁰ reported that the serum proteins of aspirin-sensitive asthmatics combined with less salicylate, *in vitro*, than did normal human sera. Lester, Lolli, and Greenberg¹¹ found that the plasma of rheumatic fever patients bound a much lower percentage of salicylate as compared to normals.

The following experiments were carried out in an attempt to clarify the mechanism of aspirin sensitivity and, perhaps, to afford a better understanding of drug allergy in general.

EXPERIMENTAL

Aspirin Antigens.—Many preparations of aspirin were tried in an attempt to enhance its antigenicity for the parenteral immunization with the drug. In the early experiments, because of the relative insolubility of aspirin in water, the drug was dissolved in an ethyl alcohol-water solution. Later experiments were conducted using the soluble sodium salt of the drug, prepared by allowing the aspirin to dissolve in an aqueous solution of sodium bicarbonate. This solution must be made up just before use for the sodium salt decomposes on standing to insoluble salicylic acid. Other preparations included aspirin adsorbed onto aluminum hydroxide and an emulsion of horse serum-mineral oil-aspirin.

Conjugates of aspirin to human serum and to casein were made by allowing aspiryl chloride to react with the appropriate protein according to the usual techniques such as described by Kabat and Mayer.¹² The acid chloride was obtained from aspirin by the action of thionyl chloride following the procedure of Riegel and Wittecoff.¹³ The test solutions of the conjugates were made up in an isotonic salt solution to a concentration of 1 per cent protein, sterilized by passage through a Berkfeld filter and stored at 4° C.

Suspensions of aspirin or conjugate in Freund's adjuvant,¹⁴ aquaphor-mineral oil-killed tubercle bacilli, were also made.

*From the Department of Internal Medicine, Division of Allergy, and Allergy Research Laboratory, Northwestern University Medical School, Chicago, Ill.

†Presented at the Sixth Annual Meeting of the American Academy of Allergy, Los Angeles, Calif., March 6-8, 1950.

Ultrafiltration Technique.—Ultrafiltration of serum-salicylate mixtures was carried out in order to determine the capacity of the serum proteins to bind the drug. The apparatus used was a Seitz filter adapted for pressure filtration. A circular piece of filter paper covered by a circle of Cellophane replaced the filter pad ordinarily used. The pressure of gas from a tank of nitrogen was the positive pressure used. Some of the preliminary dialysis experiments were carried out using a rocking dialyzer.

Salicylate Determination.—Salicylate was determined according to the method of Smith et al.¹⁵ with certain modifications. To 1 ml. of serum (or filtrate) was added 1 ml. of distilled water, 0.5 ml. of 6N HCl and 25 ml. of toluene in a 60 ml. glass-stoppered bottle. The mixture was shaken vigorously for 5 minutes and then centrifuged at 1500 r.p.m. for 5 minutes. In a 60 ml. separatory funnel was placed 20 ml. of the supernatant toluene layer, 10 ml. of distilled water, and 0.25 ml. of an aqueous 0.5 per cent FeCl_3 solution containing 0.7 ml. concentrated HCl/100 ml. This mixture was shaken for 5 minutes, the colored aqueous layer removed and centrifuged lightly, to separate any dispersed droplets of toluene. Readings were made in a Coleman spectrophotometer at 540 $\text{m}\mu$. A standard curve was prepared using known amounts of salicylate. Free or conjugated aspirin or conjugated salicylate could be determined by a preliminary acid hydrolysis.

Toluene was substituted for ethylene dichloride which is used in the conventional procedure primarily because of the lesser degree of toxicity of the vapors and partly because of the added simplicity of manipulation. The use of toluene was justified since, in preliminary trials, known amounts of salicylate added to serum were quantitatively recovered.

Active Sensitization of Guinea Pigs.—About 0.25 Gm. of aspirin in the various menstrua described above was injected into series of guinea pigs either intraperitoneally or intramuscularly every other day for a total of 3 doses. After a 2-week incubation period the animals were challenged by an intravenous injection of either 0.25 Gm. of sodium acetylsalicylate or 2 ml. of the aspirin-casein conjugate.

Another series of guinea pigs were sensitized by a single intraperitoneal injection of 2 ml. of a 1 per cent aspirin-protein conjugate solution. Challenge after 2 weeks was by the intravenous injection of sodium acetylsalicylate, sodium salicylate, or the heterologous aspirin-protein conjugate.

