THE EFFECT OF SALICYLATES ON THE PRECIPITATION OF ANTIGEN WITH ANTIBODY*

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The present study is an investigation of the effect of salicylates *in vitro* on the precipitation of protein, with special reference to the antigen-antibody reaction. Following up the statement of Marrack and Smith (1) that diphtheria toxin-antitoxin floccules are dispersed by salicylate, we undertook to determine the effect of salicylate on (1) the non-specific precipitation of protein, (2) the precipitation of an impure antigen by its antibody, and (3) the precipitation of a purified antigen by its specific antibody. In this report it will be shown that sodium salicylate modifies the precipitation of proteins, whether induced chemically or immunologically, and that the effect on specific precipitation is probably due to the inactivation of antibody.

Our interest in this subject stems from the concept that the therapeutic action of salicylate in rheumatic fever may result from the inhibition of immunological reactions in the patient. A clinical parallel involving a similar mechanism is to be found in serum sickness. As early as 1903 Hamburger and Moro (2) detected precipitin formation following the injection of horse serum in human beings and suggested a relation between the development of serum sickness and the appearance of precipitins in the circulation. Francioni (3) in 1908 showed that serum disease in man was accompanied by a diminution in circulating complement. These observations have been confirmed and have given rise to the concept that serum sickness is associated with an antigen-antibody reaction. Mackenzie and Leake (4) studying the time relations between the disappearance of precipitinogen, the appearance of precipitin, and the development of serum sickness showed that: (1) the onset of symptoms was accompanied by the appearance of circulating precipitin and the subsidence of symptoms by the disappearance of precipitin from the blood stream; (2) the failure to develop serum sickness was accompanied by the persistence of precipitinogen in the circulation and the absence of precipitin. Their findings lent further support to the concept of serum disease as an antigen-antibody reaction. Derick, Hitchcock, and Swift (5) demonstrated that the administration of acetylsalicylic acid prevented the arthritis of serum sickness (but not the other symptoms and signs of the disease as happens in rheumatic fever) and that the serum of patients treated prophylactically with this drug failed to precipitate horse serum. In their opinion, "circulating antibodies in

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the serum are kept to a low concentration by the antirheumatic drugs" and the development of symptoms is inhibited. The combined evidence shows that salicylate inhibits serum sickness and indicates that this may be due to its effect on antibody or its formation.

The similarity of some of the clinical manifestations of rheumatic fever to those of serum sickness is well recognized. Clark and Kaplan (6) have described endocardial, arterial, and other mesenchymal lesions associated with serum disease in man. These lesions were composed of fragmented collagen and cellular infiltrations comparable to the alterations of vascular tissues observed in fulminating, rapidly fatal, rheumatic fever (7). As in serum sickness, salicylates exert an inhibitory effect on the rheumatic process. Furthermore, when administered to the rheumatic subject during hemolytic streptococcal respiratory infection and the symptom-free interval following, they prevent the development of a rheumatic attack or suppress it below the clinical level (8). That this may be associated with an effect on antibody is suggested by the observations of Perry (9).

The Effect of Salicylate on the Precipitation of Normal Rabbit Serum by Sodium Tungstate

Preliminary tests showed the existence of a critical zone within which small increments of sodium tungstate produced large increases in the amount of protein precipitated from diluted rabbit serum after acidification with HCl. Rough experiments set up to explore the possible action of salicylate within this zone of tungstate concentration showed that the amount of protein precipitated was modified by the addition of small amounts of sodium salicylate, but in a somewhat irregular manner. As this system seemed poorly suited to accurate control, we undertook to study specific precipitation.

The Effect of Salicylate on the Precipitation of Horse Euglobulin by Rabbit Anti-Horse Euglobulin

In these experiments, a constant amount of antiserum and a constant volume of test solution were used throughout. The antigen dosage and the nature of the test solutions were varied.

The antigen used was horse euglobulin in a solution containing 1.3 mg. of N per cc. Antisera were obtained from three rabbits which had been immunized with horse euglobulin. Substances tested were sodium salicylate, sodium benzoate, and sodium chloride. They were used as 0.145 M solutions, (the same concentration as physiological saline). Each test mixture consisted of 1 cc. antiserum, 0.5 cc. test substance, antigen dosage as specified in Table I, and physiological saline to bring the total volume to 2 cc. Antigen, antiserum, test substance, and saline were well mixed in Wassermann tubes, incubated at 37° for 24 hours, and centrifuged at high speed. The supernatants were discarded. The precipitates, after three washings with physiological salt solution, were analyzed for nitrogen by a standard micro Kjeldahl procedure.

TABLE I

The Effect of Sodium Salicylate and Benzoate upon the Precipitation of Specific Antibody by Horse Euglobulin

Antiserum No.	Volume of antigen	Test substance	N in precipitate
	cc.		*mg.*
1	0.05	Saline (control)	0.188
		Salicylate	0.162
	0.1	Saline (control)	0.264
		Salicylate	0.240
2	0.05	Saline (control)	0.240
		Salicylate	0.226
	0.2	Saline (control)	0.296
		Salicylate	0.274
3	0.05	Saline (control)	0.828
		Benzoate	0.282
		Salicylate	0.270
	0.1	Saline (control)	0.432
		Benzoate	0.400
		Salicylate	0.368
	0.2	Saline (control)	0.520
		Benzoate	0.528
		Salicylate	0.484

TABLE II

The Effect of Increasing Concentrations of Sodium Salicylate and KI on the Amount of Precipitate Formed

Test solution	Molar concentration of test solution	N in precipitate
		mg.
Sodium salicylate	0.05	0.476
	0.10	0.484
	0.145	0.480
	0.20	0.468
KI	0.05	0.480
	0.10	0.472
	0.20	0.460

The results given in Table I show a small but definite diminution of the amount of precipitate obtained in the presence of salicylate as compared with saline controls. This loss occurred at all levels of antigen dosage tested, and

with each of three different sera. The effect of sodium benzoate was small and variable, and of doubtful significance.

Another experiment was run to test the effect of increasing concentrations of salicylate, and also to test the effect of potassium iodide.

In this case the tubes were set up as before, using 1 cc. of serum, 0.1 cc. of antigen, 0.5 cc. of test substance, and 0.4 cc. of saline. The concentration of the test solutions was varied as indicated in Table II.

These figures show a slight diminution of the amount of precipitate at the highest concentration used. Experiments to be reported below, involving much higher concentrations of salicylate, showed that the effect here indicated was progressive. Potassium iodide appeared to have about the same potency as sodium salicylate in inhibiting the precipitation of horse euglobulin.

The Effect of Salicylate on Egg Albumin Anti-Egg Albumin Precipitation at Various Levels of Antigen Dosage

Preparation of Antigen.—Crystalline egg albumin was prepared by Heidelberger's method (10). It was recrystallized three times from ammonium sulfate, dissolved in water, dialyzed against 0.85 per cent NaCl until free of ammonium salts, sterilized by filtration, preserved with merthiolate, and stored in sterile tubes at 5°C.

Preparation of Antisera.—Antisera to crystalline egg albumin were obtained from rabbits immunized with the above material. The antigen was administered as an alum precipitate suspended in saline (11). After thorough centrifugation, the serum was aged for at least 1 week at 5°C., again centrifuged, preserved with merthiolate, and stored at this temperature.

Serum I, supplied to us, was a pool of the sera of several rabbits which had been immunized with whole egg white. It contained 0.22 mg. of antibody N per cc. for crystalline egg albumin.

Serum II was a pool of the sera of two rabbits (Nos. 10 and 19) bled after a single course of immunization with alum precipitated crystalline egg albumin. It contained 0.42 mg. of antibody N per cc.

Serum III was a pool of the sera of three rabbits (Nos. 14, 19, and 22) bled after two courses of immunization with crystalline egg albumin. It contained 0.56 mg. of antibody N per cc.