Aspirin-protein conjugate incorporated in Freund's adjuvant was given intramuscularly, 0.5 ml. into each gluteal muscle, to a series of guinea pigs. The animals were challenged by the heterologous antigen and the salicylate preparations after about a 2-week incubation period.

Active Immunization of Rabbits.—Rabbits were injected with 1 ml. aspirin-protein conjugate 3 times weekly on successive days. The first dose of each week was given intraperitoneally followed by 2 intravenous injections. Trial bleedings were taken and the sera tested for precipitins to aspirin. In an attempt to enhance the antibody titer, 1 ml. of aspirin-protein conjugate in Freund's adjuvant was injected intramuscularly twice weekly and trial bleedings taken periodically.

Passive Sensitization of Guinea Pigs.—Guinea pigs were passively sensitized by the intraperitoneal injection of 5 ml. of immune rabbit serum. The animals were challenged after 48 hours by an intravenous injection of the heterologous antigen.

In addition, 3 guinea pigs were injected intravenously with 1 ml. doses of aged human serum from a patient clinically shown to be violently sensitive to aspirin. Two hours later, the animals were challenged by the intravenous injection of 1 ml. of aspirin-human serum conjugate. Seven other guinea pigs were injected intraperitoneally with 1 to 4 ml. doses of serum from 3 aspirin-sensitive patients. After a 24- to 48-hour interval, the animals were challenged with 2 ml. of the aspirin-casein conjugate given intravenously.

Skin Testing of Human Beings.—Sera from 5 aspirin-sensitive patients were tested by intradermal passive transfer on normal subjects. Twenty-four to 48 hours after the preparation, the sites were inoculated with aspirin-human serum conjugate. In 1 subject the sites were challenged by the oral administration of 20 grains of aspirin. Two patients, clinically sensitive to aspirin, were skin tested by the scratch and intradermal techniques using dilutions of aspirin and aspirin-human serum conjugate.

RESULTS

The results which Storm van Leeuwen and Drzimal¹⁰ presented on the quantity of salicylate bound to serum protein could not be substantiated by this work. The former authors used an in vitro dialysis technique, the results of which were found, in these experiments, to be markedly dependent on such variables as length of time allowed for dialysis, temperature, concentration, dilution, etc. Duplicating their experiments as closely as possible, our values for the amount of salicylate dialyzed in 17 hours averaged 1.5 times that reported by Storm van Leeuwen and Drzimal.

By exhaustive dialysis experiments we corroborated the work of others¹⁶ which indicated that salicylate was loosely bound to the serum proteins in a reversible equilibrium and that the amount bound at any given time was a function of the length of time given for the dialysis. Salicylate could be completely removed by dialyzing. In order to avoid this effect, the salicylate binding was determined on an ultrafiltrate of serum to which salicylate had been added in vitro. The results of these analyses are shown in Table I. The values given are not absolute for we corroborated the work of others¹¹ that they are dependent upon the volume of serum used, the dilution of the serum, and the amount of salicylate added. The values reported in Table I are those in which all the variable factors were kept constant so that the relative percentage for one serum could be compared with another. Patients studied were arbitrarily divided into the following groups: normal, rheumatic fever, and aspirin-sensitive. No significant difference in salicylate binding was found between these groups. Scattered sera showed less binding than the average; the explanation for this is not clear except that the patients were often taking concomitant medication. The results for a single patient with thyrotoxic heart disease showed a decreased binding.

TABLE I. BINDING OF SALICYLATE TO SERUM OF HUMAN BEINGS

GROUP	PERCENTAGE SALICYLATE BOUND	
	2 ML. SALICYLIC ACID STANDARD CONTAINING 1.455 MG./ML. ADDED TO:	
	4 ML. SERUM	3 ML. SERUM
Normal	39.3, 40.0, 56.9, 48.9, 43.0, 54.8, 55.0	40.0, 37.1, 36.5, 34.2, 41.2, 34.2, 36.4, 38.9, 41.5, 40.0, 34.6, 42.4, 36.5
Average	48.3	37.9
Rheumatic Fever	46.7, 46.0, 49.8, 49.4	
Average	47.8	
Aspirin-sensitive	38.5, 50.7, 56.9, 58.3, 52.4	
Average	51.4	
Thyrotoxic heart disease (1 patient)	34.4 (39.4, 33.8, 34.3)	
Average	35.5	
BINDING OF SALICYLATE TO ANIMAL SERUM		
	PERCENTAGE SALICYLATE BOUND	
	2 ML. SALICYLIC ACID STANDARD CONTAINING 1.455 MG./ML. ADD TO:	
	4 ML. SERUM	3 ML. SERUM
<i>Rabbit</i>		
Normal	49.7	
Sensitized	46.8, 44.5, 40.4, 34.4	
Average	41.5	
<i>Guinea Pig</i>		
Normal (pooled)	39.2	
Sensitized (pooled)	38.0	