Serum I was calibrated by the method of Heidelberger and Kendall (11), and the data are given in Fig. 1 for reference. The serum behaved in a perfectly typical way. A point was selected for study in the "equivalence zone," corresponding to 82 per cent of the dose of Ea (= egg albumin) required to precipitate the maximum amount of antibody.

Using this level of Ea (0.02 mg. Ea N), a constant amount of serum (1 cc.), a constant total volume (3.5 cc), and a constant total salt concentration (0.39 M) throughout the series, graded amounts of sodium salicylate were added, and the

precipitates analyzed after standing for 48 hours in the ice box. The results are shown in Fig. 2, curve *a*.

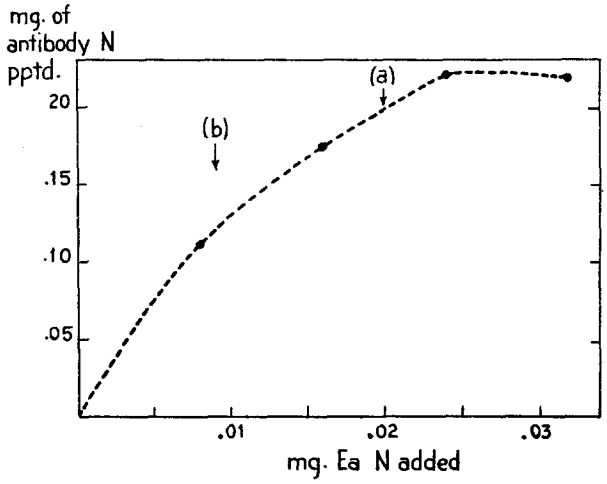

FIG. 1. Calibration curve, serum I. Ea = egg albumin N.

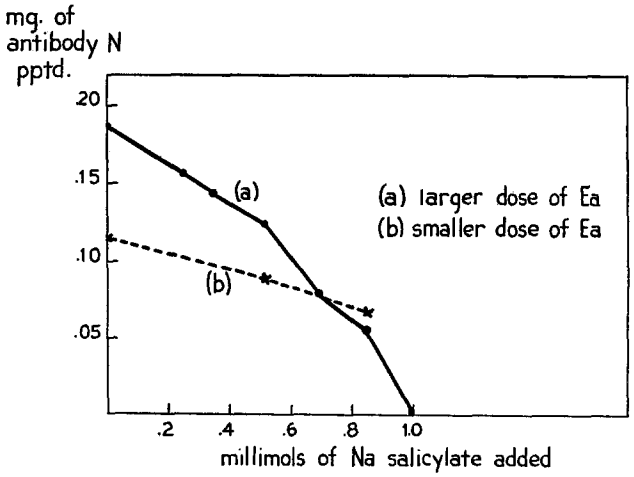

FIG. 2. The effect of graded amounts of salicylate upon the precipitation of antibody from serum I at two levels of antigen dosage.

A progressive decrease in the amount of precipitate formed was obtained with increasing amounts of salicylate.

A second level of antigen dosage was then selected in the region of antibody excess (0.01 mg. Ea N, corresponding to 42 per cent of the maximum precipitating dose of

Ea). Graded amounts of salicylate added to this system gave the result shown in Fig. 2, curve *b*.

The rate at which precipitation fell off was much slower in the second experiment. In other words, in the region of antibody excess, salicylate was less effective in preventing precipitation than in the equivalence zone.

The calibration of serum II at 0°C. is shown in Fig. 3. A calibration at 22° gave almost identical results. The effect of salicylate on the precipitation of this serum at three levels of antigen dosage, indicated as A, B, and C in Fig. 3, was determined.

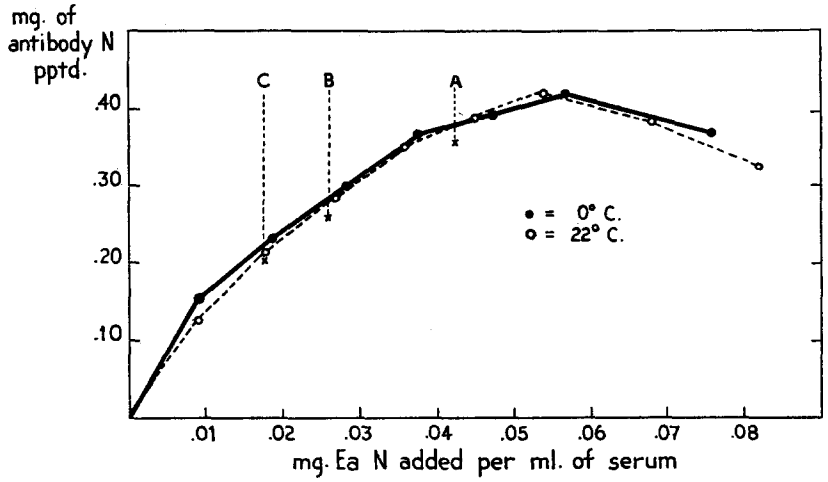

FIG. 3. Calibration curve, serum II.

A lies in the equivalence zone, B and C in the region of antibody excess. The results, expressed as mg. of antibody N precipitated against millimols of salicylate added, are given in Fig. 4.

The data substantiate the finding with serum I, that precipitation in the equivalence zone is more easily prevented by salicylate than precipitation in the region of antibody excess. They show in addition that the system becomes progressively less sensitive to the action of salicylate as the excess of antibody becomes larger.

It is seen in Fig. 4 that as the amount of egg albumin is reduced the salicylate effect diminishes (although the ratio of salicylate to Ea is increased). This indicates that the salicylate effect is not on Ea. Likewise as the total precipitate decreases, the salicylate effect diminishes. Since the only remaining component of the system is antibody, the inhibitory action of salicylate on precipitation seems referable to it.

If salicylate modifies antibody, then, as more salicylate is added, there should be less precipitable antibody present, and consequently less precipitate. If this process is continued still further, the ratio of unmodified antibody to Ea will eventually decrease to a point corresponding to the "inhibition zone," and the amount of precipitate should decline rapidly. Inhibiting proportions of Ea should ensue much sooner at level A, where the amount of Ea present

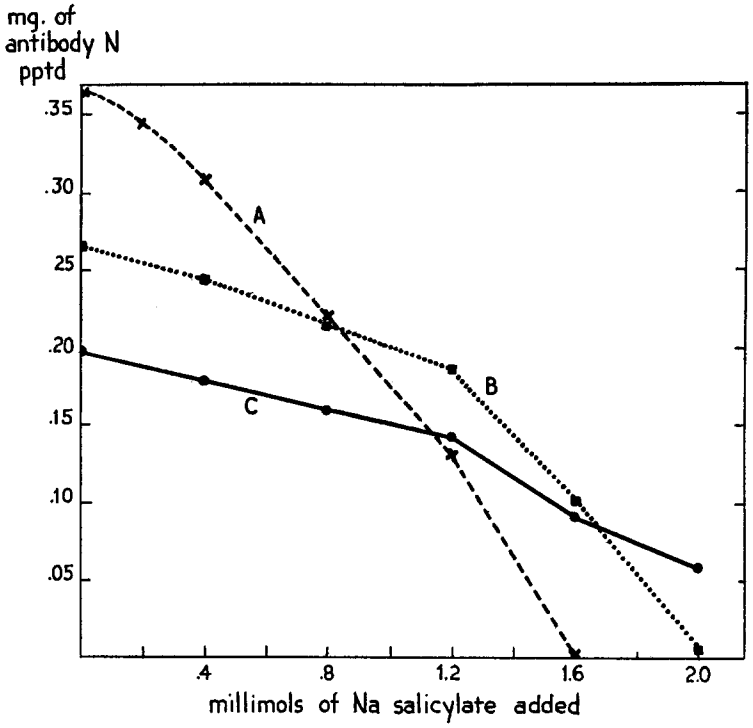

FIG. 4. The effect of graded amounts of salicylate upon the precipitation of antibody from serum II at three levels of antigen dosage.

is large, than at B and C where the amounts of Ea are progressively smaller. If we are dealing with something which progressively inactivates antibody, it should be possible to reproduce the same effect in the absence of salicylate, by merely diminishing the amount of antibody in the system.