The binding of salicylate to rabbit and guinea pig sera was also determined by this method. No significant difference was seen between normal sera and rabbit serum containing specific salicylate precipitins or pooled aspirin-sensitive guinea pig sera.

It was not possible to reproduce the results of Suzuki. Guinea pigs injected with the various salicylate preparations other than aspirin-protein conjugates were challenged with as much as 0.25 Gm. of sodium acetylsalicylate given intravenously. Aspirin-casein conjugate was also used for challenging. In none of the animals was there any evidence of an anaphylactic type reaction. Larger doses of salicylate in normal animals produced toxic symptoms which appeared somewhat similar to those of anaphylactic shock.

Guinea pigs were sensitized to salicylate by using the aspirin-protein conjugate. Aspirin in bicarbonate solution and sodium salicylate, 0.5 Gm. of each, gave mild but definite symptoms of anaphylaxis in guinea pigs sensitized by intraperitoneal injection. Heterologous conjugate, 2 ml., gave markedly more severe reactions, with prostration and severe respiratory distress but without any deaths, in a group of animals similarly sensitized. Sensitization was even more striking in the group of animals treated with aspirin-protein conjugate incorporated in Freund's adjuvant. There was no difference in the end result when either aspirin or sodium salicylate was used, for either one given intravenously produced marked anaphylactic reactions. Anaphylactic death resulted

TABLE II. PRECIPITIN TITRATIONS OF ANTIASPIRIN RABBIT SERUM ANTISERA TO ASPIRIN-CASEIN CONJUGATE

TEST ANTIGEN	SUCCESSIVE DILUTIONS OF:	RABBIT 1				RABBIT 2			
		<i>Sera Tested 29 Days After Start of Immunization</i>				<i>Sera Tested 40 Days After Start of Immunization</i>			
Aspirin (1 mg./ml.)	1:5	Interface	-	++	+	0	-	-	0
		Precipitate	++	++	++	0	+	-	0
Sodium salicylate (1 mg./ml.)	1:5	Interface	-	±	±	0	-	-	0
		Precipitate	++	+++	++	0	-	-	0
Aspirin-human serum conjugate (2 mg./ml.)	1:10	Interface	-	-	-	+	-	-	-
		Precipitate	++	+++	+++	+++	++	+++	+++
Aspirin (1 mg./ml.)	1:5	Interface	-	-	-	0	-	-	0
		Precipitate	+	±	-	0	±	-	0
Sodium salicylate (1 mg./ml.)	1:5	Interface	-	-	-	0	-	+	0
		Precipitate	+	-	-	0	-	-	0
Aspirin-human serum conjugate (2 mg./ml.)	1:10	Interface	-	-	-	-	-	-	-
		Precipitate	±	+	-	-	±	-	-
ANTISERA TO ASPIRIN-HUMAN SERUM CONJUGATE									
		TESTED 29 DAYS AFTER IMMUNIZATION				TESTED 40 DAYS AFTER IMMUNIZATION			
Aspirin (1 mg./ml.)	1:5	Interface	-	-	±	0	-	-	0
		Precipitate	±	+	±	0	±	±	0
Sodium salicylate (1 mg./ml.)	1:5	Interface	-	-	-	0	-	+	0
		Precipitate	+	±	+	0	-	+	0
Aspirin-casein conjugate (2 mg./ml.)	1:10	Interface	++	+++	+++	-	-	-	-
		Precipitate	++	+++	+++	+++	+	±	+

Interfaces read after 2 hours at 37°. Precipitates read after mixing and stirring for 24 hours at 4°. 0 indicates not tested.

when these animals were challenged with intravenous heterologous aspirin-protein conjugate. Mild reactions were even encountered in a series of animals challenged by 2 ml. rabbit serum containing salicylate as a result of parenteral salicylate administration. The salicylate content of this serum was 0.66 mg./ml.