The Effect of Progressively Reducing the Amount of Antibody at Various Levels of Ea Dosage

The above interpretation was tested experimentally so far as the limited amount of remaining serum II permitted by a procedure essentially similar to the "constant-antigen" titration of Taylor, Adair, and Adair (12). Antigen

levels A and B (see Fig. 3) were set up with graded amounts of serum (1.0, 0.75, 0.50, and 0.25 cc.) with the results shown in Fig. 5. At Ea level A, elimination of one-half of the antibody reduced the precipitate from 0.37 mg. to 0.09 mg. and at Ea level B, from 0.28 mg. to 0.169 mg. The reduction was more marked, both absolutely and relatively, at level A. Referring now to Fig. 4, curve A, it will be seen that a precipitate of 0.09 mg. of antibody N was obtained in the presence of 1.32 millimols of salicylate; and in curve B, 0.169 mg. of antibody N also corresponds to 1.32 millimols of salicylate. If this reasoning is applied again at the 0.75 cc. level (Fig. 5) we find that 0.255 mg. of antibody N was precipitated in curve A. The corresponding point in Fig. 4

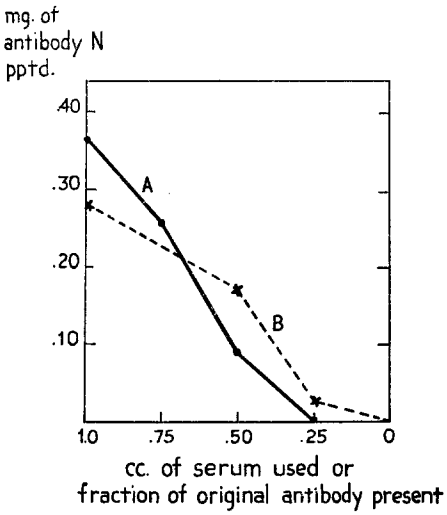

FIG. 5. The effect of reducing the amount of serum II upon the precipitation of antibody by two different doses of Ea.

gives a value of 0.62 millimols of salicylate. In brief, 0.62 millimols of salicylate had the same effect as eliminating one-quarter of the total antibody, and 1.32 millimols of salicylate had the same effect as eliminating one-half of the total antibody. This apparently direct quantitative relationship may be expressed as an averaged value of 6.2 millimols of salicylate per mg. of antibody N eliminated. Repetition of the above experiments on another serum (III) gave exactly the same value.

Observations on Reaction Time

The salicylate effect appears to involve a reaction which is complete in less than 2 hours. This can be inferred from the following experiment.

Serum and diluents were exposed to salicylate for 2 and 19 hours (in the refrigerator) before the addition of Ea. Similarly, Ea and diluents were exposed to salicylate for

2 and 19 hours before the addition of serum. In another tube all components were added at the same time (within 10 seconds). The nitrogen content of the resulting precipitates was compared with that of a salicylate-free control.

	N in precipitate mg.
Salicylate-free control	0.202
Salicylate added at time of mixing Ab and Ea	0.139
Salicylate added to serum 2 hrs. in advance of Ea	0.152
" " " " 19 " " " " "	0.146
Salicylate added to Ea 2 hrs. in advance of serum	0.153
" " " " 19 " " " " "	0.146

These figures show that previous exposure to salicylate of either antigen or antibody did not enhance the inhibitory effect of salicylate, but seemed rather to diminish it. The reason for this is still unexplained.

Reversibility of Salicylate-Antibody Reaction

The reaction between salicylate and antibody was found to be partly reversible, as evidenced by the following experiments.

(*a*) *Removal of Salicylate by Dialysis.*—

Two test mixtures were set up with the following components.

Tube No.	Serum II	NaCl 1 M	Na salicylate 1 M	Ea solution (0.096 mg. N/cc.)	N precipitated
	cc.	*cc.*	*cc.*	*cc.*	*mg.*
1	1.0	2.0	0	0.5	0.41
2	1.0	0	2.0	0.5	0

2.5 cc. of the contents of tube 2, which had remained clear after 24 hours refrigeration, was dialyzed in Visking casing against 6 changes of equimolar NaCl (a mixture of 2 parts 1 M NaCl and 1.5 parts physiological saline) until the material in the dialysis bag was free of salicylate (*i.e.* test drops gave no color with $FeCl_3$). The dialyzed material, which now contained a precipitate, was transferred to a centrifuge tube, spun, and the precipitate washed and analyzed. The amount of nitrogen found corresponded to 0.23 mg. for the whole tube. Simple dilution of 1 cc. of the contents of tube 2 with 1 cc. of saline did not produce a precipitate.

55 per cent of the amount of N precipitated from the salicylate-free control (tube 1) was precipitated in tube 2 after removal of salicylate by dialysis.

(*b*) *Resuspension of Precipitate in Salicylate Solution.*—Four equal samples of serum were precipitated as completely as possible with Ea in the absence of salicylate. The precipitates were centrifuged, freed of supernatant, washed once in saline, and resuspended in 1 cc. saline.

To each of these suspensions was added 0.5 cc. of physiological saline and a total of 2 cc. of molar salt solution, composed of graded amounts of 1 M Na salicylate plus enough 1 M NaCl to make up the rest of the volume. These mixtures were allowed to stand at 25° with frequent stirring for 1 hour, followed by refrigeration overnight. The remaining precipitates were centrifuged, washed once with saline, and analyzed for nitrogen. The results were the following.

1 M salicylate used *cc.*	N in precipitate *mg.*
0	0.147
0.2	0.129
0.4	0.112
0.8	0.104

The above figures show that increasing concentrations of salicylate dissolved increasing proportions of precipitate. Nearly one-third of the original precipitate redissolved in the highest final concentration of salicylate. It must be concluded that antibody, even though combined with antigen in a precipitate, is still susceptible to the action of salicylate.

The Effect of Other Anions in a Lyotropic Series

Since salicylate is sometimes included in "lyotropic series" of anions, we undertook to determine whether a comparable interference with immune precipitation could be produced by lyotropically powerful but chemically unrelated ions such as thiocyanate and iodide.

A comparison was made of a number of neutral salts, each of which was added in equimolecular quantities (0.7 millimols) to a standard system (1 cc. of serum I, 0.02 mg. of Ea N, total volume 3.5 cc.). Analysis of the precipitates obtained gave the following results.

	N in precipitate *mg.*	Per cent of control
NaCl (control)	0.21	100
KCl (control)	0.21	100
KBr	0.21	100
KI	0.20	95
KCNS	0.18	86
Na salicylate	0.09	43

Salicylate was much more effective than any of the other salts tried. Whether the slight action of iodide and thiocyanate involves the same mechanism as salicylate remains to be determined.

SUMMARY

1. Sodium salicylate modifies the precipitation of normal rabbit serum protein by sodium tungstate, and partially inhibits the precipitation of horse

serum euglobulin by rabbit antiserum. Sodium salicylate added to a system containing crystalline egg albumin and its antibody partly prevents the formation of precipitate, the degree of inhibition being related to the concentration of salicylate.

2. Precipitation in the equivalence zone is more readily prevented by salicylate than precipitation in the region of antibody excess, the immune system becoming progressively less sensitive to the action of salicylate as the excess of antibody becomes larger.

3. Formed precipitates were partly dissolved following resuspension in the presence of salicylate.

4. The salicylate effect on immune precipitation is reversible, and appears to be due to inactivation of antibody.

5. Salicylate was more effective in preventing specific precipitation than other anions of a lyotropic series tested.

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