Precipitin titrations on the sera of rabbits actively sensitized to aspirin conjugate were semiquantitative. The technique was to overlay 0.2 ml. of the serum, diluted 1:3, with serial dilutions of the antigen. The interfaces were read after 2-hour incubation at 37° C. and the tubes, after mixing, were kept overnight at 4° C. The amount of precipitate was then recorded. The results are shown in Table II. On prolonged immunization it was found that the titer of these specific precipitins diminished.

Guinea pigs passively sensitized with immune rabbit serum were shocked with 2 ml. of the heterologous antigen and anaphylactic symptoms were produced which were moderate to severe, but not fatal. Animals sensitized intravenously with serum from an aspirin-sensitive human being and challenged with 1 ml. of aspirin-human serum conjugate showed moderate anaphylactic symptoms. Animals sensitized by the intraperitoneal route and challenged intravenously with aspirin-casein conjugate showed mild to moderate symptoms of anaphylaxis. Passive transfers to human beings were consistently negative regardless of the antigen used for intracutaneous tests, also when tested by oral administration of aspirin. Direct skin tests with aspirin and aspirin-protein conjugates on aspirin-sensitive patients also gave negative results.

DISCUSSION

The frequency of aspirin sensitivity has been variously estimated from as infrequently as 2 per 1,000 patients seen by allergists¹⁷ to 16 out of 100 asthmatics.¹⁸ Although the number of cases of severe sensitivity is relatively small, nevertheless, these cases present a different problem because there is often a concurrent severe intractable asthma even though known salicylates have been avoided.¹⁹ Attempts to determine salicylate sensitivity by direct skin tests^{20, 21} have generally been as unsuccessful as for other drugs.²² Testing by ingesting small amounts of salicylate is frequently too hazardous.^{4, 19} Passive transfer techniques have similarly proved to be unreliable.^{20, 21}

It seemed to us that any study of the mechanism of drug sensitivity and techniques for its detection in human beings should be facilitated by experiments on the sensitization of laboratory animals to the drug under consideration. Suzuki¹ reported that guinea pigs could be sensitized to aspirin when the simple drug was given parenterally. In this study it was not found possible to confirm this work. Our observation that large doses of salicylate given intravenously produce symptoms in the guinea pig which markedly resemble anaphylactic shock may possibly be an explanation for the observations of Suzuki. We did confirm the work of Butler, Harington, and Yuill³ in which they obtained precipitins to aspirin in rabbits by the use of aspirin conjugated protein. The physiologic application of these antibodies and the interpretation of the results by the authors given to their antipyretic experiments seemed

remarkable to Marraek" who indicated that "these results are surprising considering the small amounts of haptenes bound by antibodies in vitro."

The failure to find that human-aspirin sera bound more salicylate than did normal sera may perhaps be explained on this basis too. We have corroborated the in vivo and in vitro binding of salicylate to the serum proteins and our failure to detect an increase in the amount bound to aspirin-sensitive serum may result from our experimental inadequacies to detect the additional small amount of hapten bound by the antibodies. In all this we make the assumption that antibodies are responsible for the allergic manifestations which are a result of contact with the drug. For this reason it seems difficult for us to reconcile ourselves to the results of Storm van Leeuwen and Drzimal,¹⁰ which results could not be confirmed, in which they indicate that there is decreased binding of such aspirin-sensitive sera. One explanation for these authors' results seems to be that dialysis was not allowed to come to equilibrium before making the quantitative salicylate determinations.

There is no satisfactory method for determining aspirin hypersensitivity in a patient. The oral ingestion produces a reliable and prompt response but it can scarcely be designated as a safe procedure. Direct skin testing and passive transfer techniques have been consistently negative. Guinea pigs passively sensitized by serum of human beings give distinct though mild anaphylactic reactions when challenged with heterologous aspirin-protein conjugate.

SUMMARY

1. Guinea pigs were sensitized to aspirin by the use of aspirin-protein conjugates. Uncombined aspirin even when incorporated with various adjuncts failed to produce this sensitivity.
2. Rabbits showed precipitating antibodies to aspirin when immunized with aspirin-protein conjugates.
3. The sera of aspirin-sensitive human beings as well as that of immune rabbits and guinea pigs failed to show an increase in the amount of salicylate normally bound, as determined by our technique.
4. Guinea pigs passively sensitized by serum from aspirin-sensitive patients gave distinct though mild anaphylactic reactions when challenged with heterologous aspirin-protein conjugate.

REFERENCES

1. Suzuki, M.: Experimental Anaphylaxis Induced by Acetylsalicylic Acid, *Sei-I-Kai M. J. (Abstr. Sec.)* **58**: 7, 1939.
2. Frei, W.: Further Studies in Arsphenamine Hypersensitiveness in Guinea Pigs. II. Attempts at Experimental Specific Sensitization of Guinea Pigs to Quinine, to Acetylsalicylic Acid, and to Barbitol With and Without Preceding or Concomitant Arsphenamine Sensitization, *J. Invest. Dermat.* **4**: 111, 1941.
3. Butler, G. C., Harington, C. R., and Yuill, M. E.: Studies in Synthetic Immunochemistry. 5. Observations on Antisera Against Aspirin-Protein Complexes, *Biochem. J.* **34**: 838, 1940.
4. Cooke, R. A.: Allergy in Drug Idiosyncrasy, *J. A. M. A.* **73**: 759, 1919.
5. Lamson, R. W., and Thomas, R.: Some Untoward Effects of Acetylsalicylic Acid, *J. A. M. A.* **99**: 107, 1932.
6. Dysart, B. R.: Death Following Ingestion of Five Grains of Acetylsalicylic Acid, *J. A. M. A.* **101**: 446, 1933.

7. Francis, N., Ghent, O. T., and Bullen, S. S.: Death From Ten Grains of Aspirin, *J. ALLERGY* **6**: 504, 1935.
8. Prickman, L. E., and Buckstein, H. F.: Hypersensitivity to Acetylsalicylic Acid (Aspirin), *J. A. M. A.* **108**: 445, 1937.
9. Marrack, J. R.: Immunochemistry. *Ann. Rev. Biochem.* **11**: 629, 1942.
10. Storm van Leeuwen, W., and Drzimal, H.: Ueber die Bindungsfähigkeit des Blutes für Salicylsäure in Zusammenhang mit Überempfindlichkeit gegen Salicylsäure, *Arch. f. exper. Path. u. Pharmacol.* **102**: 218, 1924.
11. Lester, D., Lolli, G., and Greenberg, L. A.: The Fate of Acetylsalicylic Acid, *J. Pharmacol. & Exper. Therap.* **87**: 329, 1946.
12. Kabat, E. A., and Mayer, M. M.: *Experimental Immunochemistry*, Springfield, Ill., 1948, Charles C Thomas, Publisher.
13. Riegel, B., and Wittcoff, H.: The Preparation of Acetylsalicylyl Disulfide and Salicylyl Disulfide, *J. Am. Chem. Soc.* **64**: 1486, 1942.
14. Freund, J., and McDermott, K.: Sensitization to Horse Serum by Means of Adjuvants, *Proc. Soc. Exper. Biol. & Med.* **49**: 548, 1942.
15. Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S.: Studies on the Pharmacology of Salicylates, *J. Pharmacol. & Exper. Therap.* **87**: 237, 1946.
16. Klotz, I. M., Triwush, H., and Walker, F. M.: The Binding of Organic Ions by Proteins. Competition Phenomena and Denaturation Effects, *J. Am. Chem. Soc.* **70**: 2935, 1948.
17. Gardner, E., and Blanton, W. B.: The Incidence of Aspirin Hypersensitivity, *Am. J. M. Sc.* **200**: 390, 1940.
18. Storm van Leeuwen, W.: *Allergische Krankheiten, Asthma Bronchioli, Heufieber, Urticaria und Andere.* ed. 2, translated by F. Verzar, Berlin, 1928, Julius Springer.
19. Friedlaender, S., and Feinberg, S. M.: Aspirin Allergy: Its Relationship to Chronic Intractable Asthma, *Ann. Int. Med.* **26**: 734, 1947.
20. Salén, E. B., and Arner, B.: Some Views on the Aspirin-Hypersensitive Allergy Group, *Acta allergologica* **1**: 47, 1948.
21. Hansen-Pruss, O. C.: Studies of Drug Sensitivity, *Ann. Allergy* **7**: 219, 1949.
22. Sherman, W. B.: Drug Allergy, *Am. J. Med.* **3**: 586, 1947.